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The Bactericidal Activities of Silver Nanoparticles (AgNPs) Produced by Cell-Free Supernatant of *Pseudomonas aeruginosa* and Sterilization by the Effect of Radiation

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Abstract: Over the past few years, a major health problem has emerged and recognized in the resistance to antimicrobial agents by pathogenic bacteria. In this regard, it is well-known that Silver bio-nanoparticles (AgNPs) have bacteriostatic and bactericidal effects. Therefore, this current research will concentrate on the synthesis of metallic bio-nanoparticles of Silver through the use of a reduction of aqueous Ag+ ion, with the culture supernatant of *Pseudomonas aeruginosa*, which has been identified by 16S-r-RNA and deposited in the Gene Bank under Accession No.: 3NP0614. Thus, the AgNPs were assessed with regard to their antimicrobial activities against the various human pathogenic microorganisms. As for the most effective antimicrobial activity of the AgNPs, it was observed to be against the following: *Staphylococcus aureus* MAM-1, *Proteus* sp. MAM-2, *Klebsiella* sp. MAM-6, and *E.coli* MAM-5; whereas only moderate antimicrobial activity was seen against *Proteus* sp., which the concentration of 0.435 ug/ml was the best one. In addition, filter paper, cotton clothes, PVA polymer and gauze were used as immobilizing materials for the AgNPs. Furthermore, gamma ray has been used as sterilizing agent for immobilized the AgNPs on gauze to be used as wound dressing. 2kGy was the best sterilization dose for gauze, immobilized with the AgNPs by a 25-mm inhibition zone.

Keywords: AgNPs, *Pseudomonas aeruginosa*, Immobilization, Gamma Ray.

1 Introduction

The environmentally friendly synthesis of nanoparticles was a revolutionary step in the field of nanotechnology [1], in which the manufactured new materials at the nano-scale level were the application of nanotechnology in the fields of science and technology [2]. In these fields, the synthesized nanoparticles showed the effectiveness of their antibacterial properties against the multi-drug resistant (MDR) microorganisms. There was no doubt that there were wide-applications for the biogenic synthesis of nanoparticles in the field of medicine, especially those produced in an eco-friendly way [3].

Out of the different nanoparticles, Silver nanoparticles were certainly the most widely used nano-materials; thus, they were used in antimicrobial agents, textile industries, water treatment, and sunscreen lotions [4]. In addition, there was another important use of the AgNPs for pathogenic bacteria that developed resistance against various antibiotics, for instance, *S. Aureus* which developed resistance to methicillin [5]. As a metal, Silver was very important; nonetheless, the silver nanoparticles also proved to be the most efficient of all nanoparticles; and that is due to the antimicrobial properties against bacteria, viruses and other eukaryotic microorganisms [6]. For long years throughout history, silver was used in the treatment of burns and chronic wounds, as well as with the water filters as early as 1000 B.C. [7]. Moreover, it was known that silver was nontoxic and safe, when used as an inorganic antibacterial agent, thus, it was capable of killing about 650 types of diseases caused by microorganisms [8]. In addition, it destroyed the bacteria without causing any toxicity to the surrounding tissue [9].

In this regard, silver nitrate was used in the eighteenth century in the treatment of venereal diseases, fistulae from salivary glands, and bone and perianal abscesses [10]. In addition, it was also used in the nineteenth century in removing the granulation tissues in order to permit epithelization and promote crust formation on the surface of wounds. Furthermore, various concentrations of silver nitrate were used in the treatment of fresh burns [7]. In the field of medicine, silver ion, metallic silver and silver nanoparticles were used in burns treatment, dental materials and sunscreen lotions; while in the field of industry, it was used in the manufacturing of coating stainless steel materials, textile fabrics and water treatment. It was advantageous for low toxicity to human tissues, low volatility and high thermal stability [11].

