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Original Article

Antibacterial Activity of Silver Nanoparticles Synthesized from *Citrus aurantium* and Its Synergistic Effect of the Combination of Silver Nanoparticles with Ampicillin against *Proteus mirabilis*

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Abstract

Background: *Proteus mirabilis* is one of the most infectious organisms that cause a various infections involving those of the skin, wounds, and urinary tract. This study describes the synthesis of silver nanoparticles (AgNps) and using it as an antibacterial agent. **Objective:** To determine the activity of AgNps and synergistic effect of combination of AgNps with ampicillin (AMP). **Materials and Methods:** Out of 205 samples, 32 isolated were identified as *P. mirabilis* and determine the inhibition zone by using AgNps and its combination with AMP. The AgNps were biosynthesized by Citrus aurantium and then characterized using Ultraviolet (UV)-visible spectroscopy, X-ray diffraction (XRD) and transmission electron microscope (TEM). The antibacterial activity of AgNps was done using well diffusion agar method and the activity was evaluated by calculating the increase in the inhibition zone and reduction in the bacterial growth. **Results:** The synthesized AgNps was first characterized by UV-visible spectroscopy which showed a peak between 400 and 500 cm^{-1}. The color changes from clear yellow to red or brown color indicate the formation of AgNps. The particle size of AgNps was calculated and found to be 21.24 nm by XRD. The TEM images showed the presence of very regular particles in shape of nanospheres with a particle size in the range of 10–28 nm. Different concentrations (25, 50, 75, 100 µg/mL) were used for determining the activities of AgNps and the combination of AgNps with AMP, and the result showed that the most efficient in inhibition zone was at concentration 100 µg/mL. **Conclusion:** This study concluded that the green synthesized AgNps from *C. aurantium* leaves extract have most potential as antibacterial against *P. mirabilis*.

Keywords: AgNps, *Citrus aurantium* extract, *Proteus* mirabilis, synergistic effects

Introduction

A member of the *Enterobacteriaceae* family, *Proteus mirabilis* is a Gram-negative bacterium with the capacity to change shape and move in swarms by using its flagella. It can cause wounds, burns, and urinary tract infections in addition to other infections.[1,2] It is a significant opportunistic bacterium that is present in soil, water, and the intestines of both humans and animals.[3,4] It has a numerous virulence factors that help in adhesion, bacterial growth, colonization, penetration into infected tissues, and developing of pathogenesis. Flagella, capsules, fimbriae, outer membrane proteins, lipopolysaccharides, the creation of biofilms, and various enzymes such as hemolysin, amino acid deaminase, and urease are among the virulence factors.[5,6]

The global health problem is the antibiotic resistance that limits the treatment options. There are numerous

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mechanisms which bacteria can resist antibiotics including: inactivation of antibiotic by bacterial enzyme, decreased antibiotic entry in to the bacteria, antibiotic efflux, as well as target site mutation.[7] Bacteria can develop multidrug resistance (MDR), which can cause ineffective antibiotic therapy and increase the risk of death and persistent infection.[8] As a result, it is crucial to develop new and effective antimicrobial treatments; one option to combat MDR organisms is the use of nanoparticles.[9]

Nanoparticles are very small particles with sizes ranging from 1 to 100 mm. Silver nanoparticles (AgNps) are currently used extensively in the medical, healthcare, and environmental fields. Antimicrobial activity of AgNps in medicine is the capacity to eliminate a variety of infections and MDR, particularly biofilms producing pathogens.[10]

Different methods, including physical, chemical, biological, and "green" synthesis, are used to produce nanoparticles. The green synthesis technique, particularly plant synthesis method, is the safest one, in which nontoxic substances are used, which are eco-environmentally friendly, economical, and renewable substances. So nowadays, green synthesis nanoparticles have gained considerable attention. For AgNps preparation, various types of plants are used.[11]

The AgNps displayed a multi-level mode of action on bacterial cells that can affect metabolic processes, disrupt cell walls and membranes, increase cell permeability, disrupt metabolic pathways with intracellular damage from AgNps, damaged biomolecules (DNA, proteins), and produce reactive oxygen species.[9]

AgNps with an average size of 10 nm or smaller exhibit electronic properties that significantly boost their bactericidal action.[12] This could also be related to the fact that as particle size reduces, reactivity increases as the surface area-to-volume ratio increases.[13] So, the current study aimed to synthesize AgNps and characterize it by using different techniques such as UV-visible spectroscopy, transmission electron microscope (TEM), and X-ray distraction (XRD). Also, this study aimed to investigate antibacterial activity of AgNps and synergetic effect of AgNps with ampicillin (AMP) against *P. mirabilis*.

