



The Antibacterial Effects of the Mixture of Silver Nanoparticles With the Shallot and Nettle Alcoholic Extracts

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Abstract

Introduction: Today, one of the most important challenges of the therapeutic system is the resistance of bacteria against different antibiotics especially in intensive care units which lead to an increase in hospitalization time and the patients' expenses. *Acinetobacter baumannii* is one of the most significant contaminating bacteria in intensive care units which has exhibited resistance against different antibiotics in recent years. The aim of this study was to investigate the synergism effect of the silver nanoparticles with the shallot and nettle alcoholic extracts against the standard and multidrug resistant *A. baumannii* isolates.

Materials and Methods: Samples were collected from intensive care units and the *A. baumannii* isolates were identified using biochemical tests. Then, the antibiogram test was carried out for each isolate. The antibacterial effect of nanoparticles, shallot and nettle extracts was evaluated singularly and in combination with each other against standard and resistant *A. baumannii* isolates. Measuring the diameter of inhibited growth zone, MIC, MBC and checkerboard tests were conducted for each isolate.

Results: The results showed that the silver nanoparticles, shallot and nettle alcoholic extracts each had antibacterial property against the standard and resistant *A. baumannii* isolates. The mixture of the nettle extract with silver nanoparticles had a synergism effect against the standard and resistant isolates and the mixture of the shallot extract with silver nanoparticles had an additive effect against *A. baumannii* isolates.

Conclusions: Due to the increase of antibiotics resistance and the resistance to the pathogenic bacteria especially in intensive care units, it is necessary to find effective and accessible substances to destroy the resistant bacteria and reduce the mortality rate of patients. The results of the present study revealed that the antibacterial property of the shallot and the nettle alcoholic extracts could increase the antibacterial property of the silver nanoparticles. As a result, these can be used for disinfecting different wards of a hospital, in particular, the intensive care units.

Keywords: *Acinetobacter baumannii*, Hospital Infection, Antibiotic Resistance, Silver Nanoparticles, Nettle Extract, Shallot Extract

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Introduction

The extensive consumption of antibiotics has undesired effects on hosts including high sensitivity, suppressing the immune system, and the allergic reactions in addition to making bacteria resistant.^{1,2} The problems raised by resistant bacteria have caused several challenges in the therapeutic system.² Nowadays, the spread of Gram-negative pathogens resistant to drugs in hospitals has become a great problem, and is rising in many countries.³ *Acinetobacter* is one of these pathogens which are found in different wards of hospitals, especially in intensive care units (ICUs). The resistance among *Acinetobacter* spp. has increased dramatically in recent years and has become a global threat.⁴ *Acinetobacter* is one important reason for pneumonia and blood infections in the ICU.⁵⁻⁷ Currently, it is necessary to find some stronger and more effective antibacterial substance against multi-drug resistant bacteria.²

From many years ago, medicinal plants have been used for the treatment of different infections.⁸ Nowadays, these plants have attracted the attention researchers because of their significant attributes including their strong antibacterial property, being cheap, and accessible.^{9,10} One of these medicinal plants which are local in Iran is the shallot (*Allium hirtifolium*) which has different therapeutic applications.¹¹ The Iranian shallot is a member of the Allium family and has bulbs (short stem with meaty leaves). This plant is rich in combinations of organosulfur and flavonoids, and due to these combinations it has a strong anti-oxidant and antibacterial activity. Conducted investigations have shown that the aqueous and alcoholic extracts of the stems and the leaves of this plant have a strong antibacterial property.¹²⁻¹⁴ The nettle has a long history in treating different diseases and has been used in traditional medicine too.¹⁵ Different studies have revealed that the nettle extract has strong antibacterial

effects especially against gram-negative bacteria.¹⁶⁻¹⁸

Nowadays, the different properties of substances can be changed by changing the size of the particles.¹⁹ Silver nanoparticles have an effective antibacterial property due to their large surface area.²⁰ It is important to study the antibacterial effect of silver and the other nanoparticle compounds against the increasing resistance to gram-negative and gram-positive bacteria.²¹ Recently, the antibacterial properties of the mixture of plant extracts and silver nanoparticles against resistant bacteria have been investigated, and new hope has been gained.²²

Therefore, it is necessary to use and find some new and effective compounds against resistant bacteria. The aim of the present study was to investigate the antibacterial effect of the shallot and nettle alcoholic extracts in combination with the silver nanoparticles against the standard and resistant *A. baumannii* isolates.