The increasing use of silver led to decreasing its antimicrobial agents, for instance, the silver dressings which were the alternative to antibiotics; if containing low level of silver ions, it might develop resistance, so it was preferred to apply high concentrations [12]. In addition, as an immobilizing matrix, the nano-fibers of Poly Vinyl Alcohol (PVA) absorbed the silver nanoparticles, providing

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an effective antibacterial property, which was used in preparing the wound dressings [13]. Furthermore, it was used to prevent the bacterial colonization on the different surfaces of devices such as catheters [14] and prostheses [15]. In this regard, we should mention that it was noticed that the nanoparticles showed antibacterial activity at low concentrations. For instance, it was found that they are cytotoxic to the bacteria, such as *E. coli* at a concentration of 8 μg/cm^2. However, it is based on the surface area to the volume ratio of nanoparticles; thus, the smaller was the size of possessed particles, the larger was the surface area to the volume ratio, hence the effective antibacterial activity [16]. Thus, with *E. coli*, silver acted by inhibiting the uptake of phosphate, releasing phosphate, mannitol, succinate, proline and glutamine from the cells of *E. coli* [17].

This current study aims to produce the AgNPs microbially, which is safer than chemically, in addition the use of gamma radiation as a sterilizing agent for gauze impregnated in the AgNPs as an immobilizing matrix, in order to get sterile wound dressing impregnated in the AgNPs to serve as antimicrobial agent.

2 Materials and Methods

2.1 Chemicals

Silver nitrate ($AgNO_3$) was purchased from Sigma/Aldrich (St. Louis, USA).

2.2 Bacterial Strains and Culture Media

The bacterial strain used in the present work for the production of AgNPs was the best isolate for the production of AgNPs (depending on the smallest size of nanoparticle that was produced and the fastest one in its productions, compared to the rest isolates in this work), identified by 16S rRNA as *Pseudomonas Aeruginosa* and deposited in the Gene Bank under Accession No.: 3NP0614 as previously mentioned [18] This strain was used in Biotechnology. The other bacterial strains were clinical isolates (human pathogens) isolated from the clinical samples (pus, urine and wounds on blood agar, CLED and MacConky agar plates) [3], identified according to [19].

2.3. Bio-Production of Silver Nanoparticles (AgNPs)

A loopful of *Pseudomonas Aeruginosa* was inoculated in 50 ml L.B. broth medium [20] in 250 ml conical flask, and incubated for 24 h. at 37 c° in a shaking (150-rpm) incubator.

The bacterial culture was centrifuged at 8000 rpm for 15 min in a cooling centrifuge. Cell free supernatant (extract) was transferred in clean and sterile flasks, and the AgNPs solution was added to a final concentration of 3mM,the best concentration as previously mentioned [18]. The flasks were incubated in water bath (80 C°) until the color changed from pale yellow to dark brown. The formed AgNPs were measured spectrophotometrically at 430 nm, using a spectrophotometer (LW-V-RS uv/VIS, Germany).

2.4. Determination of the Concentration of the Silver Nanoparticles

The concentration of the silver nanoparticles was determined through the method, previously reported by [21]. The calculations are as follows:

• To determine the average number of atoms per nanoparticle [21].

$$N = \frac{\pi \rho D^3 \times NA}{6M}$$

Where N is the number of atoms per nanoparticles, π = 3.14, ρ is the density of face centered, cubic (fcc) silver (= 10.5 g/cm3), D is the average diameter of nanoparticles (= 25nm = 25×10^{-7} cm), M is the atomic mass of silver (= 107.868 g), and NA is the number of atoms per mole (Avogadro's number) (= 6.023×10^{23}).

Thus, assuming 100% conversion of all silver ions to silver nanoparticles, then:

$$N = \frac{3.14 \times 10.5 \times (25.0 \times 10^{-7})^3 \times 6.023 \times 10^{23}}{6\times107.868}$$

atoms/nanoparticles

i.e. N = 479410.9612 atoms/nanoparticles

• To determine the molar concentration of the nanoparticle solution, we used the following formula: [21].

$$C = \frac{NT \quad M/L}{NVNA}$$

Where C is the molar concentration of the nanoparticle solution, NT is the total number of silver atoms added as (AgNO3 = 3mM), N is the number of atoms per nanoparticle (from Calculation 1), V is the volume of the reaction solution in L, and NA is the Avogadro's number (= 6.023×10^{23}).

$$C = \frac{0.194 \times 6.023 \times 10^{23}}{479410.9612 \times 0.1 \times 6.023 \times 10^{23}}$$

$$C = 4.04 \times 10^{-6} \text{ M/L}$$

2.5. The Antibacterial Activity of AgNPs

2.5.1. Determination of the Antibacterial Activity of the Silver Nanoparticles (AgNPs) for Certain Microorganisms by Using the Filter Paper Discs

The pure cultures of certain pathogenic organisms were sub-cultured on 20 ml L.B. broth in 100 ml conical flask, and were incubated at 30 °C on a rotary shaker at 150 rpm.

