JHAD
Health and Development Journal
https://jhad.kmu.ac.ir
10.34172/jhad.2022.92066
Vol. 11, No. 1, 2022, 1-8

Original Article

The Antibacterial Effects of Curcumin-Silver Nanoparticle and Curcumin-Copper Nanoparticle Loaded Niosomes

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Abstract

Background: The threat of antimicrobial resistance is increasing worldwide. Niosomes are a new drug delivery system that increases the antimicrobial potential of antibiotics. Accordingly, this study aimed to evaluate the antibacterial activity of niosomes loaded with curcumin-silver nanoparticles (Cur-AgNPs) and curcumin-copper nanoparticles (Cur-CuNPs).

Methods: Initially, a unique combination of metal and curcumin nanoparticles was prepared in free and encapsulated forms to investigate the synergistic effects of the two drugs and to evaluate them through a niosomal carrier. Particle size and polydispersity index were measured using dynamic light scattering (DLS), and entrapment efficiency (EE) was measured through indirect centrifugation. The rate of drug release from the loaded niosomes was assessed through in vitro dialysis. Finally, the antibacterial activity of Cur-AgNPs and Cur-CuNPs loaded niosomes on Staphylococcus aureus and Pseudomonas aeruginosa were measured.

Results: The curcumin-loaded niosomal formulations and the simultaneous combination of Cur-AgNPs and Cur-CuNPs had an optimum particle size of less than 200 nm and uniform dispersion. These formulations also showed high entrapment efficiencies and slow release for more than 72 hours.

Conclusion: A significant increase in antibacterial activity was observed when using curcumin in combination with metal nanoparticles loaded in niosomes, indicating that the concomitant use of metal nanoparticles and curcumin had a synergistic effect in inhibiting bacterial growth.

Keywords: Curcumin, Silver nanoparticles, Copper nanoparticles, Niosome, Antibacterial activity

Citation: Abolhassani Targhi A, Mousavi-Niri N, Jaberi Ansari F, Jafari Mansoorian H. The antibacterial effects of curcumin-silver nanoparticle and curcumin-copper nanoparticle loaded niosomes. Health Dev J. 2022;11(1):1–8. doi:10.34172/jhad.2022.92066

Received: May 29, 2022, **Accepted**: July 2, 2022, **ePublished**: July 10, 2022

Introduction

The history of microbial resistance to antibiotics dates back to the 1940s. Since then, the attention of international and national authorities has been directed to overcoming or reducing the incidence of microbial resistance. The more antibiotics are used to treat infections, the more likely microbial resistance will occur. Even antibacterial drugs with a new mechanism of action are affected by antibiotic resistance (1,2). Bacterial sensitivity to antimicrobial agents is reduced through mechanisms such as the inactivation of the drug, changing the target site, changing the metabolic pathway, and reducing the accumulation of the drug in the target site due to the reduction of drug penetration. The decreased drug penetration is probably due to the ability of some microorganisms to grow in the form of biofilm or because of the increased expression of efflux pumps that pump the drug out of the cell (3-5). Researchers are currently studying and designing new antimicrobial agents that suppress antibiotic resistance. As a result, designing effective and safe antimicrobial agents with broad antimicrobial effects is essential for treating antimicrobial infections.

Encapsulation of antimicrobial agents in nanocarrier systems is one of the alternatives and new approaches that increase the antimicrobial effect and at the same time reduce side effects (6,7). Recently, niosomes have been widely used to increase selective delivery and improve the therapeutic effectiveness of antimicrobial agents (8,9). Niosomes are double-layered structures consisting of non-ionic surfactants and cholesterol, which, due to their biocompatibility, are a suitable option for retaining large doses of drugs, especially antibiotics with very low toxicity on normal cells (10,11). Niosomes also have unique properties such as non-immunogenicity,

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biodegradability, easy storage, and safety. They can also be used for encapsulating hydrophilic and hydrophobic drugs (12-14). Recently, niosomes have found wide applications in increasing the antimicrobial effects of antibiotic agents.