Materials and Methods

Collection and isolation of clinical samples

Out of 205 were collected from different clinical source (urine, wound and burn swabs) from Kirkuk city hospitals from period of December 2021 to March 2022, 32 samples were identified as *P. mirabilis* by characteristic feature of the bacteria on culture media and using VITEK 2 compact system as a confirmatory test.

Preparation of crude extract from *Citrus aurantium* leaves

Citrus aurantium leaves were harvested and washed thoroughly with water and then washed with distilled water to remove any dust particles. The washed leaves were dried at 40ºC for 3 working days. The dried leaves were crushed and sieved for less than 220 µm. The crushed leaves were extracted using deionized water by adding 200 mL of deionized water to 20 g of crushed leaves. This mixture was then stirred for 1 h at 80ºC. The mixture was then decanted and centrifuged for 10 min at 12,000 revolution per minute. The aqueous extract was collected to use in the next step.

Synthesis of silver nanoparticles

Mixing 250 mL of deionized water with 2 g of silver nitrate and heating by hot plate and magmatic vortex to 40ºC. Following that, 10 mL of the leaf extract was added into the silver ion solution drop by drop. The mixture's color was subsequently adjusted to yellow. After 4 h of stirring at room temperature, the mixture's color changed to a deep red or brown, indicating the creation of colloidal AgNps. The obtained colloid samples were kept in dark bottle. The solutions' color was varied for 5 days, and both AgNps that produced from the mixing reactions and the active reduction of Ag+ show the brown or deep red color. The solutions color allowed examination the absorbance by UV-visible to confirm AgNps production.[14]

Characterization of silver nanoparticles

UV-visible spectroscopy initial characterizations of AgNps, including size estimate within the composition suspension were performed by UV-visible spectroscopy at center for advanced biotechnology and research (Shimadzu UV-visible 1900i, Shimadzu, Japan).[15]

X-ray distraction analysis of silver nanoparticles

Molecular and crystal structure analyses, crystallinity evaluation, isomorphous substitution, and particle size are all common applications of the analytical technique known as XRD, as well as quantitatively resolving chemical species and qualitatively recognizing distinct molecules.[16]

Transmission electron microscope imaging

TEM was used to investigate the AgNps' morphological characteristics, including size. On a copper grid covered with carbon, one drop of 52 g/mL AgNps was applied; later, it was allowed to settle for 5 min, and then allowed to air dry at room temperature.[16]

Antibacterial activity of silver nanoparticles and the combination of silver nanoparticles with ampicillin

Agar well diffusion method was used for determination of antibacterial activity of the prepared AgNps. The homogenous bacterial suspension (1.5×108 cfu/mL of *P. mirabilis*) swabbed using sterile cotton swab on Muller Hinton agar (MHA). The agar was punched with pasture pipette in 5 mm and different concentrations of AgNps, AgNps + AMP were added into each well in 0.1mL by using microtiter pipette. The agar plates were then incubated for 24h at 37°C, and the inhibition zones (in mm) were measured.[16]

Statistical analysis

Statistical analysis was carried out using SPSS (Statistical Package for the Social Sciences) version 23.0 (SPSS, IBM Company, Chicago, IL, USA). The responses with yes or no are used number and percentage by descriptive statistical analysis, Whereas, the mean and standard deviation, and the significant variance between values were done by analysis of variance test.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent from were reviewed and approved by a local ethics committee according to the document number 3657 (including the number and the date in 11/11/2021) to get this approval.

