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Original Article

Synergistic Effect of Synthesized Silver Nanoparticles Combined with Amoxicillin/Clavulanic Acid Against *Pseudomonas aeruginosa* Isolated from Clinical Samples

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Abstract

Background: *Pseudomonas aeruginosa* is one of the most prevalent nosocomial pathogenic microorganisms that affect and cause a life-threatening situation. **Objective:** The study aimed to investigate the synergistic effect of silver nanoparticles (NPs) with amoxicillin/clavulanic acid antibiotic on *P. aeruginosa* growth. **Materials and Methods:** A total of 220 clinical samples were collected. A sample has been subjected to isolation and identification by standard microbiological procedures. The extract of *Citrus aurantium* was used to synthesize silver NPs. The characterization of silver NPs was achieved at the University of Tehran, by using UV–visible spectrophotometry, X-ray diffraction (XRD), and transmission electron microscope (TEM). The antibacterial activity of AgNPs and combination with amoxicillin/clavulanic acid antibiotic were tested against bacteria using the agar well diffusion method. **Results:** Out of the 220 samples collected, 33 (15%) isolates were positive for *P. aeruginosa*. Highest resistance of *P. aeruginosa* was found to amoxicillin/clavulanic acid in percentage of 100. NPs have been characterized by a UV–visible spectrometer which revealed a broad peak at 426 cm^{-1}. XRD showed the purity of the prepared silver NPs that the particle size was equal to 21.26 nm. TEM measurement showed the presence of sphere-like structures with sizes of 20 nm for regular particles and 40 nm for irregular particles. AgNPs, amoxicillin/clavulanic acid, and mixture (amoxicillin/clavulanic acid + AgNPs) have high inhibition activity of *P. aeruginosa* in concentration of 100 µg/mL and recorded 21.33 ± 3.06, 13.00 ± 0.00, and 22.00 ± 3.46 mm, respectively. **Conclusion:** The results supported a green synthesis approach for the synthesis of AgNPs with antimicrobial. *P. aeruginosa* has synergistic combinations of AgNPs with amoxicillin/clavulanic acid, with a great inhibitory effect on the growth of the bacteria.

Keywords: AgNPs, MDR, *Pseudomonas aeruginosa*

Introduction

An organism called *Pseudomonas aeruginosa* is found in soil, water, animals, plants, and other living things.[1] It is a widely distributed ubiquitous nosocomial pathogen.[2] It is aerobic Gram-negative bacilli that do not form spores and are saccharolytic. It size ranges from 0.5–0.8 to 1.5–3.0 µm.[1] Most strains have only one polar flagellum, which is responsible for their mode of motility.[3] *P. aeruginosa* can be recognized in unidentified colony by their distinct odor, which is described as having a fruity grape-like-odor.[4] *P. aeruginosa* is a major source of bacteremia in victims of burns, catheterized patients with urinary tract infections, and nosocomial pneumonia in patients with endotracheal tube in intensive care units. Also, it is the main cause of morbidity and mortality in patients with cystic fibrosis with long-term colonization of the lungs with this bacterium, due to the abnormal airway epithelial layer.[5] It is one of the bacteria species that does not digest glucose or lactose, and it is regularly isolated from patients in medical facilities. The organism can only live as long as oxygen supplement is available, but if oxygen

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is unavailable it may use either nitrate or arginine as the final electron acceptor in the process.[6] It also produces a number of virulence factors which after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination.[7]

P. aeruginosa is a multidrug-resistant (MDR) bacterium because it has the acquired ability to resist the majority of antibiotics that are often used. MDR bacteria can be found in a variety of environments, including soil and water, among other places.[8] Worldwide, increasing resistance in *P. aeruginosa* has warranted distinct attention in hospitals and associated with early onset of ventilator-associated pneumonia, increased length of stay in the hospitals, and higher case fatality rates and complicated the selection of adequate treatment in severe infections. Thus, limiting the spread of MDR strains of *P. aeruginosa* is considered to be an infection prevention and control priority.[9]