Material and Methods

Material

All the media were purchased from Merck, Germany. The entire antibiogram discs were purchased from Patan Teb Co., Iran. The standard strain was provided by the Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran.

Collecting the Sample

In order to conduct this research, 120 Mini-BAL samples were collected from the ICUs of the Rasool-e-Akram hospital in Tehran during September 2017- September 2018. All samples were investigated after transferring to the Immunology and Infectious Disease Institute of Iran Medical Sciences University for determining the bacterial identity. Samples were cultured on blood agar, nutrient agar, and McConkey agar and were incubated for 24 hours in 37°C. *Acinetobacter* isolates were separated using microscopic and biochemical test methods.

Antibiotic Sensitivity Test

The disc diffusion test was conducted for all *A. baumannii* isolates according to the CLSI 2017 instruction.²³ The bacterial suspension was prepared with the concentration of 5×10^5 CFU/mL and was transferred to the Muller-Hilton agar medium. The antibiotic discs were placed on the Muller-Hilton agar medium and were incubated at 35°C for 18 hours. The diameter of the inhibition zone was calculated and the resistant or susceptible bacteria were characterized using the CLSI standard table. All antibiotic disks were purchased from the MAST Company, UK. The antibiotic discs included trimethoprim (25 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), sulfamethoxazole (10 µg), colistin (10 µg), imipenem (10 µg), ampicillin-sulbactam (10 + 10 µg), and piperacillin-tazobactam (100 + 10 µg). *Acinetobacter* ATCC 19606 was used as a standard.

Alcoholic Extracts

The shallot and nettle extracts were purchased from the Ebnemasouyeh Pharmaceutical Company.

Preparing Silver Nanoparticles

The silver nanoparticles were synthesized by using sodium borohydride as a reducing agent. A hundred milliliters of 0.1 (mM) silver nitrate and 30 ml of 1 (mM) sodium borohydride were prepared and chilled in an ice bath for 15 minutes. Then, the silver nitrate was added to sodium borohydride which had been stirred vigorously. Then the solution turned to light yellow.²⁴ After preparing the solution, the nanoparticles were investigated with the help of FE-SEM, UV-Vis spectrum and X-ray powder diffraction (XRD). The as-synthesized nanoparticles were characterized by field emission scanning electron microscope (FE-SEM). The FE-SEM images were recorded on a Hitachi S-4160 instrument. The XRD analysis was carried out on a Rigaku D/max 2500 V diffractometer equipped with the graphite monochromator and Cu target. The UV-Vis spectra of the nanoparticles were recorded by Perkin-Elmer Lambda 25 UV/Vis spectrometer.

Well Diffusion Test

Resistant *A. baumannii* isolates were selected and used for preparing bacterial suspension. The bacterial suspensions were cultured using a sterile swab on Muller-Hinton agar (MHA) medium. Some wells with 6 mm diameter were created in the media. An amount of 100 µL of nettle, shallot alcoholic extracts, and the silver nanoparticles were added to each well respectively. Then, they were incubated at 37°C, and the inhibition zone diameter was measured.²⁵

Determination of Minimum Inhibitory Concentration

The liquid dilution method was used to determine the minimum inhibitory concentration (MIC) of extracts and silver nanoparticles in accordance with CLSI (2011) guidelines. The MIC test was carried out through broth micro-dilution method. An amount of 100 µL of sterile Mueller-Hinton broth was added into each of the 96-well round-bottomed sterilized microtiter plates. The nettle and shallot extracts, along with the silver nanoparticles solution were each separately poured into the wells and serially diluted in the Mueller-Hinton broth. *Acinetobacter* isolates were cultured in nutrient broth at 37°C overnight. Then, 20 µL of fresh bacteria (1.5×10^5 CFU/mL) was prepared in Muller-Hinton broth and was added. Microliter plates were covered with a sterile plate sealer and incubated at 37°C for 18–24 hours. The growth of the bacteria was assessed as a function of turbidity (optical density [OD] at 600 nm).²⁶

Determination of the Minimum Bactericidal Concentration

The minimum bactericidal concentration (MBC) of samples was determined following the MIC assay. The lowest concentration that kills all the bacteria was taken as MBC. The samples without any growth were selected from wells and were poured into a Petri dish. Then, 15 mL of MHA were inserted and left to solidify. All the Petri dish were incubated at 35°C for 18–24 hours. The plate which had no bacterial growth was recorded as the MBC.²⁷