Metal nanoparticles have attracted a lot of attention due to their unique and unusual physical and chemical properties that are completely different from their metallic properties. These unique physical and chemical properties of nanoparticles are due to their small size and high surface-to-volume ratio (15-17). Copper (Cu) compounds have a high potential for application as antibacterial agents due to their relatively low cost and high environmental safety. It has been shown that copper nanoparticles can have antimicrobial properties against a wide range of microorganisms, including pathogenic bacteria (18). Another widely used metal nanoparticle is the silver nanoparticle. These nanoparticles have different properties including optical, electrical, biological, and thermal properties and high electrical conductivity. Due to their unique characteristics, these nanoparticles have various applications as antibacterial agents in industrial, household, and health products (19).

Curcumin is another new antimicrobial agent that has recently received a lot of attention in clinical research. This compound induces its antimicrobial activity by damaging the bacterial cell membrane, and as a result, it is active against a large number of gram-positive and gram-negative bacteria (20). This characteristic is probably attributed to the hydrophobic nature of curcumin (Log P 2.56 to 3.29) (21). Interestingly, curcumin has a strong synergistic effect with other antimicrobial agents (22).

The antibacterial effect of various compounds in the form of nanoniosomes alone or together with metal nanocomposites has already been investigated in several studies (9,11). In this study, the simultaneous combination of curcumin and metal nanoparticles (silver and copper) is used in free and niosomal forms to increase the antimicrobial effect and reduce antibiotic resistance. Accordingly, this study aims to prepare niosomes that are encapsulated with Cur, Cur-AgNPs, and Cur-CuNPs. After investigating the physicochemical properties of nanoparticles, the antibacterial effects of these niosomal nanocarriers are compared to the free form of drugs for the first time on gram-positive Staphylococcus aureus and gram-negative Pseudomonas aeruginosa.

Materials and Methods

The materials used in this study including cholesterol, Span 80, methanol, chloroform, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), Mueller Hinton Broth (MHB), and Mueller-Hinton Agar (MHA) were purchased from Merck (Germany). Dialysis membranes (molecular weight 12 000 Da), sodium borohydride (NaBH4), and silver nitrate (AgNO3) were purchased from Sigma-Aldrich (Germany). Copper sulfate pentahydrate (CuSO4 5H2O), starch $(C6H10O5)_n$, ascorbic acid (C6H8O6), and sodium hydroxide NaOH were purchased from Sigma Aldrich. Double distilled water was used in all experiments. Staphylococcus aureus ATCC6538 and Pseudomonas aeruginosa ATCC15442 were obtained from the microbial bank of the Pasteur Institute of Iran.

To prepare AgNPs and CuNPs, 100 mL of 2 mM AgNO3 was mixed with 200 µL of 1 M NaOH at 400 g/min for 5 minutes. Then, 33 mL of chitosan dissolved in acetic acid (0.5%) was added. Finally, the solution was exposed to ultraviolet (UV) radiation for 20 minutes with an intensity of 300 mJ/(cm^2). Silver nanoparticles (AgNPs) were diluted with double distilled water (23).

Copper nanoparticles (CuNPs) were synthesized through a chemical reduction process using copper (II) sulfate pentahydrate as a precursor salt and starch as a coating agent. The preparation process began by adding 0.1 M copper (II) sulfate pentahydrate solution to 120 mL of starch (1.2%) with vigorous stirring for 30 minutes. In the second step, 50 mL of 0.2 mM ascorbic acid solution was added to the synthesis solution. Subsequently, 30 mL of 1 mM sodium hydroxide solution was slowly added to the prepared solution with stirring and heating at 80°C for 2 hours. The color of the solution turned yellow. After the reaction was completed, the solution was removed from the heat and kept at room temperature for 24 hours. Then, the supernatant was carefully discarded. According to the instructions provided in a similar study (24), the sediments were separated from the solution by filtration and washed three times with deionized water and ethanol to remove the starch attached to the nanoparticles. The precipitates obtained were dried at room temperature. After drying, the nanoparticles were stored in a glass container for further experiments (24).