Nanotechnology is a relatively new scientific discipline that focusses on the creation and utilization of nanoparticles (NPs) with dimensions ranging from 1 to 100 nm. It is concerned with the advancement of pioneer materials in health, medicine, environment, economics, research and technology, and other disciplines, which result in a substantial shift in technologies.[10] When it comes to synthesizing different NPs, one can choose from a wide range of different approaches, including chemical, biological, and physical ones. The most environmentally friendly practices are those that use biological processes.[11] The ability of AgNPs to be synthesized by plants makes them a potentially useful source for the development of antibacterial agents. This is because their method of action targets many locations in bacteria. AgNPs have a great therapeutic value due to their unique antibacterial capabilities.[12] Due to their potent antibacterial capabilities against Gram-negative bacteria, AgNPs with a high surface-area-to-volume ratio and a size of less than 100 nm are of particular interest.[13-15] The aims of this study were to evaluate the prevalence rate of antibiotic resistance and MDR among *P. aeruginosa* and to detect the antibacterial effect of AgNPs combined with amoxicillin/clavulanic acid on the growth of *P. aeruginosa*.

Materials and Methods

Patients

The research was conducted between November 2021 and May 2022 in Kirkuk city at Azadi Teaching Hospital, Pediatrics Hospital, Kirkuk General Hospital, Gynecology and Pediatric Hospital, and Public Health Laboratory. A total of 220 patients submitted to the study under physician supervision. The medical history of each patient was recorded, including their name, age, gender, the type of specimen that was obtained, and whether or not the patient was receiving treatment at the time (provided no antibiotic intake for 3 days before collection of specimen for culture). After collection, a variety of clinical samples (including sputum, urine, bronchial washing, swabs from burns, wounds, and ears) found that 33 people were infected with *P. aeruginosa*. This number was derived from 220 specimens.

Bacterial isolation and identification

Blood agar, MacConkey agar, Cetrimide agar, and Brain Heart Broth were used to culture all the samples, which were subsequently incubated at 370°C for 24–48 h. The isolated colony was recognized by Gram stain, colony shape, oxidase production test, and non-lactose fermentation on MacConkey agar, hemolytic activity on blood agar, and pigment formation on Cetrimide agar, and identified by biochemical tests, Vitek2 system (bioMérieux, France).

Synthesis of the nanoparticles

Citrus aurantium extract

C. aurantium leaves were harvested and a thorough water wash was followed by a distilled water wash to get rid of any dust particles. The washed leaves were dried at 40°C 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. After that, the mixture was heated to 80°C for an hour. After that, the mixture was decanted and then centrifuged at a speed of 12,000 rpm for 10 min. To be used in the next stage, the aqueous extract was prepared and collected for application.

AgNPs

About 2.00 g of silver nitrate was heated to 40ºC and dissolved in 250 mL of deionized water. Thereafter, 10 mL of leaves extract was added drop wise to the silver nitrate solution. The mixture was put on a stirring hot plate at room temperature for 4 h and the color changed to deep red. UV–visible spectrophotometry was used to examine how silver nanoparticle production occurred.[16]

AgNPs-amoxicillin/clavulanic acid

AgNPs-amoxicillin/clavulanic acid was synthesized by mixing 1 g of synthesized silver nanoparticles with 1 g of amoxicillin. Reflux this mixture for 3 h and then filtered off. The filtrate was washed three times with hot ethanol and hot water to remove unreacted starting materials.

Characterization of AgNPs

UV–visible spectrophotometry of AgNPs

AgNPs were created, and their sizes were measured inside the composite solution using the UV–visible spectroscopy.