Checkerboard Assay

The FIC measurement was evaluated to determine the

interaction of the nettle alcoholic extract with the silver nanoparticles as well as the interaction of the shallot alcoholic extract with the silver nanoparticles against the selected ATCC (standard) and one of the resistant isolates (A6). The reaction of these compounds was evaluated using the checkerboard method and Sum FIC index.²⁵

Fifty microliters of the mixture of shallot extract with the silver nanoparticles were added to each well of the microplate. A hundred microliters of bacterial suspension (1.5×10^8 CFU/mL) was added in each well. Nettle extract was diluted along the vertical axis of the micro-dilution plate, and the silver nanoparticles plate was diluted along the horizontal axis serially. Each microplate contained the positive and negative control. The positive control wells contained bacterial suspension and Muller Hinton broth, and the negative control wells contained different dilutions of (combination of the nettle extract with the silver nanoparticles) and the Muller Hinton broth without bacteria. The microplate was incubated in an incubator with the shaker at 37°C for 24 hours. All the steps were also repeated for the combination of the shallot extract with the silver nanoparticles. The following formula was used to determine the interaction of the plant extract with the silver nanoparticles (FIC):

$$\text{Sum FIC}_{BC} = \frac{\text{MICB in combination}}{\text{MICB alone}} + \frac{\text{MICC in combination}}{\text{MICC alone}}$$

Sum FIC_{BC}: The total differential inhibitory concentration of the extract and the silver nanoparticles

B: Alcoholic extract

C: Silver nanoparticles

MIC_B: The minimum inhibitory concentration of the alcoholic extract

MIC_C: The minimum inhibitory concentration of the silver nanoparticles

The values below 0.9 show the synergism effect, the values between 0.9-1.1 show the additive effect and the values higher than 1.1 show the antagonistic effect.²⁸

Results

Antibiogram Test

All the samples were identified by a microscopic test. The susceptibility of *A. baumannii* isolates to antibiotics was studied using the antibiogram test. The results of the antibiogram test for some isolates are shown in Table 1. In this study, MDR *A. baumannii* isolates were resistant to at least 3 different classes of antibiotics for example meropenem, ceftazidime and trimethoprim-sulfamethoxazole.

Evaluation of the Produced Silver Nanoparticles

The UV-Vis spectrum was recorded by Perkin-Elmer Lambda 25 UV-Vis spectrometer (Figure 1). The silver nanoparticles peaked at 420 nm confidence.²⁹ The FE-SEM images were recorded on a Hitachi S-4160 instrument using a gold film for loading the dried particles on the instrument. The results have been shown in Figure 2. As shown in the FESEM image, the silver nanoparticles have a spherical shape and their average size is 50-80 nm in diameter. XRD analysis was carried out

Table 1. The Results of Evaluating the Antibiogram test for *A. baumannii* Isolates

Antibiotic	Acinetobacter Isolates					
	ATCC	AR	A6	A81	A223	A194
Amikacin (AN30)	S	R	R	S	R	R
Meropenem (MEN10)	S	R	R	R	R	R
Trimethoprim-Sulfamethoxazole (SXT)	S	R	R	R	R	R
Ceftazidime (CAZ30)	S	R	R	S	R	R
Gentamycin (GM10)	S	R	R	S	R	R
Imipenem (IMI10)	S	R	R	R	R	R
Piperacillin-Tazobactam (PTZ110)	S	R	R	R	R	R
Ampicillin-Sulbactam (SAM)	S	R	I	R	R	R
Colistin (CL10)	S	R	R	R	S	S

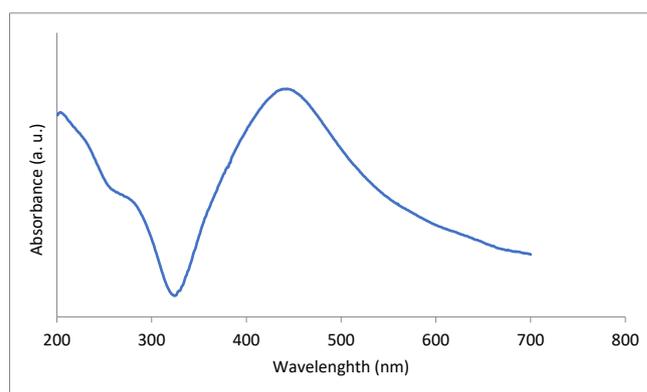


Figure 1. UV-Vis Spectrum of As-synthesized Silver Nanoparticles.