To prepare Cur-Ag and Cur-Cu nanoparticle-containing niosomes, the niosomes were prepared using the thin layer hydration method (25). Briefly, Span 80, cholesterol (1:1 molar ratio), and curcumin (1 mg/mL concentration) were dissolved in an organic solvent (chloroform: methanol 2:1 volume ratio). Then, the solvents were evaporated using a vacuum evaporator (Heidolph Instruments, Germany) and formed a thin lipid film in 60°C at 120 rpm. The thin layer was hydrated with 10 mL of phosphate-buffered saline (PBS pH 7.2). It should be noted that for curcumin-silver nanoparticle and curcumin-copper nanoparticle niosomal formulations, hydration was performed with AgNPs and CuNPs at a concentration of 200 µg/mL. Afterward, the niosomal formulations were broken by a sonicator (Hielscher Up50H Ultrasonic Processor, Germany) for 7 minutes. Finally, the formulations were stored at 4°C for further experiments.

The different groups used for the next steps are as follows:

- Empty niosome (B-Nio)
- Free curcumin (F-Cur)

- Curcumin-loaded niosome (Cur-Nio)
- Curcumin-free silver nanoparticles (F-Cur-AgNPs)
- Niosomes loaded with curcumin-silver nanoparticles (Cur-AgNPs-Nio)
- Curcumin-free copper nanoparticles (F-Cur-CuNPs)
- Niosomes loaded with curcumin-copper nanoparticles (Cur-CuNPs-Nio)

To determine the characteristics of niosomal formulations, particle size and dispersion index (PDI) were first measured using a ZetaSizer (Malvern Instrument, UK) via dynamic light scattering (DLS) at room temperature with 3 replications.

Besides, to check the size and morphology of the formulations, the surface morphology of blank (drug-free) niosomes was assessed using a scanning electron microscope (SEM) at a voltage of 200 kV.

To calculate the entrapment efficiency (EE), the Cur, Cur-AgNPs-Nio, and Cur-CuNPs-Nio containing formulations were centrifuged using an Amicon Ultra-15 MWCO 30 kDa ultrafilter membrane in a centrifuge (Eppendorf® 580R, Germany) at a speed of 400 g for 20 minutes. The free drugs moved through the filter membrane. Then, the Cur, AgNP, and free CuNP concentrations were measured using UV-Vis spectroscopy at wavelengths of 420, 415, and 480 nm, respectively (17,26). The percentage of EE was calculated with the following equation:

$$(EE) = (A-B)/A \times 100$$

where *A* is the amount of primary drug used to prepare niosome and *B* is the amount of free drug passed through the filter membrane.

The drug release rate from niosomes nanoparticles was measured through dialysis as one of the main methods used to evaluate the behavior of drug release from niosomes in vitro (27). Drug release in vitro from niosome formulations was measured with a dialysis bag (MWCO12000 Da) in a release medium containing PBS (0.5% W/VSDS) to mimic the physiological environment at 37°C for 72 hours. Two milliliters free and noisomal drugs were separately transferred to the dialysis membrane and drug release was evaluated in 50 mL PBS with continuous stirring. At predetermined intervals (1, 2, 4, 8, 24, 48, and 72 hours), one milliliter of release medium was collected and immediately replaced with an equal volume of fresh PBS to determine the drug release rate. Finally, the released drugs were measured by UV-Vis spectrometer at wavelengths of 420, 415, and 480 nm for Cur, Cur-AgNPs, and Cur-CuNPs, respectively.