Transmission electron microscope analysis of AgNPs

The technique of transmission electron microscopy (TEM), sent to Tehran, was applied in order to examine the created AgNPs in order to determine their size and

shape. For the purpose of this investigation, a JEOL-2010 ultra-high-resolution transmission electron microscope operating at 200 kV was utilized. In order to produce the TEM grids, a drop of the particle solution was positioned on a copper grid that had been coated with carbon, and the grid was subsequently dried in the presence of light.[17]

XRD analysis of AgNPs

Green synthesis and AgNPs examined by X-ray diffraction (XRD) analysis were sent to Tehran: a common analytical tool used to analyze molecular and crystal structures, identify different molecules qualitatively, resolve chemical species quantitatively, and determine crystallinity, isomorphous substitutions, and particle sizes.

Antibacterial activity of AgNPs

Five milliliters of fresh culture containing only pure isolated colonies of *P. aeruginosa* were used to test the antibacterial activities of AgNPs against multidrug-resistant *P. aeruginosa* isolated from clinical sample using agar well diffusion methods. The incubation period was 4–8 h at 37°C. To reach a comparable optical density of 0.5 McFarland and requirements (corresponding to 1.5×10^8 cells/mL), the turbidity produced by growing culture was calibrated using sterile broth. A cotton swab was used to apply the suspension. Using a sterile cork borer, wells of 7 mm in diameter were made and filled with Ag NPs solution at four different concentrations (100, 75, 50, and 25 g/mL). Following that, the plates were subjected to an additional 24 h of incubation at a temperature of 37°C. The diameter of the growth inhibition zones was measured in millimeters, so that the efficiency of the antibacterial agents could be determined.[18]

Table 1: Culture results for different clinical samples

Positive bacterial growth		Negative bacterial growth	Total
P. aeruginosa	Other bacteria	No bacterial growth	
33 (15%)	175 (79.5%)	12 (5.4%)	220 (100%)

Statistical analysis

IBM SPSS V24 (2016) was used to analyze the data frequency, percentage, and descriptive statistics (mean and standard deviation). The χ^2, one-way ANOVA, and Monte-Carlo tests were used to assess the results of this study to determine the degree of significance.[19]

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 the sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local Ethics Committee according to the document number 42471 (including the number and the date in 9/12/2021) to get this approval.

Results

Study samples

The present study included 220 samples from different clinical sites and collected at hospitals in Kirkuk City. All samples were cultured, from which 12 (5.4%) showed no bacterial growth, 33 (15%) showed growth of *P. aeruginosa*, the remaining samples 175 (79.5%) gave other bacteria [Table 1].

Isolation and identification of *P. aeruginosa*

The frequency of *P. aeruginosa* isolation was highest in the burn swabs followed by urine samples, wound swabs, ear swabs, sputum, and bronchial washing (42.4%, 21.2%, 15.2%, 12.1%, 6.1%, 3.0%), respectively [Figure 1].

P. aeruginosa colonies on MacConkey agar appeared as a pale color because non-lactose fermenter. But on blood agar, all gave β type of hemolysis.

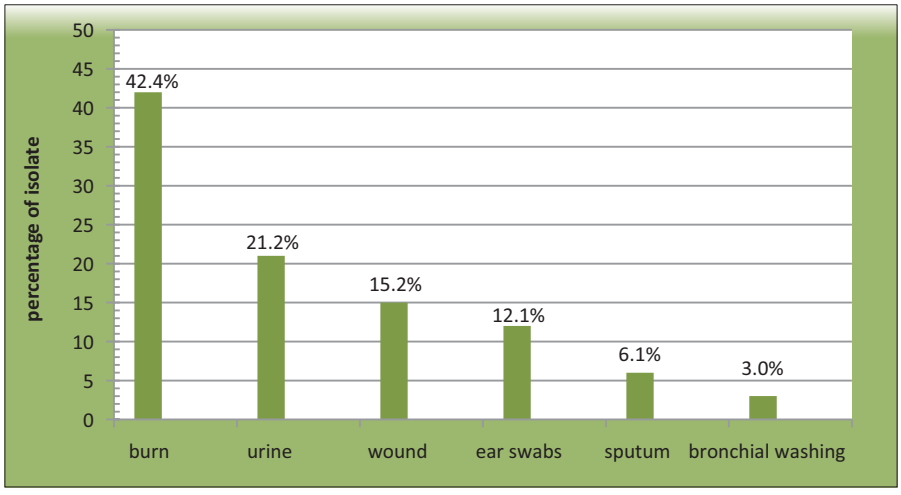

Figure 1: Frequency of *P. aeruginosa* isolates in different clinical specimens

Cetrimide agar is a selective medium used for isolation and identification of *P. aeruginosa* [Figure 2]; the single colony was streaked on media and incubated. It develops green color with sticky, big, and circular colony.