The grown strains of the bacterial suspension (0.1 ml) of 24 h. were (5.0 x 10^5 CFU/ml) swabbed uniformly by distribution onto the surface of the L.B. plate using sterile cotton swabs. Double layer sterilized paper disc (6 mm) was placed on the L.B. agar plates in equal distances; then, the AgNPs synthesized from *Pseudomonas Aeruginosa* (0.0272 – 0.0544 – 0.1089 – 0.2217 – and 0.4357 ug/ ml) were loaded into each disc to determine its antibacterial activity. In the center of the plate, the disc was loaded with sterile L.B. broth, which is used as a control. The plates were incubated at 37°C for 24 h. After the incubation period, the petri dishes were evaluated for antibacterial activity, which was measured in terms of the diameter of the inhibition zone (mm).

2.5.2. Determination of the Minimum Inhibitory Concentration (MIC) for the AgNPs

The minimum inhibitory concentration (MIC) of the synthesized AgNps was determined using one tenth serial dilution of Gram positive and Gram negative bacteria on the L.B. agar plates supplemented with AgNps, synthesized by *Pseudomonas aeruginosa* in concentrations of (0.0272 - 0.4357) ug/ml. The plates were incubated for 24 h. at 37 °C, with control, and the numbers of colonies were counted. The MIC was defined as the lowest concentration of the nanoparticles that inhibited the visual growth of the tested cultures on the tested plates [22].

2.6. Immobilization of the AgNps on Different Matrices to be used against Bacterial Pathogens

The polyvinyl amine (PVA) polymer **(**Aqueous 10 wt% of starch/PVA) solutions were prepared by dissolving 4 g of starch and 6 g PVA in 90 ml of distilled water, and stirring with a magnetic stirrer at 90 °C for 30 minutes. The mixture was poured into test tubes, then, irradiated with gamma rays from a Cobalt 60 source at irradiation dose of 30 kGy at Indian chamber. After irradiation, the obtained hydrogel was cut into discs, then, allowed to dry at 40 °C in an oven for 24 h. [23].

Cotton and gauze (1 cm diameter) were used as immobilization matrix for the AgNPs. The discs of PVA, cotton and gauze were loaded by different concentrations of AgNPs (0.0272 -0.4357) ug/ml. The loaded matrices were placed on the surface of the pre-inoculated L.B. agar plates [(100 ul of 5 x 10^6 CFU/ml) of each bacterial pathogenic strain in duplicate for each and matrix]. The inoculum was spread uniformly by cotton swabs. Then, the control discs were loaded with the same volume of sterile L.B. broth. The inoculated plates were incubated at 37 °C for 24h, thus, the diameter of the clear zone around the discs had been determined.

2.7. Sterilization of the Immobilizing Matrix by Gamma Radiation

Gauze matrices loaded with AgNPs (100ul) were exposed to gamma radiation at doses of (2.0, 5.0 and 10.0 kGy) in order to get sterilized matrices loaded with (AgNPs) to be used as wound dressing. Thus, the inhibition zone diameter (mm) of the AgNPs with different concentrations at different gamma radiation doses against the pathogenic bacterial strains has been determined.

3 Results and Discussion

In the present study, the AgNPs showed remarkable antibacterial activity against Gram negative (*E. coli, Proteus* sp., *Klebsiella* sp. and *Pseudomonas* sp.), and Gram positive (*Staph. aureus*) which registered multi-drug resistance (MDR) against several antibiotics.

