Original Article

Green Synthesis of Silver Nanoparticles Using *Nasturtium officinale* L. Extract and Analysis of Their Antibacterial Activity Against Nosocomial Pathogens

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Abstract

Background & Objective: The green synthesis of the silver nanoparticles (AgNPs) is an eco-friendly and straightforward synthesis method. This study aimed to investigate the green synthesis of AgNPs using *Nasturtium officinale* leaf extract and analysis of their antibacterial activity against some nosocomial pathogens.

Materials & Methods: The obtained AgNPs were characterized using UV-visible spectroscopy, X-ray diffraction (XRD), and scanning electron microscope (SEM). In addition, the antibacterial activity of synthesized AgNPs and *N. officinale* leaf extract were performed against five bacterial strains *(Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa)* by agar disk diffusion assay and minimum inhibitory concentration test.

Results: The green synthesized AgNPs had significant antimicrobial activity against all studied bacteria. In addition, greater inhibition activity was observed against *E. coli* and *P. aeruginosa* rather than other bacteria.

Conclusion: The synthesized AgNPs revealed a special antibacterial effect against selected bacteria, compared to leaf extract of *N. officinale*. Identifying new antibacterial agents with profound efficacy against antibiotic-resistant opportunistic pathogens is essential. These data provide an eco-friendly and rapid green approach for AgNPs synthesis and the potential of AgNPs for use in drug development against nosocomial infections.

Keywords: Nosocomial pathogens, Silver nanoparticles, *Nasturtium officinale*

Introduction

Nosocomial infections are infections usually acquired after hospitalization and manifest 48-72 hours after admission to the hospital. For nosocomial infections, responsible pathogens include bacteria, fungi, and viruses. In opportunistic bacterial infections, common organisms include *Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas aeruginosa* (1, 2). Studies have shown that 5% –10% of hospitalizations in Europe and North America result in nosocomial infections, in Asia and Africa, it increased to 40% (3). Based on National Healthcare Safety Network with the Center for Disease Control (CDC) there are 13 types of nosocomial infection with 50 infection sites (4, 5). Antibiotic-resistant bacteria strains are considered

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a significant threat to health care systems worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common resistant Gram-positive bacterium responsible for serious nosocomial infections leading to death. MRSA is resistant to nearly all beta-lactam antibiotics, due to the expression of penicillin-binding protein 2a (PBP2a). PBP2a is persistent in the action of all Beta-lactam antibiotics. MRSA is now considered a community-related infection (CA-MRSA) due to the prevalence of antibiotic resistance (6, 7). Recently, nanoparticle-based approaches such as nanoliposomes and metal nanoparticles have been developed to overcome these problems. Nanoparticles are made up of dozens or hundreds of atoms or molecules and are found in a wide range of sizes (8, 9). Silver nanoparticles have engrossed a great deal of interest due to potential advantages in various research areas, especially pharmaceutical problems and biomedical applications, such as antimicrobial and anticancer activities (10, 11). Many studies have shown that surface active oxygen species formed on nanosilver are released in certain conditions and may have antimicrobial and antifungal activity (12, 13). Several techniques have been reported for the preparation of nanoparticles. The facet, well-defined shapes, sizes, and controlled synthesis of nanoparticles are crucial in their activity (14, 15).

Various physical and chemical processes have been reported for synthesizing AgNPs such as gamma irradiation, laser ablation, chemical reduction and synthetic biological techniques (16). Due to the growing need for safe, lower toxicity, environmental-friendly and better size controlling aspects, green synthesis of nanoparticles from various plant extracts and essential oils is the preferred path of nanoparticle preparation over various physical and chemical methods (17). Also, biosynthesis methods do not require high pressure, temperature, energy, or toxic and dangerous chemicals. Besides, plants represent highly stable nanoparticles compared to other techniques and are straightforward to measure (18). Medicinal plants have a great value for detecting medical drugs and people have been using them for various aims from the beginning of the history of humans (19, 20). *Nasturtium officinale* L. (Watercress) is a perennial herb native to Asia and Europe and belongs to the Brassicaceae family that can be grown around and in water (Figure1). The plant is used fresh or as an ingredient in different food (21). It contains vitamins especially A, B, and C, phenolic compounds, folic acid, minerals, fibers, proteins, iron, and calcium, and a high level of glucosinolates which is the highest concentration compared to all other vegetable plants, along with high carotenoids concentration, for example, beta-carotene and lutein (20, 21, 22). Traditionally, the plant has numerous medical uses, such as anti-diabetes, antibacterial, stopping hemorrhages, chest pain, purifying blood, anemia, iron deficiency, throat expectorant, gallbladder, bronchitis, tumors and tuberculosis (23, 24). Previous studies have displayed antioxidant, nephroprotective, anti-inflammatory, hepatoprotective, and anti-hyperlipidemia features of the watercress in vitro and in vivo conditions (20, 23, 24).