Synthesis and characterization of AgNPs

Green synthesis is the optimal method for the synthesis of AgNPs. Plants and plant extracts are favored, particularly in the green synthesis process due to the rich bioactive ingredients that they contain, and plant metal atoms can be oxidized in plant extracts. These bioactive ingredients can bind to the surface of metal oxides during the process of synthesis and that give antibacterial properties by the metal oxides.[20]

UV–Vis spectrum of AgNPs

This type of spectra has been widely used to study the spectra of nanomaterials in the solutions, especially the change in color. The UV–Vis spectrum of the as-prepared silver nanoparticles solution showed a broad peak at 426 cm^{-1}, which is the characteristic peak of the silver NPs. This peak does not express an electronic transmission within the silver orbital, but rather is a peak that is result of surface plasmon resonance (SPR)[20-25] [Figure 3].

XRD of silver nanoparticles

The XRD technique of the powder gives complete information about the type of substance under analysis as well as information about the purity of the compound or metal in addition to its ability to determine the crystal planes and thus determine the crystal system.

Based on this, XRD measurement of the prepared silver NPs was carried out. The measurement showed a peak at 38.559°, which is attributed to the crystal level 111, a peak at 44.761° for the 200 level, and a peak at 65.013° for the 211 level, whereas the peak at 77.904° is attributed to the 311 planes, as shown in Table 2 and Figure 4.[26,27] These values provide the formation of the cubic-faced center system. Furthermore, the XRD measurement showed no additional peaks, proving the purity of the prepared silver NPs. The Scherrer equation proves that the particle size was equal to 21.26 nm.

TEM of silver nanoparticles

TEM showed the presence of geometric structures that were mostly regular, as they are sphere-like structures with sizes not exceeding 20 nm. Moreover, the measurement showed the presence of irregular sphere-like geometric structures with diameters not exceeding 40 nm [Figure 5].

The antibacterial activity of AgNPs, amoxicillin/clavulanic acid, and combination of amoxicillin/clavulanic acid with

