


Antibacterial Activity of Silver Nanoparticles Synthesized by *Aspergillus flavus* and its Synergistic Effect with Antibiotics

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Abstract

Microbial antibiotic resistance is rapidly increasing as a result of overuse or misuse of antibiotics, as well as a lack of new, effective antibiotics. Alternative antimicrobial treatments, such as nanoparticles, and their potential for stronger synergetic effect when paired with other active chemicals, could be a viable option. This study is prepared to estimate the antibacterial activity of silver nanoparticles (AgNPs) that have been synthesized using the biomass-free filtrate of *Aspergillus flavus*. The formation of AgNPs was reported by color changed to a dark brownish-black after 72 hours of incubation. The AgNPs surface plasmon resonance peak was indicated in the UV-Vis spectrum at 427 nm. The synthesis of AgNPs with a nanoparticle size of 10 to 35 nm was validated using transmission electron microscopy. The increase in folding area was calculated to detect the synergistic potential of the combined AgNPs with a broad range of conventional antibiotics. AgNPs have broad-spectrum activity against all strains tested. The most sensitive strain was *Escherichia coli* (11 mm), whereas the most resistant strain was *Pseudomonas aeruginosa*, as indicated by the lowest inhibition zone (7 mm). The lowest Minimum Inhibitory Concentration indicated was against *K. pneumonia* and *Enterobacter cloacae* (0.025 mg/mL, each), followed by *Staphylococcus epidermidis* (0.05 mg/mL), *E. coli* and *Shigella* sp. (0.075 mg/mL, each), and then *S. aureus* (0.1 mg/mL). Notable synergy was reported between AgNPs and either ampicillin, erythromycin, ceftriaxone, vancomycin, azlocillin, or amoxicillin against *S. aureus* in the range between 29.3-fold to 8-fold. In addition, synergy was seen between AgNPs and either vancomycin, clindamycin, or erythromycin against *P. aeruginosa* (31.1-8.0-fold). Also, a maximum increase in IFA when erythromycin and vancomycin were synergized with AgNPs against *E. cloacae* was reported (IFA of 10.0 and 9.0, respectively). Similarly, AgNPs with either aztreonam or azlocillin against *E. coli* and amoxicillin, ciprofloxacin, or ceftriaxone against *Shigella* sp. caused an increase in the fold area of inhibition of between 5.3-3.7-fold. This result may have an advantage in encouraging the use of combined AgNPs with conventional antibiotics in treating infectious diseases caused by antibiotic-resistant bacteria.

Keywords: AgNPs, *Aspergillus flavus*, Antibacterial, Synergy

INTRODUCTION

Antibiotic overuse accelerates microbial resistance and represents a major threat to the world community.¹ As a result, it appears that the discovery of novel and efficient antibacterial drugs is becoming increasingly important.²

Nanoparticles (NPs) have captivated researchers for the past two decades because of their unique physio-chemical properties, controlled size and shape, and ability to interact with other molecules.³ When compared to bulk materials, nanomaterials have a distinguishing advantage in terms of surface area and chemical reactivity, giving them a distinct advantage over currently used antibiotics.⁴ The antimicrobial action of NPs is based on the generation of reactive oxygen species and the release of metal ions.⁵ Due to their small particle size, NPs adhere to the cell wall and cause damage without diffusing into the cell.⁶ Consequently, NPs are less likely than antibiotics to cause bacterial resistance. Apart from the numerous applications of various

nanoparticles, antimicrobial applications of AgNPs are anticipated to be successful in the treatment of bacterial diseases.⁷ AgNPs demonstrated broad-spectrum antibacterial activity.^{8,9} However, it is possible to synthesize nanoparticles from chemical precursors, which could be risky and toxic for humans. Biogenic approaches are required to continue producing eco-friendly and nontoxic nanoparticles.

