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Antibacterial Activity of Silver Nanoparticles and Their Combination with *Zataria multiflora* Essential Oil and Methanol Extract

Shirin Sheikholeslami,[1] Seyyedeh Elaheh Mousavi,[2,3] Hamid Reza Ahmadi Ashtiani,[4] Seyed Reza Hosseini Doust,[1] and Seyed Mahdi Rezayat[2,3,*]

[1]Department of Microbiology, Faculty of Advanced Sciences & Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, IR Iran
[2]Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran
[3]Department of Pharmacology & Toxicology, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, IR Iran
[4]Department of Basic Sciences, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, IR Iran

[*]*Corresponding author*: Seyed Mahdi Rezayat, Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, P.O. Box: 13145-784, Tehran, IR Iran. Fax: +98-2166402569, E-mail: rezayat@tums.ac.ir

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Abstract

Background: Against a variety of antimicrobial resistant pathogens, the scientists attempted substitution of antimicrobial medicine with various nanoparticles and plant-based antibacterial substances.
Objectives: The aim of this study was to assess the antibacterial effects of silver nanoparticles solely and in combination with *Zataria multiflora* essential oil and methanolic extract on some photogenic bacteria.
Methods: Minimum inhibitory concentrations (MICs) and fractional inhibitory concentrations (FICs) of plant essential oil, methanolic extract, and silver nanoparticles against bacteria were evaluated using the broth microdilution method and check board microtiter assays.
Results: The results of the experiment showed that the MIC and minimal bacterial concentration (MBC) values of Ag-NPs against all strains were in the range of 15.625 - 500 μg/mL, and values for the essential oil and plant extract were in the range of 1.56 - 100 mg/mL.
Conclusions: Silver nanoparticles were observed to have additive effects with essential oil against *Staphylococcus epidermidis* and *S. aureus*. The obtained results suggest the need for further investigations of the antibacterial effects of the combination of silver nanoparticles with other plant extracts and essential oils.

Keywords: Nanoparticles, Silver, Antibacterial Susceptibility, Essential Oil, Plant Extracts, Bacteria

1. Background

The wide spectrum of nanotechnology plays a significant role in major areas of the biological sciences. Nanotechnology deals with the investigation of nano-sized materials (1). Synthesis of nano-sized medicinal components with characteristic chemical and physical qualities is of great importance in the advancement of new pharmaceutical products (2). Because of their antimicrobial activity, the uses of metal ions such as those of silver have been examined for a long time. Such activities are generally attributed to oligodynamic action (3).

Silver nanoparticles (Ag-NPs) have a broad range of uses in the biomedical sciences, including antibacterial effects, treatment of burns, and targeted drug delivery (4). Silver nanoparticles are known for their higher surface to volume ratio and smaller size in comparison to common metallic silver, which permits them to interact closely with bacterial membranes, partially because of the diffusion of silver ions in solution (5).

Zataria multiflora Boiss (Avishan-e-shirazi in Persian) belongs to the *Laminaceae* family, which grows wild in the central part of Iran (6, 7). This plant is traditionally utilized as a spice in a variety of Iranian foods; it has also been applied as a diuretic agent, analgesic, antiseptic, and an antispasmodic, as well as in traditional folk remedies for the treatment of premenstrual pain, jaundice, sore throat, asthma, and edema (7). The antibacterial activity of *Z. multiflora* has been shown against a number of Gram-positive and Gram-negative bacteria (8). This plant has positive effects in controlling some microbial diseases because of its antibacterial, antifungal, and anti-inflammatory properties, as well as its immunostimulation activity in humans and in some animal models (9).

Staphylococcus aureus is a normal bacterium found on human skin, but when it enters in the body, it can

cause skin infections, such as cellulitis, furuncles, and impetigo. It is also responsible for nosocomial infections (10). Some of the life-threatening diseases produced by *S. aureus* include bacteremia, pneumonia, osteomyelitis, endocarditis, empyema, sepsis, scalded-skin syndrome, and toxic shock syndrome (11). Methicillin-resistant *S. aureus* (MRSA) exhibits a broad range of resistance against penicillin and other β-lactam antibacterial drugs. Patients suffering from MRSA may need antibiotics that are less toxic and more potent for the treatment of infections with drug-resistant microorganisms. This group of microorganisms has led to serious concern in human medicine (12).

Staphylococcus epidermidis is a part of the skin's normal flora, but it is also an opportunistic pathogen that exploits immunodeficiency in the host's innate defenses. It causes nosocomial infections associated with catheters and other foreign bodies (13). *Pseudomonas aeruginosa* is an environmental bacterium with minimal nutritional requirements for survival. It is an opportunistic pathogen in humans and causes nosocomial infections, fatal infections in patients with compromised immune defense, cystic fibrosis, burns, and hosts with cancer (14).