Figure 1. *Nasturtium officinale.*

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In the present study, a simple and rapid approach was applied for the green synthesis of AgNPs using *Nasturtium officinale* L. leaf extract. Furthermore, the antimicrobial activity of *N. officinale* leaf extracts and nanoparticles synthesized via leaf extracts were evaluated against common nosocomial pathogens.

Materials & Methods

Source of chemicals and bacteria

Ethanol, Antibiotics, Dimethylsulfoxide (DMSO), Silver nitrate ($AgNO_3$) were taken from Sigma-Aldrich (St Louis, MO, USA). Mueller Hinton broth (MHB) was obtained from Oxoid, Basingstoke, United Kingdom. Mueller-Hinton agar (MHA) was purchased from Merck, Germany. All other reagents were of analytical grade and used as received. All strains of bacteria were obtained from the Pasteur Institute of Iran.

Preparation of plant samples

Nasturtium officinale L. (Watercress) was collected from the mountains of Mako city in the West Azerbaijan province, Iran. Botanists identified the plant parts at the biology department, Islamic Azad University. Plant materials were washed thoroughly with distilled water to remove any earthy matter or remove the dirt and any other contaminations. The plant leaves were dried at room temperature under shade to retain their fresh green color and prevent the loss of active compounds. Dried leaves of *N. officinale* were powdered by a grain mill and kept in glass containers for further use.

Preparation of plant extract

The powder of *N. officinale* leaves was subjected to ethanol extraction whereby 30g of powder leaves were macerated in 300 ml of ethanol 80% for 72h at room temperature. The resulting mixture was filtered (Whatman No. 2) and then evaporated at 33°C using a rotary evaporator with an 80 rpm rotation speed. The stock solution is prepared in 10% dimethylsulfoxide (DMSO) and was stored at 4°C.

Synthesis of silver nanoparticles

6 ml of the plant extract and 40 ml of 10 mM aqueous silver nitrate solution were mixed with continuous stirring for 5 minutes at room temperature to synthesize silver nanoparticles. The colorless silver nitrate solution turns to brown color that reveals the formation of AgNPs. Silver nanoparticles were collected by centrifugation (13,000 rpm for 20 min) and dried at 40°C for two h, before evaluation of the characterization.

Characterization of silver nanoparticles UV–visible spectroscopic characterization of AgNPs

Silver ions (Ag^+) reduction to metallic particles (Ag^0) and formation of AgNPs were identified by measuring the UV-Vis spectrum using UV–visible spectrophotometer (JASCO V-670 Spectrophotometer) within the wavelength ranges of 300–700 nm (25).

X-ray diffraction analysis

The X-ray diffraction (XRD) analyses were recorded for AgNPs to evaluate the crystal and nano-structural of AgNPs. The spectra were obtained on a Philips PW-1730 X-ray diffractometer (XRD) in the range of $2\theta = 0°–110°$, using Copper Kα radiation.

Scanning electron microscopy

The surface characteristics and morphology of the prepared AgNPs were investigated by scanning electron microscopy (SEM) (XL30, Philips, Eindhoven, Holland).

Bacterial strains

For further use, all organisms were maintained on Mueller Hinton broth (MHB) containing 30% (v/v) glycerol at -20°C for further use. The antibacterial properties of ethanolic extracts of *N. officinale* leaves were evaluated using the following strains of bacteria, Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603),

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and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228).