To measure the rate of bacterial lethality by niosomes formulations over time, the antibacterial activity of niosomes enclosed with drugs (including curcumin, AgNPs, and CuNPs) was measured for 72 hours using the 96-wall plate procedure on the *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains. Curcumin-free forms and curcumin alone, Cur-AgNPs, and Cur-CuNPs were prepared with a concentration of 100 µg/mL. Then, 100 µL of the tested sample was added to each well of the 96-well plate, including (*i*) free curcumin, (*ii*) niosomal curcumin, (*iii*) free Cur-AgNPs, (*iv*) curcumin-niosome silver nanoparticles, (*v*) Cur-CuNPs. free, (*vi*) curcumin-niosome copper nanoparticles, and (*vii*) niosome blank. Then, 100 µL of diluted bacterial suspension to a final concentration of 105 CFU/mL was added to each well. Afterward, the 96-well plate was incubated at 37°C and the optical absorbance (OD) at 600 nm was measured at specific time intervals (0, 2, 4, 6, 24, 48, and 72 hours) using a microplate reader (EPOCH, Japan) (28). The growth curve of Staphylococcus aureus and Pseudomonas aeruginosa was considered a positive control.

GraphPad Prism 5.0 software was used for the statistical analysis of data through one-way analysis of variance (ANOVA). Each value was expressed as mean ± SD and a *P* value less than 0.05 was considered statistically significant.

Results

To prepare and determine their properties, AgNPs and CuNPs were successfully synthesized by the chemical reduction method. The niosome formulations containing Cur-AgNPs and Cur-CuNPs had optimal particle sizes of 188.10 and 197.53 nm, respectively, and particle dispersion indices of 0.170 and 0.117 (Table 1). As can be seen in Table 1, the particle dispersion index of niosomal nanoparticles is less than 0.3, indicating the homogeneity of the particle size. The analysis of the EE of curcumin and AgNPs and CuNPs nanoparticles showed that curcumin was loaded with high efficiency (≥90%) in niosomal nanoparticles, while the EE of AgNPs and CuNPs was 12.24 and 20.45, respectively.

SEM images of the blank niosome formulation in Figure 1 used to analyze the size and morphology of niosome formulation indicated almost spherical niosome vesicles with a smooth surface in the range of 174-197 nm particle size, confirming that the parameters selected for preparing niosomes significantly affected the morphology

Table 1. The particle size, dispersion index, and entrapment efficiency of different niosomal formulations: Cur-Nio, Cur-AgNPs-Nio, and Cur-CuNPs-Nio

Formulation	Size (mm)	Particle dispersion index	Curcumin entrapment efficiency (%)	AgNP/CuNP entrapment efficiency (%)
Cur-Nio	174.30±5.57	0.182±0.012	95.59±1.23	
Cur-AgNPs-Nio	188.19±10.21	0.170±0.005	94.57±0.88	12.24±1.55
Cur-CuNPs-Nio	197.53±6.42	0.117±0.011	0.117±1.22	20.45±2.31

The data were replicated 3 times and each data is displayed as mean ± standard deviation (SD).

of nanoparticles.

The release of curcumin and metal nanoparticles from the niosome formulations was analyzed at 37°C for 72 hours. In this study, the dialysis membrane containing the treatment groups was immersed in the buffer solution and sampling was performed in 1 mL of the solution at the predetermined intervals. The removed volume was replaced with the same amount of buffer solution. Figure 2 shows the release of curcumin alone and its simultaneous combination with AgNPs and CuNPs from niosomal formulations. As can be seen, up to 8 hours from the start of the release, the drug is released fast followed by a slow and controlled release up to 72 hours, indicating the release of the antimicrobial agents through the two lipid layers.

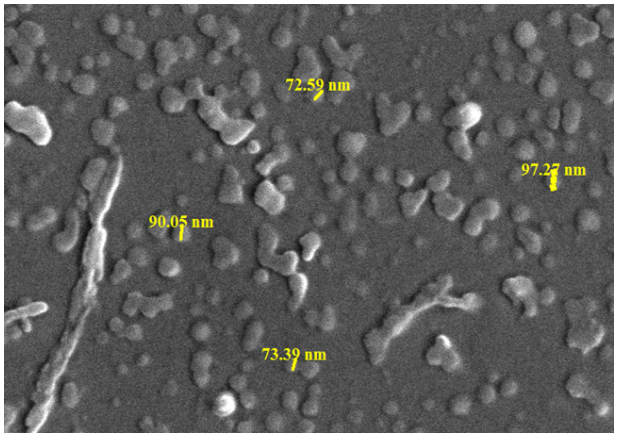

Figure 1. The SEM image of the blank niosomal formulation.