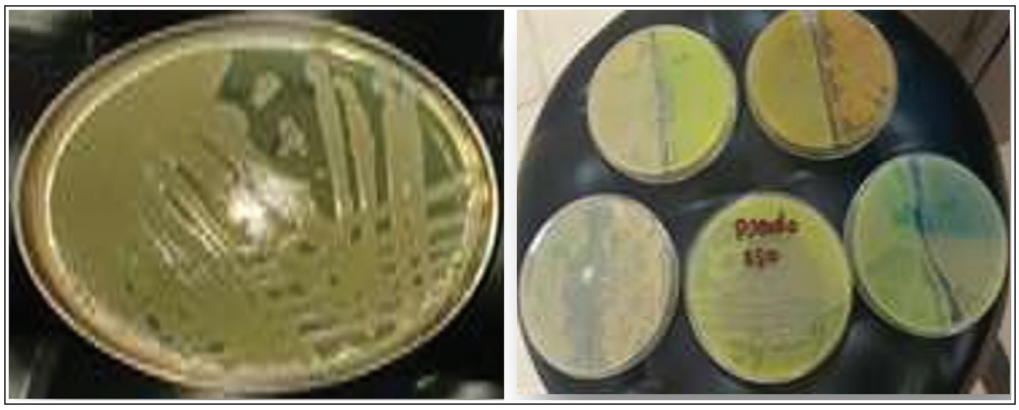

Figure 2: Culture characteristics of *P. aeruginosa* on Cetrimide agar

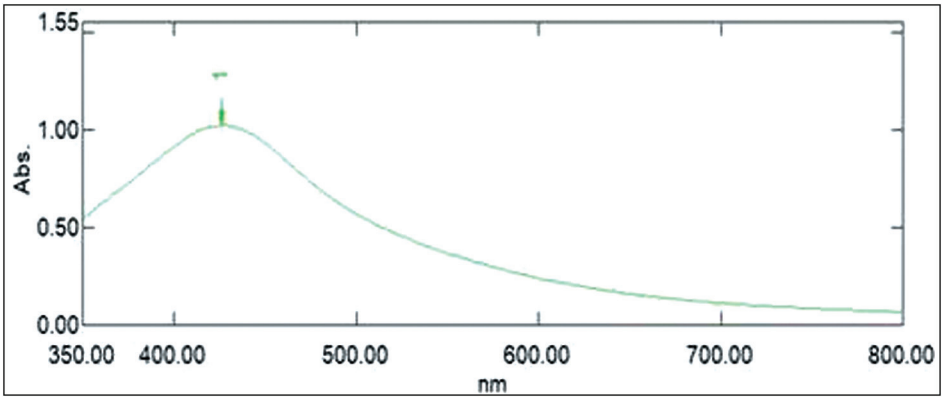

Figure 3: UV–Vis spectrum of AgNPs

AgNPs against *P. aeruginosa* was determined by the agar well diffusion method.

Figure 6 shows antibacterial activity and combination effect of AgNPs-amoxicillin/clavulanic acid and AgNPs-ampicillin. *P. aeruginosa* resistance to amoxicillin/clavulanic acid decreased after combination with AgNPs. This is evidence of a synergistic effect of combination antibiotic and NPs [Figure 7].

Table 3 showed the concentration of 100 µg/mL of AgNPs and amoxicillin/clavulanic acid; also the mixture (amoxicillin/clavulanic acid + AgNPs) has inhibition activity against *P. aeruginosa* where the inhibition zone recorded 21.33 ± 3.06, 13.00 ± 0.00, and 22.00 ± 3.46 mm, respectively. For a concentration of 75 µg/mL, the inhibition zones were 19.67 ± 2.52, 11.33 ± 0.577, and 20.67 ± 3.21 mm, respectively. The concentration 50 µg/mL has an inhibitory activity of 18.00 ± 2.00, 9.67 ± 0.577, and 19.00 ± 2.65 mm, respectively, whereas for a concentration of 25 µg/mL, inhibition was 16.33 ± 2.52, 8.33 ± 0.577, and 17.67 ± 2.31 mm.

Table 2: XRD data and average particle size of AgNPs

Pos. [°2Th.]	Index	FWHM [°2Th.]	*d*-spacing [Å]	Particle size (nm)	Average particle size (nm)
38.559	111	0.36000	2.35148	24.44	21.26
44.761	200	0.45998	2.03151	19.52	
65.013	220	0.45001	1.44110	21.88	
77.904	311	0.5555	1.22873	19.22	

Discussion

Study samples

Thirty-three (15%) were positive for *P. aeruginosa*, which was similar to the results of Farhan *et al.*,[28] which reported that out of 530 samples 150 (28.3%) were *P. aeruginosa* isolates, which in turn agree with ALmusawi.[29]

Isolation and identification of *P. aeruginosa*

The frequency of *P. aeruginosa* isolates was highest in the burn swabs followed by urine samples, wound swabs, ear swabs, sputum, and bronchial washing (42.4%, 21.2%, 15.2%, 12.1%, 6.1%, 3.0%), respectively [Figure 4]. The current results showed high incidence (42.4%) of *P. aeruginosa* among samples collected from patients with burns. Burn patients are more liable to get infections in