3.1. Determination of the Antibiotic Activity of the Bio-Nanoparticles (Silver Nanoparticles) for Certain Microorganisms Using Filter Paper Discs

The results revealed that 0.4357 ug/ ml of the AgNPs produced large inhibition zones against all the Gram +ve and Gram –ve bacterial strains used in the present study, in which the antibacterial activity of the silver nanoparticles against *Staph. aureus* and *Proteus* sp. was maximum (35.0 mm), followed by *Klebsiella* sp. (26.0 mm), *E.coli (*25.0 mm*),* and *Pseudomonas* sp. (21.0 mm), so *Staph. aureus* and *Proteus* sp. were sensitive to AgNPs than the other clinical bacterial strains as indicated in Table (1). In spite of the well documentation of the antimicrobial effects of silver derivatives, the mechanisms through which it exerts its bioactivity are yet unknown [24].

The amount of silver and the rate of its release determine the antimicrobial activity of silver. However, in its metallic state, silver is inert; it reacts with the skin's moisture and the wound's fluid as it gets ionized , which is very reactive, as it binds with the tissue proteins making structural changes in the wall and nuclear membrane of the bacterial cell, which in turn results in the cell's distortion and death. In addition, the silver's binding with the bacterial DNA and RNA leads to their denaturation, and inhibits the replication of bacteria [22]. In this regard, Nanotechnology is considered as the newest and one of the most interesting fields of research in the modern medical science; and that is due to the fact that the nanoparticles exhibit new and improved properties in relation to size, distribution and morphology than larger particles [25]. This is attributed to the surface-to-volume ratio of nanoparticles, which is proportional to their biological effectiveness. Therefore, its surface area and catalytic reactivity were increased [26], in which the bioactivity of the AgNPs is based on their ability to anchor

Table (1): Effect of AgNPs immobilized on filter paper disc against pathogenic bacterial strains in means of inhibition zone diameter.

Concentrations / Strains	Inhibition Zone Diameter(mm)			
	0.0544 ug/ml	0.1089 ug/ml	0.2178 ug/ml	0.4357ug/ml
***1-S.aureus* MAM-1**	12	12	16	**35**
***2- Proteus* sp. MAM-2**	-ve	12	17	**35**
***3-Pseudomonas* sp. MAM-3**	12	14	15	17
***4-Pseudomonas* sp.MAM-4**	-ve	12	15	21
5-E.coli MAM-5	13	14	15	25
6-*Klebsiella* sp. MAM-6	13	14	14	26
***7-Staph. aureus* MAM-7**	15	17	19	21

and penetrate the bacterial cell wall, and to modulate cellular signaling. For example, it was shown that the production of silver nanoparticles by the *Pseudomonas aeruginosa* cell free supernatant in the range of 15-35 nm was spherical in shape as indicated previously by AFM [18]. While chemical and physical processes were used for the formation of AgNPs, a recently developed biological method is underway, which is considered as a more advanced method than chemical and physical methods. This new method is cost-effective and environmentally friendly [27], with a minimum time required [1].

On the other hand, the physical method produces low yields, and the chemical method causes contamination because of the precursor chemicals, through the use of toxic solvents and the generation of hazardous by-products, as indicated by [28]. There is an agreement between the results of the current study and the results of [28], who found the silver nanoparticles' antimicrobial and bactericidal activity on Gram-positive and Gram-negative bacteria, including multi-resistant strains such as methicillin resistant *S. aureus*. At the size of 25 nm, the silver nanoparticles' antibacterial activity was found at its highest antibacterial activity. In addition, for *E. coli* (ATCC 10536) and *S. aureus* (ML 422), the silver nanoparticles showed higher bactericidal efficiency than penicillin [26]. Nonetheless, the synthesis of the nanoparticles using *Pseudomonas aeruginosa* can possibly eliminate the problem of chemical agents which could have adverse impacts in application, hence making the nanoparticles more biocompatible.

3.2. Determination of the Minimum Inhibitory Concentration (MIC) for AgNPs

Figure (1 and 2) illustrates the values of **log N** and **MIC** in the current research. The AgNPs showed strong antibacterial activity against all human pathogens, even at the lowest concentrations used.