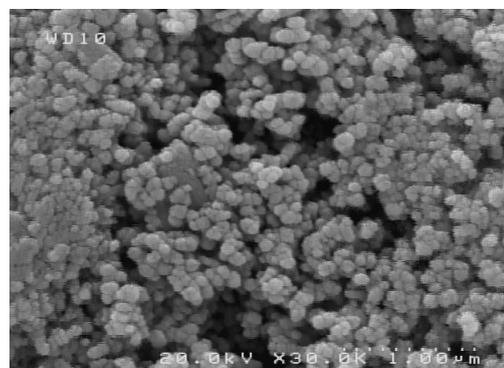


Figure 2. FESEM Image of As-Synthesized Silver Nanoparticles.

on a Rigaku D/max 2500 V diffractometer equipped with the graphite monochromator and Cu target. Figure 3 shows the XRD pattern of the as-synthesized silver nanoparticles. The strong intensity and no additional peaks in the pattern indicated the high crystallinity and purity of the product. Furthermore, all the diffraction peaks are in correspondence with a cubic structure with the JCPDS reference code 01-087-0597.

Well Diffusion

The diameter of the inhibition zone of the nettle extract, shallot extract and the silver nanoparticles against *A. baumannii* isolates have been presented in Figure 4. The maximum size

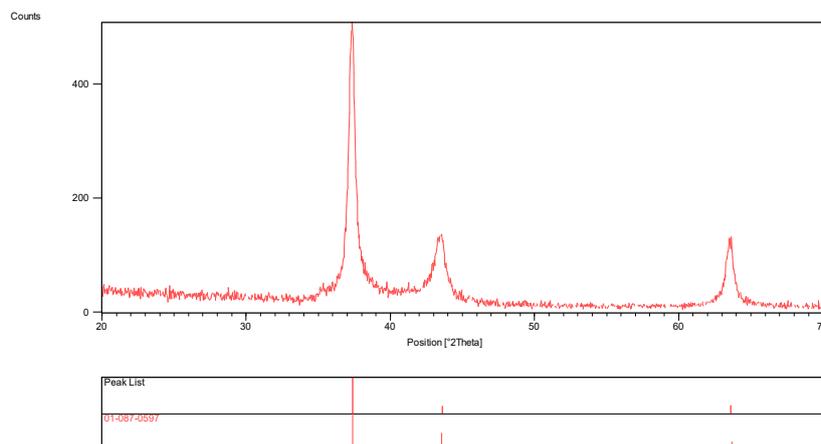


Figure 3. XRD Pattern of As-synthesized Silver Nanoparticles.

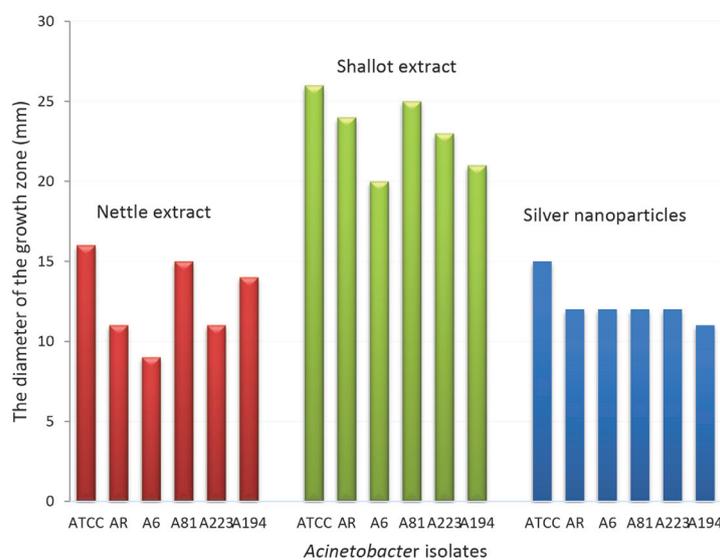


Figure 4. The Results of Evaluating the Antibacterial Effect Using the Well-Established Methods.

of the inhibition zone was seen on the *A. baumannii* ATCC sample. The diameter of the inhibition zone for the nettle extract, shallot extract and the silver nanoparticles was 16, 26 and 15 mm, respectively. The minimum size of the inhibition zone was seen on the A6 sample. The diameter of the inhibition zone for the nettle extract, shallot extract and silver nanoparticles plates was 9, 20 and 11 mm respectively.