Antibacterial Activity Assay

Bacterial suspensions equivalent to 0.5 McFarland (1.5×10^8 CFU/ml) were made in sterile normal saline solution. A sterile cotton swab was dipped into a test tube containing bacterial suspensions and then was cultured on the Mueller-Hinton agar (MHA). These plates were incubated for 24 h at 37°C.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Briefly, The extract and green synthesized nanoparticles MICs, separately, were determined using the broth microdilution assay following recommendations of the CLSI guidelines 2018 (26). The MIC test was carried out using the standard broth microdilution method. It was done in a sterile 96-well round bottom microtitre plate.

A total of 95 µl of MHB and 5 µl of every bacterial strain suspension were added to the 96 wells.100 µl of plant extract (800 mg/ml DMSO) was then added into the first wells of each column and serial dilution was prepared. A sterility control well and a growth control well were also studied for each strain. The same was done for green synthesized AgNPs (2000µg/ml MBH). The plate was incubated for 24 h at 37 ◦C. The last well which had no bacterial growth was defined as the MIC.

MBC values were determined using sub-culturing of bacterial suspension (10^8 CFU/mL) from the MIC tubes into Mueller-Hinton agar plates and then incubated at 37°C for 24h. The diameter of the growth inhibition zone (mm) surrounding the disks for plant extracts, AgNPs and antibiotics showed the inhibitory effect.

Disk Diffusion Assay

The antibacterial activities of both ethanolic extract and green silver nanoparticles synthesized were tested for antibacterial properties against bacterial strains and then compared with antibiotics (Tetracycline, Erythromycin, Chloramphenicol, and Ciprofloxacin) using the agar disk diffusion techniques according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2018 (26). They are the most common antibiotics in treating nosocomial infections (27-29). Briefly, each strain was spread on a sterile Mueller-Hinton agar plate using a sterile cotton swab. For testing the antibacterial properties of extract, the sterile 6 mm blank paper disks (Padtan Teb Inc., Tehran, Iran) were loaded with 50 µL of filter-sterilized plant extracts and four disks with antibiotics as the positive control, and the corresponding volume of ethanol was used as the negative control (30, 31). For evaluation of the antimicrobial property of synthesized nanoparticles, the sterile paper disks were impregnated with 50 µL of AgNPs (62.5µg/ml), and four disks with antibiotics and one disk were as negative control. After 24 h of incubation at 37°C, the inhibitory zone was measured for extract, AgNPs, and antibiotics. For each sample, three replicate trials were conducted against each organism.

Statistical Analysis

The statistical analyses were conducted using SPSS statistics applying the student's t-test and presented as mean ± SD. A p-value less than 0.05 was considered statistically significant.

Result

Synthesis of AgNPs

The formation of AgNPs was visually indicated by color change (from light yellow to dark brown) in the mixtures of silver nitrate solution and leaf extract (Figure 2). The confirmation of NPs synthesis was characterized by UV-vis spectroscopy, XRD, and SEM.

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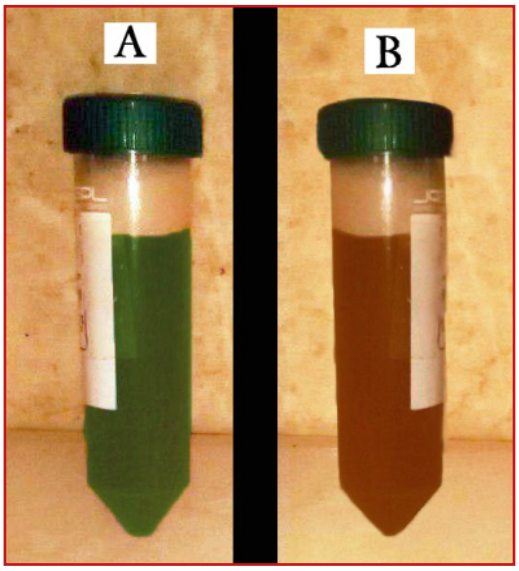