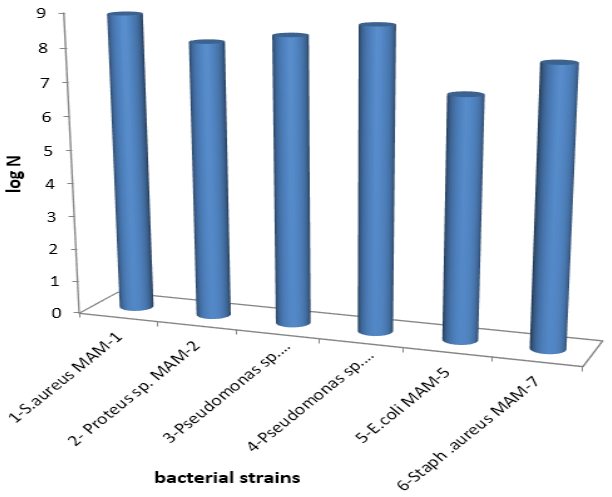

Figure (1): The growth of different bacterial pathogens.

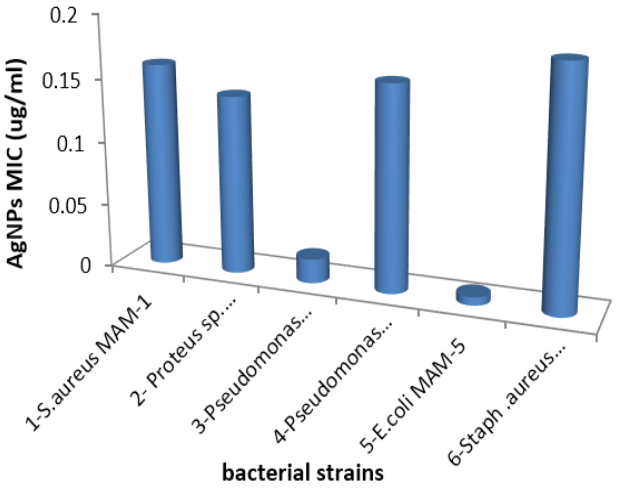

Figure (2): The minimal inhibitory concentration of silver nanoparticles for different bacterial pathogens.

As for the *Staph aureus* sp.MAM-1 without AgNPs

(control), the results revealed that its count was 8.0 x 10^8 CFU/ml. The lowest concentration of AgNPs presents the growth of *Staph aureus* sp. MAM-1 was 0.160 ug/ml. However, in case of *Proteus* sp., the count of the control was 1.6 x 10^8 CFU/ml, while the MIC of AgNPs was 0.140 ug/ml. In addition, the serial dilution indicated that the *Pseudomonas* sp. MAM-3 control count was 3.6 x 10^8 CFU / ml, and the AgNPs MIC was 0.019 ug/ml; whereas the results of another *Pseudomonas* sp. MAM-4 revealed that the control count was 8.0 x 10^8 CFU/ml, while the MIC of AgNPs was 0.160 ug/ml.

The results also revealed that the count of *E.coli*. MAM-5 control was 1.2 x 10^7 CFU/ml, and the MIC of AgNPs was 0.0065 ug/ml .However, another *Staph. aureus* sp. MAM-7 control count was 1.4 x 10^8 CFU/ml, and the MIC of AgNPs was 0.185 ug/ml.. From all of the previously mentioned results, it was clear that the *E. coli* recorded the lowest MIC (0.0065 ug/ml) among all of the tested Gram positive and Gram negative pathogenic MIC strains in the present study. There is an agreement between the results of other examinations and these results as demonstrated by [29], who found that the 20–25 nm silver nanoparticles have MICs ranging from 0.4 to 1.7 µg/mL, and that they are the same as the MICs of commercial antibiotics.

Nonetheless, [30] illustrated that the silver nanoparticles' antibacterial activity controls the growth of Gram positive and Gram-negative bacteria at (10 µg/mL) as (MIC) a minimal inhibitory concentration. In this regard, the work of [22] proved the concept of evaluating the bacterial cell count as a fast method to determine susceptibility.

3.3. The Antibacterial Effects of AgNPs Immobilizing on Polyvinyl Amine (PVA)

As for the applications of the nanoparticle effect, PVA was loaded with AgNPs, and the antibacterial effect was examined.