In this study, the antibacterial activity of the free and niosomal forms of curcumin and the combination of Cur-AgNPs and Cur-CuNPs was evaluated as a function of time on *S. aureus* and *P. aeruginosa* bacteria (Figure 3). The results indicated that the forms encapsulated with niosomes had more lethality due to reduced optical density (OD) absorbance on the target bacteria ($P < 0.05$). The OD absorbance was greater in the bacteria exposed to CuNP-containing niosomes than in those exposed to AgNP-containing niosomes. Indeed, the higher EE of CuNPs inside niosomes (20%) compared to AgNPs (12%) can be one of the reasons for the higher lethality of niosomes loaded with Cur-CuNPs. Moreover, as displayed in Figure 3, all three niosomal carriers containing Cur, Cur-AgNPs, and Cur-CuNPs have significantly higher EE compared to the control group on gram-negative *P. aeruginosa* bacteria ($P < 0.05$).

Discussion

Niosomes are vesicles based on nonionic surfactants that are usually used to release drugs, genes, proteins, etc. The structure of a niosome is almost similar to a liposome, but due to the difference in its raw materials, it has many advantages such as higher stability, lower cost, and more biocompatibility than liposome. In general, drug release from niosomal nanoparticles has two phases:

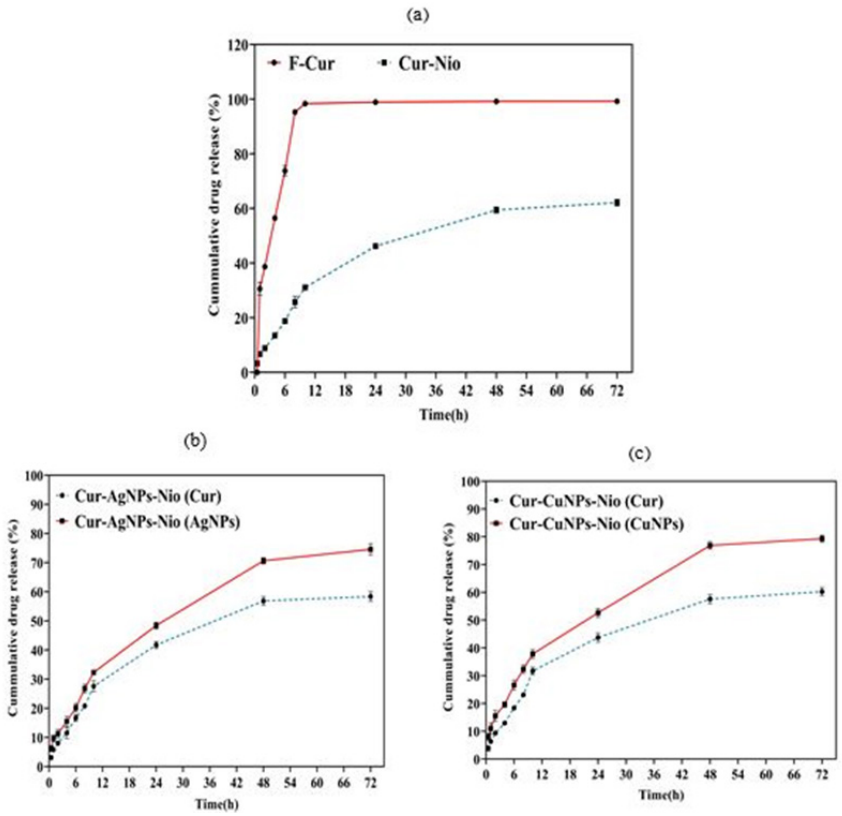

Figure 2. Release of curcumin and metal nanoparticles from niosomal formulations: (a) release of free curcumin and niosome, (b) release of curcumin and silver nanoparticles and (c) release of curcumin and copper nanoparticles from niosome.