Figure 2. Visual observation of synthesis of AgNPs. A) *N. officinale* leaf extract, B) *N. officinale* leaf extract treated

UV-visible Spectroscopy

The UV–visible spectroscopic analysis is one of the valuable and reliable techniques to confirm the formation of nanoparticle synthesis (18). A broad absorption peak at around 421.5 nm confirmed AgNPs were successfully gained by plant extract (Figure 3, A)

X-ray diffraction analysis

X-ray diffraction (XRD) proved the crystalline nature of silver nanoparticles. The XRD patterns for synthesized AgNPs revealed diffraction peaks at 2θ degrees = 38.31, 44.43, 64.73, and 77.74 corresponding to (111), (200), (220), and (311) reflections, respectively (Figure 3, B).

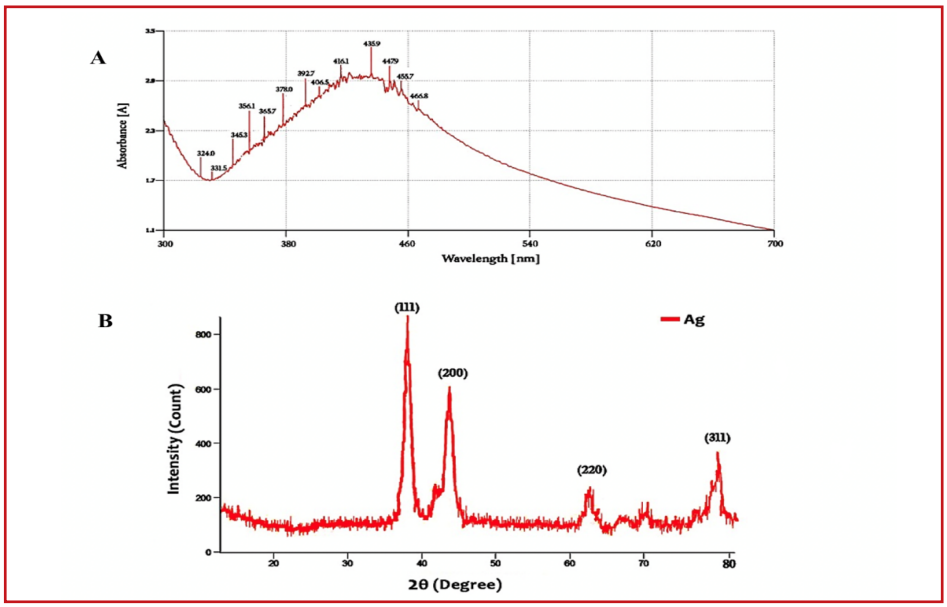

Figure 3A. UV-visible absorption spectrum of AgNPs synthesized using *N. officinale* leaf extract treated with 10mM $AgNo_3$.

B. XRD pattern of AgNPs synthesized by *N. officinale* leaf extract

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Scanning electron microscopy analysis of silver nanoparticles

The morphology and particle size of the AgNPs were evaluated by scanning electron microscopy. This technique magnifies the image by using electrons instead of light. The high-density AgNPs synthesized by treating *N. officinale* extract are observed in the SEM image. The average silver nanoparticle size was 22.64 nm and had a spherical shape (Figure 4, A).

Antibacterial Activity

Disk Diffusion Assay

A disk diffusion test was performed to evaluate the sensitivity of standard strains to plant ethanolic extracts and synthesized silver nanoparticles. For assessing the antibacterial activity of extract of *N. officinale* leaves, biosynthesis AgNPs, and antibiotics (Tetracycline, Erythromycin, Chloramphenicol, and Ciprofloxacin) on bacteria strains, diameters of inhibition growth zones were measured and the results are presented as shown in Table 1, Figure 4, B. and Chart 1.