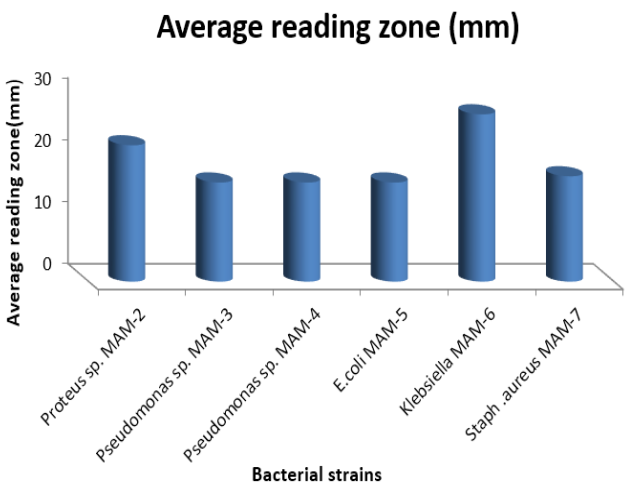

Figure (3): Effect of AgNPs immobilized on PVA polymer against pathogenic bacterial strains.

The results are shown in Figure (3). The zones were measured, and the results indicated that *Klebsiella* sp. was the most inhibited, then *Proteus* sp.by 27 and 22 mm respectively. Therefore, (PVA) polymer can be used as an immobilizing material for the silver nanoparticles.

3.4. The Antibacterial Activities of AgNPs Immobilized on Cotton Filter Paper (1 Cm) with Concentrations

In the present study, the immobilization of silver nanoparticles on cotton (1 cm-diameter) with different concentrations was used on different microorganisms. The zones were measured as indicated in Table (2), showing that the affected concentrations were 0.2178, 0.4357 ug /ml. Therefore, cotton can be used as an immobilizing material for silver nanoparticles, thus, we recommend using PVA and the cotton as well.

Table (2) Effect of AgNPs immobilized on cotton clothes matrix against pathogenic bacterial strains in means of inhibition zone diameter.

AgNPs conc.(ug/ml)	Diameter of inhibition zone diameter(mm)			
	Pseudomonas sp. MAM-3	*Pseudomonas* sp.MAM-4	*E.coli* MAM-5	*Staph.aureus* MAM-7
0.0136	-ve	-ve	-ve	-ve
0.0272	-ve	-ve	-ve	13
0.0544	-ve	30	-ve	20
0.1089	-ve	30	13	20
0.2178	13	35	13	25
0.4357	15	40	14	50

3.5. The Antibacterial Activities of AgNPs Immobilized on Gauze (1 Cm) and Sterilized by Different Radiation Doses

The effect of radiation on the biocidal effect of silver nanoparticles on the four microorganisms (*Pseudomonas* sp., *staph*. *aureus* and *E.coli*) using gauze is illustrated in Tables from 3a to 3d. From these results, it was found that the factor which affects the biocidal ability for silver nanoparticle is the concentration of the silver nanoparticles, not the radiation dose; and the effect of radiation as an antiseptic agent is maintained over long time up to over two months at least. In a related context, other researchers intend to conduct similar works using other microbes; and that is in order to examine their applicability to synthesize other metallic nanostructures, in an attempt to understand the biochemical and molecular mechanisms of the nanoparticles formation by the cell filtrate, with the purpose of achieving better control over the size of the nanoparticles.

In addition, it is intended to develop nano-medicines against several human pathogens through means of the AgNPs synthesized by various microbes, in which the exact mechanism of the silver's impact on the microbes is still unknown; however, the possible impact mechanism of metallic silver, silver ions and silver nanoparticles have been proposed in accordance with the morphological and structural changes found in the bacterial cells [14]. Thus, the silver's impact mechanism is connected to its interaction with thiol group compounds, which is found in the respiratory enzymes of the bacterial cells, as silver binds to the wall and cell membrane of the bacterial cell in this manner, while inhibiting the respiration process [31]. Furthermore, it is also proposed that when DNA molecules are in a state of relaxation, the replication of DNA can be conducted efficiently. Nonetheless, when the DNA is in form of condensation, it loses its ability of replication. Therefore, after the silver ions penetrate inside the bacterial cell, the DNA molecule turns into the condensed form and loses its ability of replication resulting in the cell's death. Moreover, it was proved that heavy metals react to proteins through the interaction with the thiol group, thus the proteins get inactivated [24]. The silver ions' bactericidal action is basically caused through the interaction of silver ions with ribosome, in addition to the suppression of the expression enzymes, thus, the impact on proteins is necessary for the ATP production [28].