Results

Synthesis and characterization of silver nanoparticles

UV-visible spectroscopy of silver nanoparticles

UV-visible spectrophotometer is considered one of the most important techniques for studying the spectra of solutions of nanomaterials, specially the colored ones, the yellow color of leaf extract, when mixed with $AgNO_3$ turned dark brown after 24h, at the same time, no color change was detected in the control [Figure 1].

This color change is due to the reduction of Ag^+ (silver ion) to Ag^0 (AgNps) in the aqueous solution that occurs due to excitation of surface plasmon resonance (SPR) in AgNps. Initial diagnosis of nanomaterials, as it gives a characteristic peak of SPR within a specific area which is strongly dependent on the morphology of nanoparticles. Accordingly, this measurement was carried out, which gave a peak at the site 442 cm^{-1} [Figure 2], which proves the validity of the reaction of forming AgNps.[17,18]

Figure 1: Green synthesis of AgNps and changes in color

X-ray distraction of silver nanoparticles

This technique was used to determine the purity of AgNps, as well as to determine the crystalline pattern of AgNps in addition to determine the formation of AgNps.

The measurement showed the presence of four peaks at (38.56, 44.76, 65.01, and 77.91°C), which are attributed to the crystal levels (111, 200, 220, and 311) respectively. These peaks indicate the formation of AgNps with high purity as they do not contain other peaks,[17,19] as shown in Figure 3.

From Scherrer equation,[20] the particle size of AgNps was calculated and found to be 21.24 nm[20] [Table 1].

Transmission electron microscope of silver nanoparticles

TEM measurement of AgNps proves the success of the formation of silver metal in nano scale which 1–100 nm. The TEM showed the presence of very regular particle in shape which was nanospheres with a particle size in the range of 10–28 nm. It is worth noting that these sizes agree with those calculated by the XRD technique, and this is due to the fact that the Scherrer equation used is an equation that applies usually to spherical particles [Figure 4].

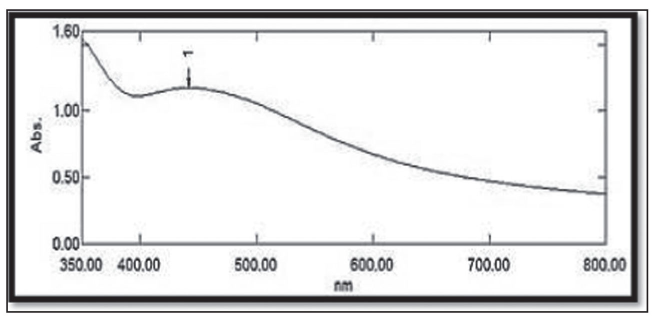

Figure 2: UV-visible spectroscopy of silver nanoparticles

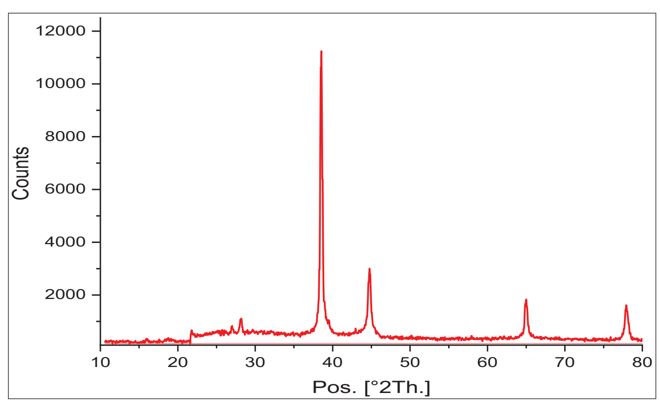

Figure 3: XRD of silver nanoparticles

Table 1: XRD data and average particle size of silver nanoparticles

Pos. [°2Th.]	Index	FWHM [°2Th.]	d-spacing [Å]	Particle size (nm)	Average particle size (nm)
38.56	111	0.35271	2.35148	24.94	21.24
44.76	200	0.46361	2.03151	19.37	
65.01	220	0.45037	1.44110	21.86	
77.91	311	0.56828	1.22873	18.79	

FWHM: Full Width at Half Maxima.