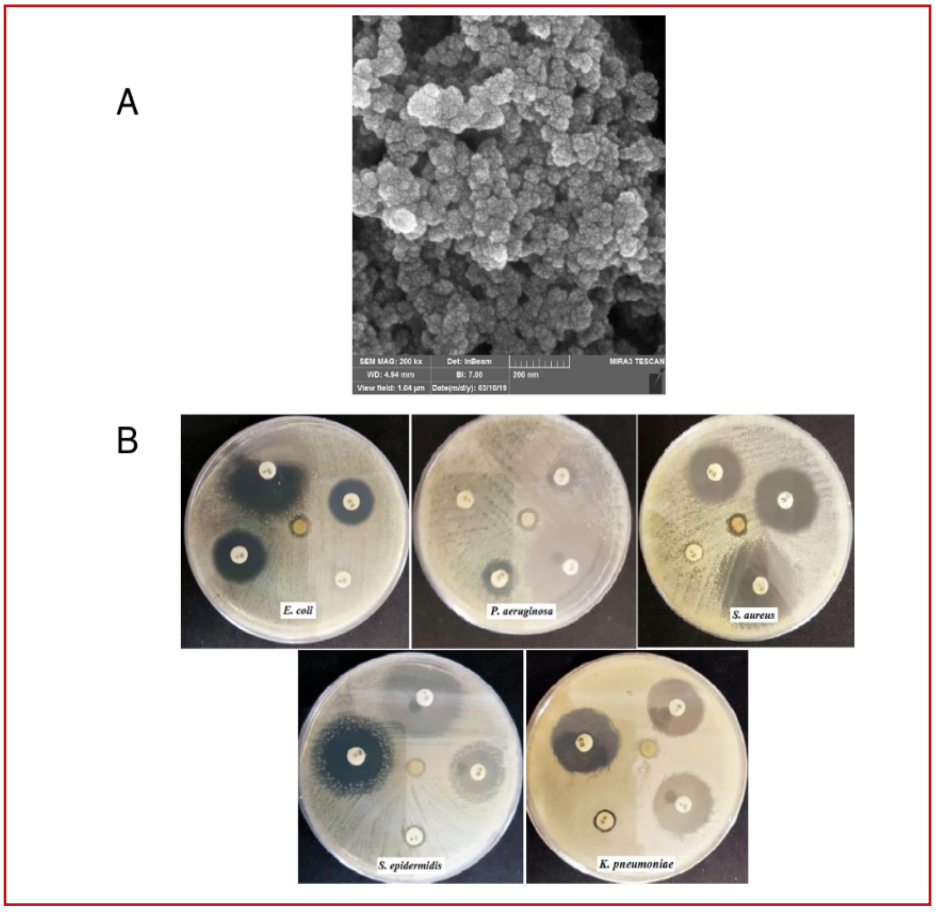

Figure 4. A SEM micrograph showed the morphology of synthesized AgNPs using N. officinale leaf extract. B. Antibacterial activities of silver nanoparticles *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus and Staphylococcus epidermidis* compared with antibiotics (Tetracycline, Erythromycin, Chloramphenicol, and Ciprofloxacin)

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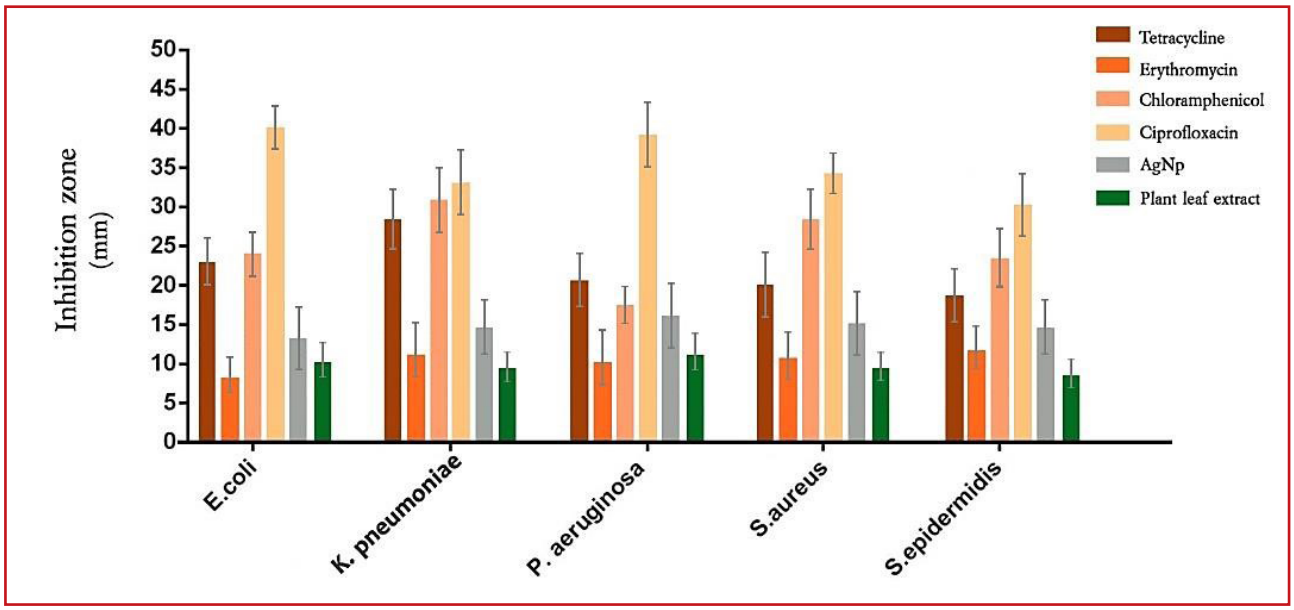