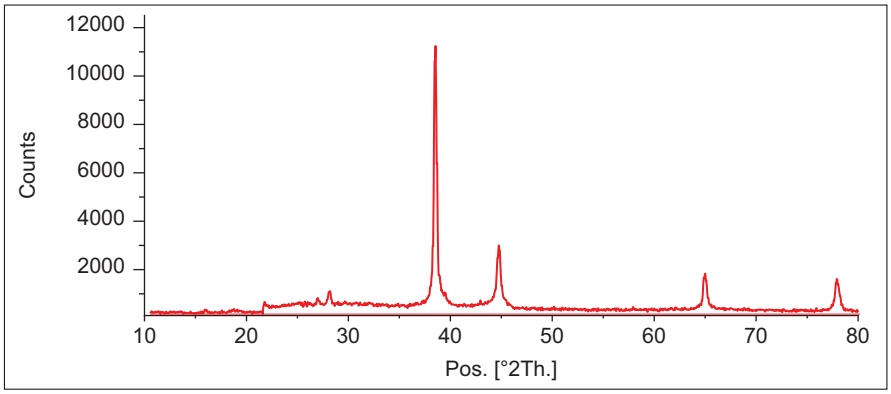

Figure 4: XRD of AgNPs

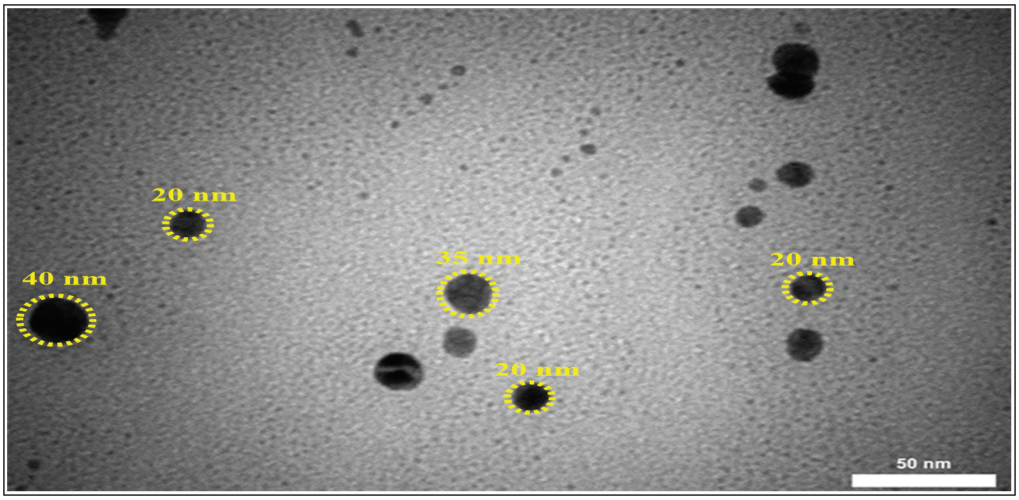

Figure 5: Transmission electron microscope of AgNPs

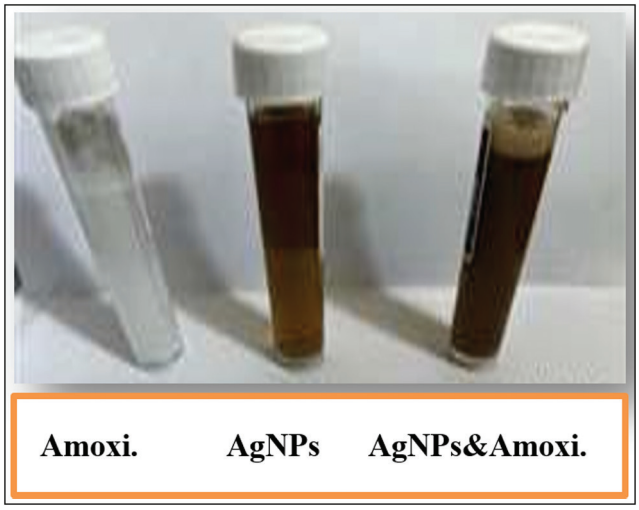

Figure 6: Combinations of AgNPs with amoxicillin/clavulanic acid

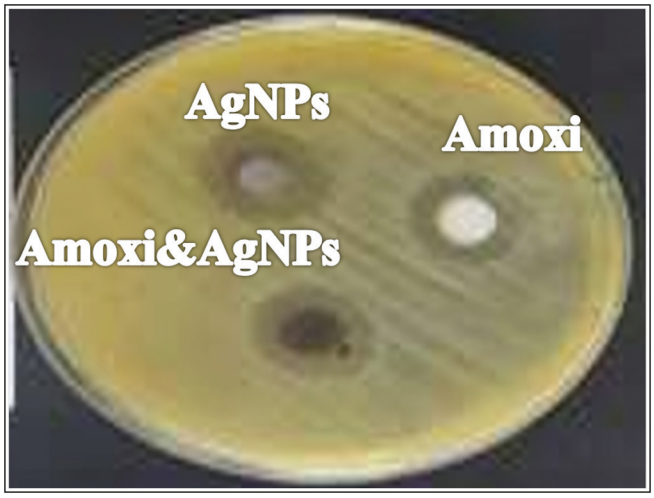

Figure 7: Antibacterial activities of AgNPs, amoxicillin/clavulanic acid, and amoxicillin/clavulanic acid combination with AgNPs (agar well diffusion method)

Table 3: Inhibition zone of AgNPs, amoxicillin/clavulanic acid, and amoxicillin/clavulanic acid combined with AgNPs against *P. aeruginosa*

Treatment concentration (μg/mL)	AgNPs	Amoxicillin/ clavulanic acid	Amoxicillin/ clavulanic acid +AgNPs
100	**21.33±3.06a**	**13.00±0.00g**	**22.00±3.46a**
75	**19.67±2.52b**	**11.33±0.577g**	**20.67±3.21b**
50	**18.00±2.00d**	**9.67±0.577g**	**19.00±2.65d**
25	**16.33±2.52f**	**8.33±0.577h**	**17.67±2.31f**

*Different small letters in rows indicate significant differences (P <0.05)

comparison with other patients because of their damaged skin barrier and suppressed immune system, in addition to extended hospital stay and invasive therapeutic and diagnostic procedures.[30] The study agreed with the rate of infection with bacteria which is very high in wound and burn patients; this is due to many reasons, including the ease of obtaining low quality and ineffective antibiotics without a prescription, or due to the contamination of the hospital environment and permanent crowding of patients.[31]

Antibacterial activity of AgNPs, amoxicillin/ clavulanic acid and combination of amoxicillin/ clavulanic acid with AgNPs against *P. aeruginosa*