In addition, other significant changes occur in the membrane structure of bacteria due to the interaction with silver cations, resulting in an increase in the membrane's permeability of the bacteria [23]. Therefore, the AgNPs showed destabilization in the outer membrane of the bacterial cells, consequently rupturing its plasma membrane, leading to a depletion of the intracellular ATP, as illustrated by [32].

Generally speaking, as illustrated by [7], the main mechanism for the AgNPs is their incorporation in the cell membrane, leading to a leakage of the intracellular substances which eventually causes the cell's death.

In this regard, the stability of the AgNPs generated using the cell-free culture supernatants might be attributed to the presence of a proteinaceous capping agent, preventing the nanoparticles' aggregation. As proposed by [33] and [34], the supernatants were found to be stable for 6 months or more.

4 Conclusion

As for the synthesis of silver nanoparticles through a reduction of aqueous Ag+ ion with the culture supernatant of *Pseudomonas aeruginosa*, the antimicrobial activities of AgNPs against the different human pathogenic microorganisms were proved; and the concentration of 0.435 ug/ml was the best one as for the antimicrobial effect. The effect of the radiation of 2kGy was the best sterility for gauze, immobilized with the AgNPs by a 25-mm inhibition zone.

Table 3(a) The effect of radiation on the biocidal effect of silver nanoparticles using gauze on *Pseudomonas* sp.MAM-3

Conc. of AgNP (ug/ml)	**Inhibition zone diameter (mm)**			
	Non-radiated	**2kGy**	**5kGy**	**10kGy**
0.0136	15.0	18.5	15.5	17.0
0.0272	17.5	17.0	16.5	20.0
0.0544	17.0	14.0	16.0	18.0
0.1089	**18.5**	18.5	18.0	19.0
0.2178	18.0	18.5	17.0	**21.0**
0.4357	**18.5**	**25.0**	**21.0**	14.0

Table 3(b) The effect of radiation on the biocidal effect of silver nanoparticles using gauze on *pseudomonas* sp. MAM-4

Conc. of Ag NP (ug/ml)	Inhibition zone diameter (mm)			
	Non-radiated	2kGy	5kGy	10kGy
0.0136	19.0	18.0	20.0	19.0
0.0272	17.5	16.0	16.5	18.5
0.0544	16.0	18.0	19.0	20.0
0.1089	20.0	20.0	17.5	**22.5**
0.2178	**21.0**	**22.0**	**24.5**	19.0
0.4357	20.0	18.5	20.0	17.5

Table 3(c) The effect of radiation on the biocidal effect of silver nanoparticles using gauze on *staph. aureus*. MAM-7

Conc. of AgNP (ug/ml)	Inhibition zonediameter(mm)			
	Non-radiated	2kGy	5kGy	10kGy
0.0136	14.5	15.0	19.0	17.5
0.0272	15.0	17.5	17.0	15.5
0.0544	16.0	15.0	18.5	12.0
0.1089	15.5	15.5	15.0	17.0
0.2178	20.0	20.0	22.0	**18.5**
0.4357	**24.0**	**30.0**	**24.0**	15.5

Table 3(d) The effect of radiation on the biocidal effect of silver nanoparticles using gauze on *E.coli* MAM-5

Conc. of Ag NP (ug/ml)	Inhibition zone diameter(mm)			
	Non-radiated	2kGy	5kGy	10kGy
0.0136	19.5	18.0	**23.0**	18.5
0.0272	16.0	16.0	16.5	18.0
0.0544	17.0	18.0	20.0	16.5
0.1089	19.0	17.0	22.0	**21.5**
0.2178	17.0	17.5	19.0	18.0
0.4357	**20.0**	**20.0**	20.0	16.5

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