Chart 1. Comparative antibacterial analysis of *Nasturtium officinale* L. extracts with some common antibiotics based on the disk diffusion method

The disc diffusion assay showed *N.officinale* extract has antibacterial activities against all the tested bacteria. The highest activity of the plant extract was found against Gram-negative bacteria *P. aeruginosa* (11± 0.37 mm) and *E.coli* (10 ± 0.22 mm). The growth inhibition was moderately active against bacteria S. *aureus* (9 ± 0.34 mm), K. *pneumoniae* (9 ± 0.23 mm) and S. *epidermidis* (8 ± 0.16 mm).

The in vitro test for antibacterial activity revealed that AgNPs synthesis by *N. officinale* leaf extract inhibited all of the growth of Gram-negative and Gram-positive, with an inhibition zone of 13 ± 0.36 mm to 16 ± 0.32 mm. The positive control (Tetracycline, Erythromycin, Chloramphenicol, Ciprofloxacin) showed different inhibition zone depending on Bacteria strains (Table 1).

Table 1. Inhibition zone (IZ) of leaf extract of *Nasturtium officinale* and biosynthesized silver nanoparticles (AgNPs) against five pathogenic bacteria

Bacteria	Antibiotics	IZ of antibiotics(mm) (mean ± SD)	IZ of AgNPs (mm) (mean ± SD)	IZ of extract (mm) (mean ± SD)
Escherichia coli	TE ER CH CI	22 ± 0.23 8 ± 0.31 24 ± 0.44 40 ± 0.19	13 ± 0.36	10 ± 0.22
Staphylococcus epidermidis	TE ER CH CI	18 ± 0.41 12 ± 0.18 23 ± 0.43 30 ± 0.31	14 ± 0.25	8 ± 0.16

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Staphylococcus aureus	TE ER CH CI	20 ± 0.24 10 ± 0.41 28 ± 0.47 34 ± 0.34	15 ± 0.47	9 ± 0.34
Klebsiella pneumoniae	TE ER CH CI	28 ± 0.32 11 ± 0.37 31 ± 0.23 33 ± 0.25	15 ± 0.25	9 ± 0.23
Pseudomonas aeruginosa	TE ER CH CI	20 ± 0.21 10 ± 0.26 17 ± 0.36 39 ± 0.26	16 ± 0.32	11 ± 0.37

*TE: Tetracycline, ER: Erythromycin, CH: Chloramphenicol. CI: Ciprofloxacin

MIC and MBC of ethanolic extract and silver nanoparticles

MIC and MBC of each strain were specified for both biosynthesis AgNPs and *N. officinale* extract. MIC and MBC of the plant extracts were assessed against all the experimented bacteria (Table 2). To compare the effect of the plant extract and green synthesis nanoparticles, the MIC and MBC were remarkable. Table 3 showed that AgNPs exhibited the lowest MIC and MBC against *S. aureus* (62.5µg/ml), and plant extract had the lowest value for MIC against *S. aureus* and *S. epidermidis* (25mg/ml). Also, the highest MBC was related to *E. coli* with a concentration of 250 µg/ml for biosynthesis AgNPs.