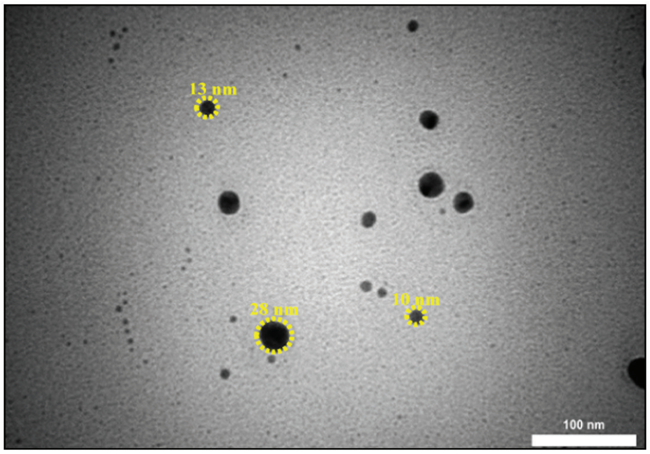

Figure 4: TEM of silver nanoparticles

Antimicrobial activities of silver nanoparticles, ampicillin, and synergistic effect of combination of silver nanoparticles + ampicillin against *Proteus mirabilis* isolates

The antibacterial activity of AgNps that prepared by green synthesized method from *C. aurantium* leaf extract against *P. mirabilis* by using agar well diffusion method shown in Table 2 and Figure 5. The results indicate that the AgNps give a highest inhibition zone at concentration 100 μg/mL (19 mm), whereas the lowest inhibition zone at 25 μg/mL (11.333 mm) and intermediate inhibition zone at 75 and 50 μg/mL (18.333, 17.667) respectively.

Using of AMP alone in this study showed resistance of antibiotic against isolated bacteria, whereas the combined AgNps + AMP exhibit the efficacy of them against bacteria. As shown in Table 3, the inhibition zone of the combination is ranged from (12.33, 18.333, 18.667, and 20.333) at (25, 50, 75, and 100 μg/mL) concentration the higher inhibition zone is at (100 μg/mL) whereas the low concentration zone is at (25 μg/mL).

Discussion

In this study *P. mirabilis* show inhibition zone to AgNps at all used concentrations and the inhibition zone increases with an increase in concentration; that is, mean the efficacy of AgNps also increases against bacteria. This result was compatible with[21] who found that AgNps was able to inhibit bacterial growth at all used concentration.

The previous study[22,23] evaluated that *P. mirabilis* exhibit a highest inhibition zone (21 mm) which agree with current study which showed that the inhibition zone at 100 μg/mL was 20.33 mm. Due to thin peptidoglycans layer in gram negatives cell wall that make the AgNps penetration in to the bacteria be more easily which inhibit the bacterial growth and increase inhibition zone this lead to decrease the resistance of bacteria.

AgNps have many mechanism, increase cell permeability, and damage cell wall and membrane of bacteria; cellular penetration and damage that effects on metabolic pathways; DNA and protein damage; and production of reactive oxygen species.[24]

The synergistic effect of combined AMP and synthesized AgNps increases the susceptibility among the isolated bacteria. In this study, AMP was selected as an antibiotic for detection of synergistic effect by combining it with AgNps because it was the most common resistance antibiotic against *P. mirabilis* isolates. The effect of AMP alone appeared with very low inhibition zone, which means bacterial resistance to AMP. Also, AMP efficacy increases with high AgNps concentration. However, synergistic activity of the combination (AgNps + AMP) was more than the activity of antibiotic alone and when compared with other antibiotics.[25,26]

The increase in synergistic effect may be caused by the bonding reaction between AMP and AgNps. Bacterial cell wall lysis occurred by The AgNps + AMP complex binding to their wall and inhibits cross links formation in the peptidoglycans layer. Therefore, the penetration of the complex into the bacteria increases. The AgNps + AMP complex react and prevent the unwinding of DNA that causes serious destruction of bacterial cell.[27]