Microorganisms exhibit unique and promising characteristics that make them ideal for designing nanomaterials.¹⁰ AgNPs mediated by fungi are superior to bacteria due to their high growth rate, ease of cultivation, and large amounts of extracellular metabolite and enzyme secretions that help to reduce silver nitrate salts and cap or stabilize produced nanoparticles.¹¹ Several fungi species have been used to synthesize preferable AgNPs, such as *Aspergillus niger*, *Trichoderma resei*, *Fusarium oxysporum*, and *Phytophthora infestans*.¹² The advantages beyond using these fungi in nanoparticles syntheses are their broad biological activities,

including antibacterial, antifungal, antioxidant, and anticancer.¹² Therefore, the current study aims were to evaluate the inhibitory effect of AgNPs synthesized using biomass free filtrate of *Aspergillus flavus* against Gram-positive and Gram-negative bacteria. Also, to explore the synergistic effects of AgNPs with currently used antibiotics.

MATERIALS AND METHODS

Fungus Strain

Aspergillus flavus was isolated for this project from soil samples collected at an olive oil mill in Al-Karak province, south of Jordan. ITS sequencing was used to identify the fungal strain to the species level (GENWIZ, USA). After performing a sequence similarity analysis against the NCBI database, the sequence was registered in the NCBI database, and an accession number was obtained (Accession no. MK028996).

Culture Condition and Preparation of *Aspergillus flavus* Biomass Free Filtrate

Aerobic growth of *Aspergillus flavus* was achieved in a broth medium containing 1.0 percent glucose, 1.0 percent yeast extract, and 0.5 percent sodium chloride. One hundred ml of growth medium was inoculated with 2.0×10^6 fungal isolate spores and orbitally shaken at $33 \pm 4^\circ\text{C}$ and 150 rpm. After 72 hours of incubation, the fungal biomass was filtered using filter paper (Whatman No.1) and washed extensively with deionized distilled water. Then, 10 g wet weight of the collected biomass was added to 100 ml of water and incubated at 33°C with an agitation rate of 150 rpm. After three days (72 hours), the filtrates were collected through filtration using filter paper (Whatman No.1).

Synthesis of Silver Nanoparticles

A freshly collected *Aspergillus flavus* biomass-free filtrate was used to synthesize AgNPs. This was performed by mixing AgNO_3 with 100 mL of the biomass-free filtrate to reach a 1.5 mM final concentration of AgNO_3 . The mixture was incubated in the dark at 27°C and 150 rpm. At 72 hours of incubation, aliquots of the mixture solution were obtained for characterization of AgNPs.

Characterization of AgNPs

A UV-vis spectrophotometer (SPUV-19, Sco-TECH, Germany) was used to monitor the absorption spectrum of color change in the reaction medium as an initial indicator of AgNP formation. The TEM image was acquired using a Morgagni (Philips, Netherlands) 268 FEI electron microscope equipped with a Mega View G2 Olympus Soft Imaging Solutions. TEM grids were prepared by drop-casting ten microliters of purified nanoparticles distributions onto Formvar-coated copper TEM grids (300 mesh, Ted Pella Inc., Redding, CA) and allowing them to dry aerobically.

Antibacterial Activity and Combination Effect of AgNPs with Standard Antibiotics

Bacterial Strains

Seven clinically isolated bacteria were used in this study. These bacteria were collected from two different hospitals in Jordan, Karak Governorate Hospital and Al Bashir Hospital, from 9/2019 to 2/2020. Among them, five Gram-negative bacteria and two Gram-positive bacteria. The gram-positive bacteria were Beta-lactamase producing *Staphylococcus aureus* and *Staphylococcus epidermidis*. The gram-negative bacteria were Beta-lactamase producing *E. coli*, *Enterobacter cloacae* complex, Beta-lactamase producing *Klebsiella pneumoniae*, *Shigella sp.* and Beta-lactamase producing *Pseudomonas aeruginosa*. *S. aureus*, *S. epidermidis*, *E. coli*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa* were obtained from urine samples of patients who were diagnosed with urinary tract infections whereas *Shigella sp.* was obtained from stool sample of patient who was diagnosed with enteritis. These species and the antibiotic profile were characterized by BIOMERIEUX VITEK® 2 SYSTEM. Antibiotics

In order to evaluate the synergistic effect of AgNPs with standard antibiotics, 16 types of antibiotics were used in this study. The antibiotics used in this study are listed in Table 1.