One study applied the combination of AgNPs with β-lactam antibiotics such as ampicillin and penicillin, quinolone antibiotics, aminoglycoside, and tetracycline where the quinolone antibiotics, aminoglycoside, and tetracycline showed the inhibition effect against *Salmonella,* whereas penicillin and ampicillin did not show effect.[32] The study[33] showed the effects of green biosynthesis AgNPs of banana extract with five antibiotics against *P. aeruginosa, Escherichia coli, Proteus mirabilis*, and *Salmonella typhi*. The increase of inhibition effect is experimented with tobramycin, clavulanic acid, nitrofurantoin, nalidixic acid, and chloramphenicol in the presence of AgNPs; this inhibitory effect is due to increase in availability of drug after binding with the bacterial cell membrane and due to the absorbance effectively for components.[34]

However, the current study is compatible with other studies, which used AgNPs and AgNPs mixed with antibiotics which indicate the efficiency of mixture of AgNPs with neomycin against *P. aeruginosa* isolated from burns.[35] There are several advantages of the combination of NPs with antibiotics, such as lowering the dose of each agent, which can reduce the toxicity effects and improve the antimicrobial characteristics.[36] Moreover, the combination of antibiotics with AgNPs assists the antibiotics to take the location of the antibiotic–microbes contact which stimulates the efficiency of antibiotics.[37,38]

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Nil.

Conflicts of interest

There are no conflicts of interest.

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