MIC and MBC Results

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) results of the nettle alcoholic extract, the shallot alcoholic extract and the silver nanoparticles have been shown in Table 2.

Checkerboard Test

The FIC index was calculated using formula 1 to determine the kind of interaction. In this study, the amounts of differential inhibitory concentration were determined through the modified dilution checkerboard method. The resulted FIC values for the nettle and the shallot alcoholic extracts have

been presented in Tables 3 and 4. The interaction of nettle extract and silver nanoparticles had a synergistic effect and the interaction of shallot extract and silver nanoparticles had additive effects.

Discussion

The spread of the multi-drug resistant pathogens is a great threat in the therapeutic system all over the world. The extensive consumption and inappropriate prescription of antibiotics leads to bacterial resistance. Today, the spread of multi-drugs resistant bacteria in the ICU is increasing significantly. Among these multi-drug resistant bacteria, *A. baumannii* is one of the most important factors for creating hospital infections, especially in the ICU.⁵

Silver nanoparticles as well as different combinations with the base of silver show extensive antibacterial effects against pathogens.³⁰ These substances prevent the colonization of bacteria on prosthetics, catheters, etc.²¹ Silver nanoparticles make changes in morphology and permeability in the bacterial membrane by attaching to the proteins of the

Table 2. The Results of the MBC and the MIC of the Nettle Alcoholic Extract and the Silver Nanoparticles and the Shallot Alcoholic Extract

Sample	Nettle MIC (mg/mL)	Nettle MBC (mg/mL)	Silver Nanoparticles MIC (mg/mL)	Silver Nanoparticles MBC (mg/mL)	Shallot MIC (mg/mL)	Shallot MBC (mg/mL)
ATCC	1.81	3.62	0.65	1.3	1.4	2.81
AR	3.62	7.25	0.65	1.3	2.81	2.81
A6	3.62	3.62	0.65	1.3	2.81	2.81
A81	3.62	7.25	0.65	1.3	2.81	2.81
A223	3.62	3.62	0.65	1.3	2.81	2.81
A194	3.62	3.62	0.65	1.3	2.81	2.81

Table 3. The Interaction of the Nettle Alcoholic Extract With the Silver Nanoparticles

Isolates	FIC of the Nettle Extract	FIC of the Silver Nanoparticles	FIC _{indexA}	The Kind of Interaction
ATCC	0.5	0.0625	0.5	Synergism
A6	0.25	0.25	0.5	Synergism

Note: The FIC_{indexA} represents the total differential inhibitory concentration of the nettle extract and the silver nanoparticles.

Table 4. The Interaction of the Shallot Alcoholic Extract With the Silver Nanoparticles

Isolates	FIC of the Nettle Extract	FIC of the Silver Nanoparticles	FIC _{indexB}	The Kind of Interaction
ATCC	0.5	0.5	1	Additive
A6	0.5	0.5	1	Additive

Note: The FIC_{indexA} represents the total differential inhibitory concentration of the shallot extract and the silver nanoparticles.

surface of the bacterial membrane. They also inhibit the DNA synthesis that lead to the cellular death.^{31,32} Karimi-Poor et al showed that silver nanoparticles have antibacterial effect against *A. baumannii* and it can prevent the growth of this bacterium.³³ Behdad et al demonstrated that the silver nanoparticles have an effect on the efflux pumps of *A. baumannii* and through this process it can destroy the cell membrane of *A. baumannii*.³⁴ The results of this study were in consistency with the above mentioned studies. Actually, the synthesized silver nanoparticles can inhibit the growth of *A. baumannii* isolates and lead to the bacterial death. The effect of silver nanoparticles on *A. baumannii* isolates showed that the maximum diameter of growth inhibition zones in ATCC isolate was 15 mm and the minimum diameter of growth inhibition zones in A194 isolate was 12 mm. The minimum inhibitory concentration was 0.65 mg/mL in all isolates, and the minimum bactericidal concentration was 1.3 mg/mL in all isolates.