Disc Diffusion Method

The antibacterial activities of AgNPs and standard antibiotics were evaluated using the disc diffusion method according to Qaralleh et al.¹³ with some modifications. Briefly, 250 mL of bacterial

suspension adjusted to 10⁶ was mixed with 30 mL of melted Mueller-Hinton agar. After solidification, a sterilized disc (6 mm) containing AgNPs (0.5 mg/mL), standard antibiotics (Table 1), or negative control (DMSO) was transferred aseptically to the surface of the inoculated agar. Then, the plates were incubated at 37°C for 24 h and the inhibition zone diameter was measured as mm in diameter. Each sample was tested in triplicates.

Minimum Inhibitory Concentration of AgNPs (MIC)

The minimum inhibitory concentration of AgNPs (MIC) was evaluated using the disc diffusion method. Briefly, 250 mL of bacterial suspension adjusted to 10⁶ was mixed with 30 mL of melted Mueller-Hinton agar. After solidification, a sterilized disc (6 mm) containing different volumes of AgNPs or negative control (DMSO) was transferred aseptically onto the surface of the inoculated agar. Then, the plates were incubated at 37°C for 24 h and the inhibition zone diameter was measured as mm. The lowest concentration that gave less than 7 mm inhibition zone was reported as MIC. Each sample was tested in triplicates.

Synergistic Effect of AgNPs with Standard Antibiotics

The synergistic effect of AgNPs with standard antibiotics was evaluated using the disc

diffusion method.¹³ Briefly, 250 mL of bacterial suspension adjusted to 10⁶ was mixed with 30 mL melted Mueller-Hinton agar and left to solidify. Each of the standard antibiotic discs (Table 1) was impregnated with 10 µL AgNPs (0.5 mg/mL) and transferred to the surface of the inoculated agar. Then, the plates were incubated at 37°C for 24 h and the inhibition zone diameter was measured as mm in diameter. Each sample was tested in triplicates.

The synergistic effect was determined using the IFA (Increase in Fold Area) equation.¹⁴

$$IFA = \frac{B^2 - A^2}{A^2}$$

Where A and B Stand for:

A is the inhibition zone (mm) of the antibiotic alone.

B is the inhibition zone (mm) of the antibiotic in combination with AgNPs.

RESULTS AND DISCUSSION

Characterization of Biosynthesized AgNPs

The synthesized AgNPs were characterized using a spectrophotometer and TEM. In this study, the formation of AgNPs was initially indicated by visualizing the formation of a dark brown color. The shifting of the solution color was noted after 72h. The formation of AgNPs was confirmed using UV-vis spectroscopy analysis and an SPR peak was recorded. As shown in Figure 1, the UV spectra indicate a broad absorbance response with flat SPR peak with maximum absorbance recorded at 427 nm. In fact, the size, shape, and number of the formed nanoparticles are reflected in the intensity of the absorption peak.¹⁵ The

Table 1. Standard antibiotics used in this study

Antibiotic	Abbreviation	Concen. (µg/disc)
Tigecycline	TGC	15
Clindamycin	CD	2
Erythromycin	E	15
Gentamicin	CN	10
Chloramphenicol	C	30
Ampicillin	AMP	10
Amoxicillin	AML	25
Ertapenem	ETP	10
Azlocillin	AZL	75
Aztreonam	ATM	30
Vancomycin	VA	30
Cefoxitin	FOX	30
Ceftriaxone	CRO	30
Ciprofloxacin	CIP	5
Nitrofurantoin	F	300
Colistin	CS	10

Table 2. Inhibition zones (mm) and MIC (mg/mL) of AgNPs Synthesized by Biomass Free Filtrate of *A. flavus*

Bacteria Strain	Inhibition zone (mm)	MIC (mg/mL)
<i>S. aureus</i>	10.5±0.5	0.10
<i>S. epidermidis</i>	9.5±0.0	0.05
<i>E. coli</i>	11.0±0.0	0.075
<i>K. pneumonia</i>	10.0±0.5	0.025
<i>E. cloacae</i>	9.5±0.5	0.025
<i>Shigella sp.</i>	10.5±0.0	0.075
<i>P. aeruginosa</i>	7.0±0.0	>5

Each disc contains 0.50 mg/mL AgNPs.

formation of AgNPs appears to occur by one of two possible mechanisms, either by NADH-dependant nitrate reductase or by the shuttle quinine process.¹⁶ The results of the present study indicate that the formation of nanoparticles occurs in the extracellular media due to the action of extracellular nitrate reductase.