In this study, the TEM showed very regular particle in shape which was nanospheres with a particle size in the range of 10–28 nm. TEM measurement of AgNps proves the success of formation of silver metal in nano scale which 1–100 nm, and XRD showed the particle size of AgNps to be 21.24 nm. The size of AgNps determines the antibacterial activity, in which large AgNps permit huge surface area to attach bacterial cells; this means tiny particles will actually have higher percentage of interaction comparative with large particles and the smaller Nps show higher toxicity to bacterial pathogen.[28]

Table 2: The effect of AgNps against *P. mirabilis*

Nps	Mean ± SD of diameter of inhibition zone (mm)			
	Concentration			
	25 μg/mL	50 μg/mL	75 μg/mL	100 μg/mL
Citrus leaf AgNps	11.333±0.577	17.667±0.577	18.333±0.577	19.000±1.000

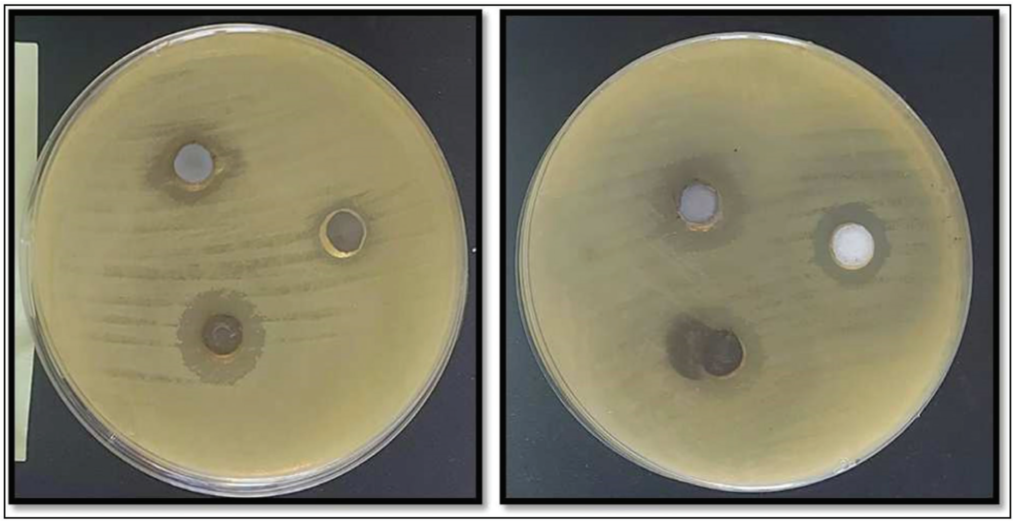

Figure 5: Antimicrobial activities of AgNps, ampicillin, AgNps + ampicillin against *P. mirabilis* isolates in Muller Hinton agar

Table 3: The synergistic effect of AgNps + AMP and AMP alone against *P. mirabilis*

Type	Mean ± SD of diameter of inhibition zone (mm)			
	Concentration			
	25 μg/mL	50 μg/mL	75 μg/mL	100 μg/mL
AMP	6.333±1.528	9.333±0.577	11.333±1.528	13.667±0.577
AgNps + AMP	12.333±1.155	18.333±0.577	18.667±0.577	20.333±0.577

A using of *C. aurantium* leaf in this study as an extract for AgNps preparation gives a significant result when using it as an antibacterial agent and as gives a synergistic effect when combined the AgNps with AMP which makes the bacteria more sensitive and inhibit bacterial growth. The researcher initiated that the AgNps synthesized by using plant extracts exhibited significantly highest antibacterial efficacy than AgNps synthesized from other sources, such as bacteria, fungi, and algae. This is because AgNps synthesized by plant extracts have low toxicity, are cost-effective, and are easy to prepare.[29-31]

Conclusion

1. The green synthesis of nanoparticles is considered eco-environmentally friendly, safe, cheaper, and easy to prepare.
2. Green synthesized AgNps have a high effect on *P. mirabilis* as anti-bacteria agent.
3. The synergistic of combination of AgNps with AMP could increase the inhibition zone that means this combination reduces the resistance of bacteria.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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