2. Objectives

In this study, we determined the antibacterial potential of silver nanoparticles and that of their combination with essential oil and methanolic extract of *Z. multiflora* against Gram-positive and Gram-negative bacteria.

3. Methods

3.1. Silver Nanoparticles

A stock solution of commercially available water soluble Ag-NPs (~ 40 nm) was procured from Nano Lotus Pasargad, Inc. (Tehran, Iran) with the trade name LNP-CS.

3.2. Plant Material

The aerial parts of *Z. multiflora* were collected from Isfehan, Iran, and the taxonomic identification of plant materials was confirmed by a senior plant taxonomist. A voucher specimen of the plant was deposited at the herbarium of the faculty of pharmacy at the Tehran University of Medical Sciences under number PMP-404.

3.3. Preparation of the methanol extracts

Aliquots of dried powder of the plant were extracted with 85% methanol using percolation for 48 hours and filtered with cloths. The methanolic extract was concentrated by a rotary evaporator apparatus, and the methanol was removed to produce extracts. The extracts were kept in clean vials in a dark, cool place for further tests (15).

3.4. Essential Oil Preparation

The plant was cut into small pieces (100 g) and exposed to hydrodistillation for six hours using a Clevenger type apparatus. The oil was collected and dried using anhydrous sodium sulfate and stored in a tightly closed dark vial at +4°C until use. The essential oil was prepared by hydrodistillation, and the major oil components were analyzed by a combination of capillary gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS) (16, 17).

3.5. Bacterial Strains

Strains of the following bacteria were purchased from the institute of standard and industrial research of Iran: *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Staphylococcus epidermidis* (ATCC 14990), and Pseudomonas aeruginosa (ATCC 27853). Bacterial strains were grown overnight on Mueller-Hinton agar (Merck, Germany) plates at 37°C before use.

3.6. Determination of Minimum Inhibitory Concentrations

The minimum inhibitory concentration (MIC) values of Ag-NPs, oil, and extract were determined by broth microdilution assay. The Ag-NPs were serially diluted two-fold with deionized water in concentrations ranging from 5.00 to 7.812 μg/mL. The oil and extract were serially diluted two-fold with 10% dimethyl sulfoxide (DMSO) (Merck, Germany) containing 1.00 - 1.56 mg/mL of oil. After shaking, 100 mL of diluted Ag-NPs, oil, and extract was added to each well of 96-well microtiter plates. Cation-adjusted Muller-Hinton broth (Merck, Germany) was used as the broth medium. Microbial suspensions were adjusted to 0.5 MacFarland and diluted to 1×10^6 CFU/mL, then 100 mL of the suspension was added to each well and incubated at 35 ± 2°C for 24 hours. MIC values were determined as the lowest concentration of compound that inhibited bacteria after 24 hours (17, 18).

3.7. Determination of Minimum Bactericidal Concentrations

After MIC determination, aliquots of 50 μL from all wells that showed no bacterial growth on Mueller-Hinton agar (Merck, Germany) plates were incubated at 35 ± 2°C for 24 hours. The minimal bacterial concentration (MBC) endpoint was defined as the lowest concentration of antimicrobial agent that killed 100% of the initial bacterial population (18).

3.8. Check Board Microtiter Assay

Eight serial, two-fold dilutions of Ag-NPs and oil/extract were prepared and used in the MIC tests. Fifty μL of each dilution of oil/extract was vertically added to the wells of the 96-well microtiter plates, and 50 μL of Ag-NPs dilution was added horizontally to the wells of the 96-well microtiter plates. One hundred μL of microbial suspension (10^6 CFU/mL) was added to each well and incubated at 35 $\pm$ 2°C for 24 hours. Fractional inhibitory concentrations (FICs) were calculated using the MIC of the combination of Ag-NPs and oil/extract divided by the MIC of Ag-NPs or oil/extract alone. Our interpretation of the FIC results, according to the accepted criteria, is as follows: if the FIC index is $\leq$ 0.5, the combination is interpreted as being synergistic; if the FIC index $>$ 0.5 and $\leq$ 1.0, the combination is interpreted as additive (19); if the FIC index is between 1 and 4, the combination is interpreted as indifferent; and if the FIC index is $>$ 4, the combination is interpreted as antagonistic (20).