Table 2. MIC and MBC (mg/ml) values of *Nasturtium officinale* ethanolic extract against studied bacteria

Bacteria	MIC(mg/ml)	MBC(mg/ml)
Escherichia coli	50	200
Klebsiella pneumoniae	50	100
Pseudomonas aeruginosa	50	100
Staphylococcus aureus	25	100
Staphylococcus epidermidis	25	200

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Table 3. MIC and MBC (µg/ml) of the AgNPs against selected bacteria

Bacteria	MIC(mg/ml)	MBC(mg/ml)
Escherichia coli	125	250
Klebsiella pneumoniae	125	125
Pseudomonas aeruginosa	125	125
Staphylococcus aureus	62.5	62.5
Staphylococcus epidermidis	125	125

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Discussion

Recently, the increase in opportunistic bacterial infections and the development of antibiotic-resistant infections associated with high mortality are becoming worrisome. Therefore, detecting novel antibacterial agents against opportunistic pathogens is urgently required (32). Advances in nanobiotechnology, novel applications of nanoparticles, and research on natural products toward detecting affective antibacterial agents have become extensive attention to the synthesis of nanoparticles using plants (18, 16). The chemical synthesis of AgNPs requires the use of harmful chemicals that may affect the environment and human health. The synthesis of nanoparticles using plants offers several advantages. It presents the best manner to synthesize silver nanoparticles due to the numerous active biomolecules such as alkaloids, phenolic acids, polyphenols, terpenoids found in plants (33, 34).

This study assayed the silver nanoparticles produced by,*N. officinale* leaf extract and their antibacterial effects. AgNPs from *N. officinale* were successfully synthesized. Silver nanoparticles display a brown color in an aqueous solution due to a surface plasmon excitation in AgNPs that agrees with previous studies (35, 36). Similar color changes have also been observed in different studies, confirming the chemical reaction between leaf extract and silver nitrate. Also, UV–Vis spectroscopy, SEM, and XRD analysis were carried out to characterize the nanoparticles. Following previous studies, the maximum absorption peak in the UV-Vis spectra around 421.5 nm confirms the formation of Ag nanoparticles (36, 37). The XRD pattern revealed the purity of synthesized AgNPs without additional diffraction peaks. The crystalline structure of synthesized AgNPs was

characterized through X-Ray comparison to standards. The spherical shape of silver nanoparticles was displayed by SEM image. The bioactive compounds found in plant extracts can be helpful for reducing metal ions and resulting in the synthesis of silver nanoparticles (38, 39). The SEM image displayed that the size of AgNPs synthesized was 16 - 35 nm. A similar result of the size of the biosynthesized silver nanoparticles by *N. officinale* extract has been reported in Sadeghi, (2014) study (40). The disk diffusion test and MIC / MBC assays were performed against both Gram-positive and Gram-negative bacteria to assess the potential of green nanosilver bactericidal effects. It should be attended that the antibacterial properties of AgNPs depend on their structure including the size, shape, colloidal state, preparation method, and the bacteria with which they interact (37, 38, 41). The exact mechanisms by which nanosilver performs antibacterial efficacy are still under verification. It was considered that AgNPs form pits on the bacterial cell wall surface leading to the demolition of the cell structures and damaging cell function (42-44). Besides, it seems ionic Adhesion of the AgNPs onto the bacteria cell wall and plasma membrane, owing to the interaction between positively charged Ag ions and negative surface charge of the cell membrane, causes structural variations in the membrane and the disorder of proton motive force and thus destruction of membrane. Moreover, silver nanoparticles by manufacturing free radicals upon contact with bacteria harm the membrane (45-48). The large sizes of zones growth inhibition produced by *N. officinale* extract, AgNps, and the four antibiotics (Tetracycline, Erythromycin, Chloramphenicol, and Ciprofloxacin) against all bacteria indicated the potency of the active constituents in *N. officinale*, silver nanoparticles, and antibiotics. Ethanolic extract of *N. officinale* revealed the highest zone of inhibition in *P. aeruginosa* and *E. coli*. The Gram-negative bacteria have extra tolerant against a broad range of antibiotics. The presence of lipopolysaccharides, mutations in porins and changing the hydrophobic properties in the outer membrane can cause this resistance (49-51). The extract gives a robust inhibitory effect against Gram-negative bacteria; this may demonstrate the more function of *N. officinale* extract specifically against the Gram-negative cell membrane and cell wall.