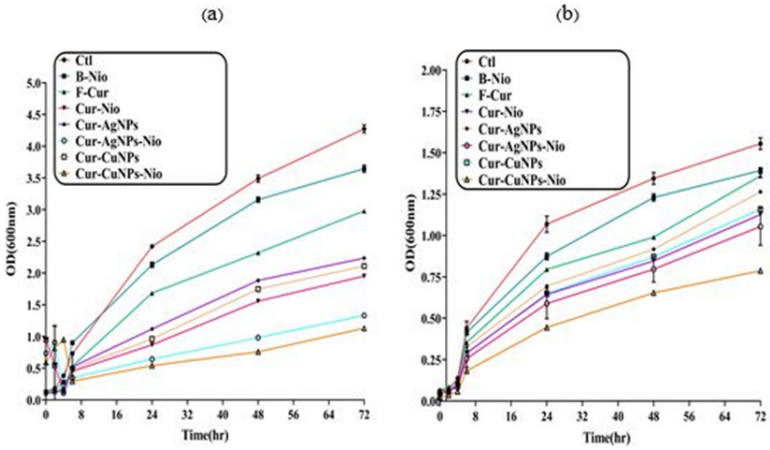

Figure 3. Anti-bacterial activity of free drugs and niosomes on (a) *S. aureus* and (b) *P. aeruginosa* bacteria by measuring the optical density (OD) absorbance as a function of time. As can be seen, the drug niosomal forms (Cur, Cur-AgNPs, and Cur-CuNPs) showed more anti-bacterial activity on the studied bacteria over time ($P<0.05$).

The rapid release in the early hours followed by slow and controlled release. The rapid release may be due to the release of the drug from the surface of niosomes while the slower phase is mainly induced due to the diffusion of the drug through two lipid layers, which indicates the high stability of the encapsulated drug (29,30,31). The release rate of curcumin alone from the niosome nanostructure (Figure 2a) was similar to the release of curcumin from niosome containing Cur-AgNP and Cur-CuNP-containing niosomes. However, AgNPs and CuNPs had a faster release (Figures 2b and 2c), so that after 72 hours, about 80% of these nanoparticles were released from the niosome membrane, which is probably due to the partial separation of two adjacent layers and the increased diffusion in the samples loaded with AgNPs and CuNPs (32).

Curcumin is known as a natural antimicrobial compound and has effective biological and medicinal activity. To overcome the hydrophobicity of curcumin, Liao et alused the simultaneous combination of tannic acid and metal to coat curcumin nanoparticles (CurNPs) and investigate their antibacterial effect. The introduction of metal ions increased the bacterial inhibition efficiency compared to curcumin and metal ions alone. The results showed that the minimum inhibitory concentration of the CuNP-containing complex was 7.5 times lower than that of curcumin alone on *S. aureus* (33). Loo et al also used the simultaneous combination of AgNPs and CuNPs to increase the anti-biofilm activity on *S. aureus* and *P. aeruginosa*. The results of the SEM and confocal laser scanning microscope showed that the simultaneous treatment of AgNPs and CuNPs is the most powerful method to destroy the biofilm of the mentioned bacteria compared to each of these factors alone (34). In another study, Huang et al investigated the effect of silver-decorated polymeric micelles combined with curcumin on enhanced antibacterial activity of *S. aureus* and *P. aeruginosa* and observed that due to the cooperative antibacterial effects of silver nanoparticles and curcumin, the lethal effect of this polymer structure increased compared to the polymer micelles containing silver nanoparticles and micelles encapsulated with curcumin alone (35).