4. Results

Four different strains of bacteria *S. aureus*, MRSA, *S. epidermidis*, and *P. aeruginosa* were used to evaluate the possible antibacterial activity of silver nanoparticles, *Z. multiflora* essential oil, and methanolic extract. The silver nanoparticles, oil, and methanolic extract exhibited antibacterial activity against all strains with the MIC and MBC values shown in Table 1. The MIC and MBC values of Ag-NPs against all strains were observed in the range of 15.625 - 500 μg/mL, and those of the essential oil and plant extract were in the range of 1.56 - 100 mg/mL. In comparison with all bacterial strains, the MIC and MBC value of Ag-NPs against MRSA was found to be very high, but the MIC and MBC values of essential oil and plant extract against *P. aeruginosa* were also high. The lowest MIC and MBC values of Ag-NPs were against *S. epidermidis* and *S. aureus*; essential oil and methanol extracts of *Z. multiflora* also had the lowest MIC and MBC values against these bacteria.

The antimicrobial effects of silver nanoparticles in combination with essential oil and methanolic extract of *Z. multiflora* are shown in Tables 2 and 3. The combination of silver nanoparticles with essential oil against *S. epidermidis* and *S. aureus* caused an additive effect, as defined by FICI values of 0.6248 and 1, respectively. The combination of silver nanoparticles with plant extracts against *S. epidermidis* produced an additive effect, as defined by an FICI value of 1.

5. Discussion

The traditional use of plants as medicines provides the basis for indicating which essential oils and plant extracts may be useful for specific medical conditions. The antimicrobial properties of *Z. multiflora* extracts have been utilized in traditional medicine to overcome infections (21). *Z. multiflora* essential oils rich in carvacrol and thymol have gained importance for their antibacterial activity (22, 23). Sharififar et al. (2007) (24) reported that essential oil and methanolic extract of *Z. multiflora* are effective bactericides against a number of Gram-positive and Gram-negative bacteria. In our results, the MIC value of essential oil showed antimicrobial activity against *S. epidermidis* that was similar to that found by Sharififar et al. (24). In contrast, Saei-Dehkordi et al. (2010) (25) have reported that *Z. multiflora* essential oil exhibits inhibitory effects against *S. epidermidis* and *P. aeruginosa*. Rahman et al. (2010) (26) reported MIC and MBC values of methanolic extract of 2.344 mg/mL and 6.250 mg/mL, respectively for *S. aureus*. In our study, the MIC and MBC values of methanolic extracts against *S. aureus* were observed to be 1.56 mg/mL and 6.25 mg/mL, respectively. It is well known that the outer membrane of Gram-negative bacteria is primarily constructed from tightly packed lipopolysaccharide molecules, which provide an effective permeability barrier. Thus, these bacteria were the most resistant (27).

Reactive metal oxide nanoparticles have been shown to possess excellent bactericidal effects (28). Development of nanobiotechnology compounds is an important field that has potential applications in the fight against pathogenic bacteria. Silver ion and silver-based compounds, such as silver nanoparticles, are extremely toxic to microorganisms and demonstrate strong biocidal effects against microbial species because these are highly reactive species with a large surface area (29). In addition, a number of studies have demonstrated the antimicrobial activity of silver nanoparticles.

In this study, the MIC value of silver nanoparticles against *S. aureus* and *S. epidermidis* was 62.5 μg/mL, for MRSA it was 125 μg/mL, and for *P. aeruginosa* it was 15.625 μg/mL. Jain et al. (2009) (30) reported that silver nanoparticles (mean size of 16 nm) were an effective bactericidal against *P. aeruginosa* at concentrations of 6.25 μg/mL and at concentrations of 12.5 μg/mL for *S. aureus*. Ansari et al. (2011) (31) demonstrated that the values of MIC and MBC for silver nanoparticles (mean size 5 - 10 nm) against *S. aureus* and MRSA were in the range of 12.5 - 50 μg/mL and 12.5 - 100 μg/mL, respectively. The reported MIC results are lower than those obtained by us in the present study, which suggests that the antimicrobial activity of nanosilver may be influenced by particle size. Our results indicate better an-

$$FIC\quad of\quad Ag-NPs = \frac{MIC\quad in\quad combination\quad with\quad Z.multiflora\quad oil/extract}{MIC\quad of\quad Ag-NPs\quad alone} \tag{1}$$

$$FIC\quad of\quad Z.multiflora\quad oil/extract = \frac{MIC\quad in\quad combination\quad with\quad Ag-NPs}{MIC\quad Z.multiflora\quad oil/extract\quad alone} \tag{2}$$

tibacterial activity compared to the earlier work of Ayala-Nu-ez et al. (2009) (32) in terms of the MIC and MBC values of silver nanoparticles (size ~ 100 nm) against MRSA (1,800 μg/mL and 2,700 μg/mL, respectively).