Synthesized AgNPs are effective against Gram-negative and Gram-positive bacteria with the highest inhibitory zone in *P. aeruginosa* (Chart 1). Some studies have shown that the formed AgNPs using plant extract were effective against negative and positive pathogens. Silver nanoparticles synthesis by *N. officinale* had appreciable antibacterial activity and the higher concentration was essential for growth inhibition of *E. coli*. (52-54). Compared to the antibiotics, the plant extract was more effective than Erythromycin on *P. aeruginosa* and *E. coli*. In contrast, synthesized AgNPs demonstrated a greater effect on all five bacteria strains than Erythromycin. Ciprofloxacin and Chloramphenicol had the greatest and lowest effect on all bacteria. The lowest MIC value was achieved for *S. aureus* exhibiting that both extract and AgNPs have the maximum toxicity to this bacteria (25mg/mL and 62.5 µg/mL, respectively). *S. epidermidis* is another sensitive Gram-positive microorganism to plant extract its MIC value was 25mg/ml. The results indicated AgNPs have considerably higher antibacterial activity than plant extract and 62.5 µg/ml was sufficient to inhibit *S. aureus* growth. Different amounts in MIC of extracts may be due to various chemical components and the volatile temper of the constituents (55). Several studies have shown the inhibitory effects of synthesized AgNPs on the Staphylococcus aureus, which was consistent with the present study's findings (56, 57). Mahdavi et al. (2019) showed that *E. coli* had a high resistance to *N. officinale* essential oil as this study revealed resistance *E. coli* to leaf extract (58). Also, another study revealed that natural hexanol in the plant extracts,

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including *N. officinale*, affected bacteria such as *S. aureus* and *E. coli* (59). This is one of the components in *N. officinale* extract that causes inhibiting the growth of bacteria.

Conclusion

The presence of adverse microorganisms in uman health has become a significant concern, due to the variety of infections and diseases. Thus, there is a need for better effective and nontoxic treatment options with more antimicrobial activity against infectious diseases, incredibly opportunistic bacterial infections and resistant strains. Plant-based silver nanoparticles (AgNPs) have specified antimicrobial properties that deem them fit for use as an alternative to antibiotics and have many advantages like cost-effectiveness, efficiency, safety and less toxicity to living organisms. The antimicrobial properties shown by the AgNPs reported here represent a great alternative to achieving this objective. The synthesis reported here provides a green and eco-friendly method to rapidly synthesize AgNPs through implementing Nasturtium officinale leaf extract at ambient temperature. The study's findings propose that the leaf extract of *N. officinale* is an appropriate reducing and capping agent for the biosynthesis of AgNPs. Initially, the observation of changing the color of Ag + solution from colorless to dark brown confirmed the formation of AgNPs. The synthesized AgNPs were characterized using UV-Visible, XRD and SEM analysis. In addition, the antibacterial properties of both ethanolic extract and nanoparticles obtained by extract were assessed against five tested bacteria by disc-diffusion and MIC/MBC methods. The results suggest the synthesized AgNPs are more effective than extract and low concentrations are required to kill both Gram-negative and Gram-positive bacteria. Thus, this rapid, environmentally friendly, and cost-effective method and significant antibacterial activity may potentially suggest using such nanoparticles in future drug development and the food industry. Nevertheless, research in pharmacological and toxicological studies, especially in vivo, is needed to develop and design future antimicrobial therapeutic agents for in vivo application.

Conflicts of interest

We declare that we have no conflict of interest.

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