TEM images of the formed AgNPs were taken to verify the formation of AgNPs (Figure 2

and 3). The TEM image reveals that the formed nanoparticles are spherical in shape and the diameter of the nanoparticles is between 10 to 35 nm.

Antibacterial Activity of AgNPs

New antibacterial agents are still considered necessary due to the increasing prevalence of infections caused by resistant

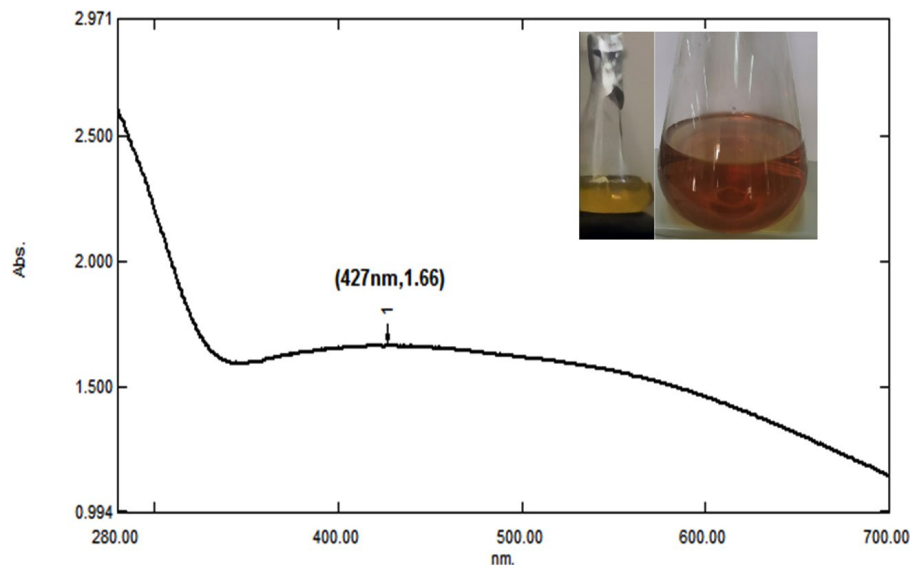


Figure 1. Biosynthesis of AgNPs using 1 mM AgNO₃ and fungal biomass free filtrate prior to the optimization process. (a) color change and (b) Ultraviolet-Visible spectra.

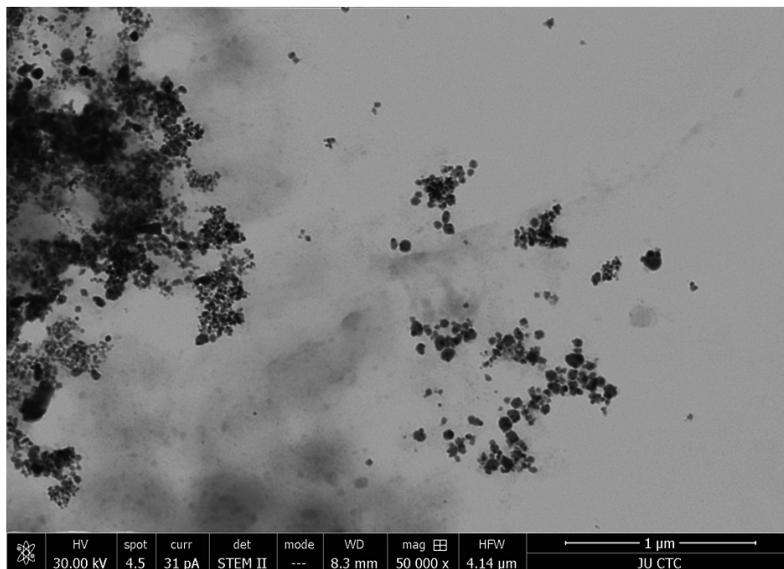


Figure 2. TEM image of silver nanoparticles. Magnification (1µm)

bacteria. Several protocols have been proposed to address the problem of antibiotic resistance. One of the most regularly used approaches is to use active ingredients derived from natural sources. Additionally, the use of nanoparticles is considered as one of the most favorable drug development strategies. The drug combination approach, which may present an agent with multiple target sites, is extremely beneficial in reducing antibiotic resistance.