Different patterns of antibacterial activity related to free and encapsulated drugs with niosomes observed in the present study were consistent with previous reports (36-38). In these studies, growth inhibition was observed as an increase in the lag phase, a decrease in the growth rate, or a decrease in the OD of the bacterial strains, and the encapsulated drugs were gradually released over time, leading to the "slower and longer" pattern of antibacterial activity. This effect may be because vesicles, apart from interacting with the outer bacterial membrane, can release a large amount of drug near the surface of the bacteria and cause a concentration gradient of the drug to facilitate its entry into the cell (39). Interestingly, the inhibitory effect of the simultaneous combination of metal nanoparticles with curcumin in the niosomal formulation was more than curcumin encapsulated in the niosome alone, indicating that the simultaneous use of metal nanoparticles and curcumin has a synergistic effect in inhibiting bacterial growth.

Previous studies have shown that the mechanism of antibacterial activity of curcumin involves disrupting the GTPase activity of FtsZ protofilaments, which play an important role in bacterial cytokinesis. This disturbance is fatal for bacteria and by inhibiting the FtsZ community in the Z ring, it prevents the proliferation of bacterial cells. However, the membrane structure of bacteria is not disturbed by curcumin in any way (40). Another study showed that curcumin inhibits the surface protein Sortase A and prevents cell adhesion to fibronectin. Indeed, the mechanism through which curcumin nanoparticles exert their antibacterial properties is that they are anchored to the bacterial cell wall, break it, then penetrate the cell and disrupt the structure of the internal components of the

bacteria (41).

The exact mechanism of action of AgNPs on bacteria is still unknown. However, some researchers have suggested that the action of AgNPs on bacteria may be due to their ability to penetrate the cell (42), formation of free radicals (43,44), inactivation of proteins in the cell by silver ions (45), and production of reactive oxygen species (46). In addition, other factors such as AgNP concentration, bacterial type (47), shape (48,49), size (50), and combination with different antibiotics (51,52) may also be effective on the bactericidal performance of nanoparticles. CuNPs also have different effects on the killing of bacteria. The copper ions released by the nanoparticles are probably attached to the negatively charged bacterial cell wall and by destroying the cell wall, they lead to cellular death. Furthermore, copper ions inside bacterial cells are attached to deoxyribonucleic acid molecules and interfere with the bond inside and between nucleic acid strands, and as a result, an irregular spiral structure is formed. Furthermore, the absorption of copper ions by bacterial cells also disrupts important biochemical processes (53,54).

This study showed all three niosomal carriers containing curcumin, Cur-AgNPs, and Cur-CuNPs were more effective against gram-negative *P. aeruginosa* possibly due to the difference in the cell walls of gram-negative and gram-positive bacteria. Gram-positive bacteria have a thick cell wall (20-80 nm) as the outer shell of the cell, while gram-negative bacteria have a relatively thin layer (less than 10 nm) of the cell wall and an outer membrane with several pores and appendages. These differences in the cell envelope give different characteristics to the cell, especially when reacting to external stresses, including exposure to antimicrobial agents (29).

Conclusion

The simultaneous use of AgNPs and CuNPs along with curcumin in niosomal formulations can be an effective solution to inhibit the growth of bacteria, which is caused by the synergistic effect of both compounds together compared to each of the compounds alone. The death rate of bacteria that were exposed to CuNP-containing niosomes was higher than that of bacteria that were exposed to AgNP-containing niosomes. In fact, the higher EE of CuNPs inside the niosomes compared to NgNPs can be one of the reasons for the higher lethality of niosomes loaded with Cur-CuNPs. Besides, the use of niosomal formulation causes more and longer inhibition of bacterial growth. This formulation can be used directly as a solution to prevent the spread of bacterial infections or as a coating on medical equipment to achieve a long-term and continuous antibacterial effect.

Acknowledgments

This article was extracted from a master's thesis by Arefa Abolhassani Targhi, a graduate of Islamic Azad University, Medical Sciences Branch, Tehran.

Authors' Contribution

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Writing–review & editing: Hossein Jafari Mansoorian, Neda Mousavi-Niri.

Competing Interests

The authors declared no conflict of interest.

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