In this study, the application of silver nanoparticles as an antimicrobial agent in combination with *Z. multiflora* essential oil and methanolic extract was investigated by growing *S. aureus*, MRSA, *S. epidermidis*, and *P. aeruginosa* on Mueller-Hinton agar plates. Bioactive essential oil or plant extracts supplemented with silver nanoparticles is a novel concept and could be beneficial (as a synergistic or additive interaction) or deleterious (as an antagonistic or toxic outcome). Thus, it may prove to be more effective than individual agents used as monotherapy (33).

This is the first report describing the antibacterial activity of silver nanoparticles combined with essential oil and methanolic extract of *Z. multiflora*. Our results confirm that these compounds exerted additive effects against *S. epidermidis* and *S. aureus* when silver nanoparticles were combined with essential oil. However, no significant difference in effects was observed against MRSA and *P. aeruginosa*. In addition, the combination of silver nanoparticles with *Z. multiflora* extracts demonstrated indifferent effects, except against *S. epidermidis*, against which the combination exhibited additive effects.

Many investigations have shown that metal nanoparticles combined with various antimicrobial agents have antibacterial effects. The enhanced or decreased activity and the extent of efficacy of silver nanoparticles in combination with various antibacterial agents also depends on the type of antibacterial components and bacterial strains used for study (34). The comparison of our results with other investigations indicates that antibacterial concentrations are different and dependent on size, shape, and mode of action (35).

The antibacterial properties of nanoparticles having a size between one and 100 nanometers are ascribed to their small size and increased specific surface area. It is reasonable to assume that the antibacterial effects of nanoparticles are dependent on their size (36). It appears that plant extract components could lead to the aggregation of silver nanoparticles, which may change the size and shape of the nanoparticles and thus greatly affect cell particle interactions. Large accumulation of particles can considerably prevent the effects of special particle size and shape on antibacterial activity. Taken together, reducing the colloidal stability of the nanoparticles caused a decrease of the concentration of effective silver in the broth medium (36, 37).

These results suggest that the additive effect of silver nanoparticles with *Z. multiflora* essential oil and methanolic extract can be used as effective growth inhibitors in various microorganisms, making them applicable to antimicrobial control systems. In light of this, although all antimicrobial agents do not have synergistic or additive effects with silver nanoparticles, it is necessary to conduct further investigations of other combinations of silver nanoparticles with natural antimicrobial agents in which the check board and time-kill methods are used to determine additive or synergy effects.

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Authors' Contribution: Shirin Sheikholeslami and Seyed Mahdi Rezayat contributed equally as co-first authors of this study.

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$$FIC\ \ index = FIC\ \ of\ \ Ag-NPs + FIC\ \ of\ \ Z.multiflora\ \ oil/extract \quad (3)$$

Table 1. Antimicrobial Activity of Silver Nanoparticles, Essential Oil, and Methanol Extracts of *Zataria multiflora*

Strains	Ag-NPs, μg/mL		*Z. multiflora* Oil, mg/mL		*Z. multiflora* Methanolic Extract, mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	62.5	125	3.125	6.25	1.56	6.25
MRSA	125	500	3.125	12.5	12.5	25
S. epidermidis	62.5	62.5	6.25	6.25	12.5	12.5
P. aeruginosa	15.625	31.25	12.5	50	50	100

Table 2. Antimicrobial Activity of Silver Nanoparticles in Combination With Essential Oil of *Zataria multiflora*

Strains	Agent	FIC	FICI	Interaction
S. aureus	Ag-NPs	0.5	1	Additive
	Essential oil	0.5		
MRSA	Ag-NPs	0.25	1.25	Indifferent
	Essential oil	1		
S. epidermidis	Ag-NPs	0.5	0.6248	Additive
	Essential oil	0.1248		
P. aeruginosa	Ag-NPs	1	3	Indifferent
	Essential oil	2		

Table 3. Antimicrobial Activity of Silver Nanoparticles in Combination With Methanol Extracts of *Zataria multiflora*

Strains	Agent	FIC	FICI	Interaction
S. aureus	Ag-NPs	1	3	Indifferent
	Methanolic extract	2		
MRSA	Ag-NPs	0.5	1.5	Indifferent
	Methanolic extract	1		
S. epidermidis	Ag-NPs	0.5	1	Additive
	Methanolic extract	0.5		
P. aeruginosa	Ag-NPs	1	1.125	Indifferent
	Methanolic extract	0.125		

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