In this study, the antibacterial activity of AgNPs was evaluated using the disc diffusion method (Table 2). In general, the AgNPs exhibited broad antibacterial activity against all strains tested except *P. aeruginosa*. The most sensitive strain was *E. coli* with an inhibition zone of 11 mm, followed by *S. aureus* (10.5 mm), *Shigella* sp. (10.5 mm), *K. pneumonia* (10 mm), *S. epidermidis* (9.5 mm), and *E. cloacae* (9.5 mm). The most resistant

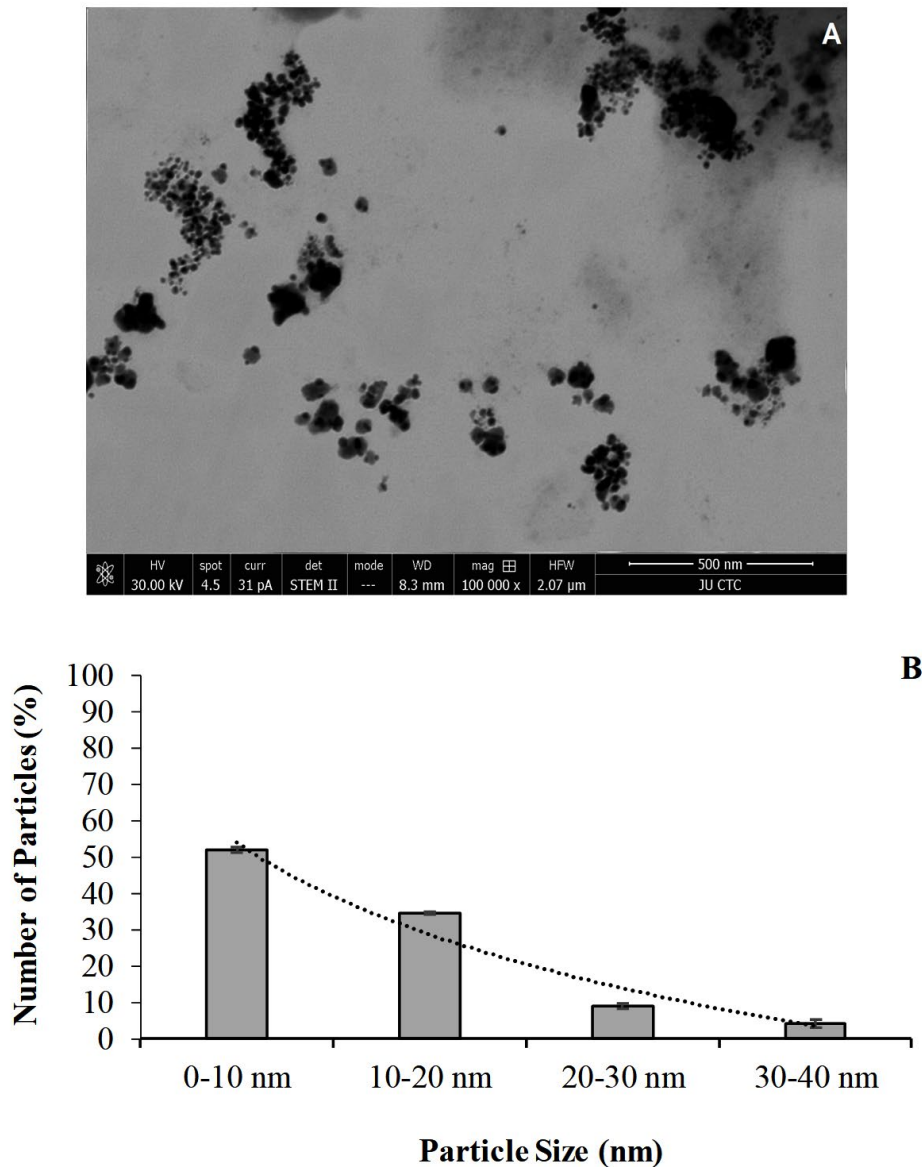


Figure 3. TEM image of silver nanoparticles and size-distribution histogram: A, TEM image (Magnification 500 nm); B, Size distribution histogram.

strain was *P. aeruginosa*, as indicated by the lowest inhibition zone (7 mm).