One medicinal plant which is used in traditional medicine is the Iranian shallot with the scientific name of *Allium hirtifolium* Boiss and has different properties including antibacterial property.¹⁴ A research conducted by Hasani et al showed that the shallot essence has a high potential to destroy *Listeria monocytogenes*.³² Alebrahim-Dehkordy et al demonstrated that the shallot extract had an effective antibacterial property against *Enterococcus faecalis* which was resistant to penicillin. In this study, the shallot extract had an effective antibacterial property against both standard and resistant isolates.³⁵ The maximum size of growth inhibition zones in the ATCC isolate was 26 mm and the minimum size of growth inhibition zones in the A6 isolate was 20 mm. The minimum inhibitory concentration of shallot extraction on ATCC isolate was 1.4 mg/mL, and was 2.81 mg/mL

in the other resistant isolates. The minimum bactericidal concentration was 2.81 mg/mL in all isolates. The results indicated that the shallot extract had an antibacterial effect against both standard and resistant *A. baumannii* although it had a better effect on standard isolate.

A research conducted by Modaresi et al showed that the nettle alcoholic extract has an inhibitory effect on the bacterial growth of *Vibrio parahaemolyticus* and *Bacillus cereus*.³⁶ In another study, the antibacterial effect of this extract was seen on clinical resistant gram-positive and gram-negative bacteria such as *Staphylococcus epidermidis* and *Escherichia coli*. The alcoholic extract of nettle destroys the integrity of the cell wall of bacteria and finally leads to the bacterial death.¹⁵ This study also showed that the alcoholic extract of the nettle has antibacterial property on standard and resistant *A. baumannii* isolates. The best effect was seen on ATCC isolate and the least effect was seen on resistant A6 isolate. The diameter of growth inhibition zones was 16 mm and 9 mm respectively. Nettle extract inhibits the growth of all *A. baumannii* isolates. It can inhibit the growth of ATCC isolate in the concentration of 1.81 mg/mL and the other resistant isolates in the concentration of 3.62 mg/mL. The MBC of nettle extract against ATCC, A6, A223, and A194 isolates was 3.62 mg/mL and was 7.25 mg/mL for the AR and A81 isolates. In general, the extract of shallot against *A. baumannii* isolates was more effective than the extract of nettle.

Recently, the combination of different drugs has been used for the treatment of many infectious diseases to reduce toxicity and increase medicinal effect against resistant bacteria.³⁷ A study which has been conducted in 2014 showed that the combination of *Drosera binate* extract with the silver nanoparticles was more effective compared to singularly using the silver nanoparticles or *Drosera binate*

extract against resistant *Staphylococcus aureus* isolates.²² In another research, the antimicrobial property of the oil essence of *Mentha piperita* with the silver ions against *S. aureus*, *E. coli*, and *Candida albicans* was investigated. The antibacterial synergism activity was found between the metal ions which has hydrophobic property, and oil essence which had hydrophobic activity.³⁸ Smekavola et al investigated the antibacterial property of the combination of some antibiotics with the silver nanoparticles. Their results showed that some bacteria which were intrinsically resistant to specific antibiotics could become susceptible to them through the use of the combination of these antibiotics with silver nanoparticles.³⁶ The results of these studies were in consistency with the results of the present study. In this study, the checkerboard method was used to measure the synergism effect of these two compounds and it was determined that the combination of silver nanoparticles with the nettle alcoholic extract against ATCC and resistant *A. baumannii* isolates had a synergistic effect. The combination of silver nanoparticles with the shallot alcoholic extract against ATCC and resistant *A. baumannii* isolates had the additive effect. It seems that the extracts of nettle or shallot can make changes on the bacterial cell wall. Then the permeability of the cell wall increases and the silver nanoparticles can enter through it. Finally, the silver nanoparticles inhibit the cell division cycle by inhibiting the DNA synthesis.

Conclusions

It seems that the rise of multidrug-resistant bacteria has led to an increase in the mortality of patients, hospitalization time and expenses. Due to the rise of antibiotics resistance, it is necessary to find some accessible and affordable substances with minimum side effects. The medicinal plants which have different properties have attracted the attention of scientists from long time ago. Using these natural resources in combination with nanobiotechnology products can provide more effective drugs against multidrug resistant bacteria.

Authors' Contributions

SM and ASTB designed and supervised the research, SZA and MAK performed the experiment and wrote the manuscript. SM and ASTB made the final revising of the manuscript. All the authors approved the final revision of the manuscript.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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