Also, the MIC was determined using the disc diffusion method (Table 2). The result of MIC is not in parallel with the result of the disc diffusion

method. The lowest MIC indicated was against *K. pneumonia* and *E. cloacae* (0.025 mg/mL) followed by *S. epidermidis* (0.050 mg/mL), *E. coli* (3 µL), *Shigella* sp. (0.075 mg/mL) and *S. aureus* (0.10 mg/mL). Based on the above results, it can be said

Table 3. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *S. aureus*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles	
Tigecycline (TGC)	20±0.5	30±0.5	1.3
Clindamycin (CD)	6±0.0	15±0.5	5.3
Erythromycin (E)	6±0.0	31±1.0	25.7
Gentamicin (CN)	13±0.5	25±0.0	3.8
Chloramphenicol (C)	20±1.0	24±0.0	0.44
Amoxicillin (AMP)	10±0.0	30±1.0	8.0
Ampicillin (AML)	6±0.0	33±0.0	29.25
Ertapenem (ETP)	15±1.0	15±0.0	0.00
Azlocillin (AZL)	6±0.0	20±1.5	10.1
Aztreonam (ATM)	15±1.5	40±1.0	6.1
Vancomycin (VA)	6±0.0	23±0.5	13.7
Cefoxitin (FOX)	22±1.5	45±0.5	3.2
Ceftriaxone (CRO)	6±0.0	25±0.5	16.4
Ciprofloxacin (CIP)	20±1.5	38±0.5	2.61
Nitrofurantoin (F)	10±0.0	23±1.0	4.3
Colistin (CS)	10±0.0	10±1.0	0.00

Table 4. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *S. epidermidis*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	6±0.0	20±0.5	10.1
Clindamycin (CD)	6±0.0	6±0.0	0.0
Erythromycin (E)	6±0.0	10±0.5	1.8
Gentamicin (CN)	15±0.5	20±0.0	0.8
Chloramphenicol (C)	6±0.0	10±0.0	1.8
Amoxicillin (AMP)	6±0.0	15±0.5	5.3
Ampicillin (AML)	6±0.0	15±1.5	5.3
Ertapenem (ETP)	13±0.5	15±0.5	0.3
Azlocillin (AZL)	6±0.0	10±0.5	1.8
Aztreonam (ATM)	6±0.0	17±0.0	7.0
Vancomycin (VA)	6±0.0	10±0.0	1.8
Cefoxitin (FOX)	20±0.5	22±0.5	0.2
Ceftriaxone (CRO)	6±0.0	10±0.5	1.8
Ciprofloxacin (CIP)	22±1.5	35±1.5	1.5
Nitrofurantoin (F)	7±0.0	17±0.5	4.9
Colistin (CS)	10±0.5	15±0.5	1.3

Table 5. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *E. coli*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	17±1.0	20±1.0	0.4
Clindamycin (CD)	6±0.0	10±1.0	1.8
Erythromycin (E)	6±0.0	10±0.0	1.8
Gentamicin (CN)	14±1.0	16±0.5	0.3
Chloramphenicol (C)	10±0.5	10±0.5	0.0
Amoxicillin (AMP)	6±0.0	6±0.0	0.0
Ampicillin (AML)	6±0.0	6±0.0	0.0
Ertapenem (ETP)	13±0.5	20±0.0	1.4
Azlocillin (AZL)	6±0.0	13±1.0	3.7
Aztreonam (ATM)	6±0.0	15±0.5	5.3
Vancomycin (VA)	6±0.0	10±0.5	1.8
Cefoxitin (FOX)	11±0.5	14±0.0	0.6
Ceftriaxone (CRO)	6±0.0	10±0.5	1.8
Ciprofloxacin (CIP)	20±0.5	24±0.5	0.4
Nitrofurantoin (F)	14±0.5	15±0.5	0.3
Colistin (CS)	13±0.5	16±0.5	0.5

that AgNPs possess broad-spectrum antibacterial activity. The inhibition of gram-positive and gram-negative bacterial species at low concentrations ranging from 0.025-0.10 mg/mL indicated the remarkable antibacterial activity of AgNPs.

AgNPs have long been recognized for their broad-spectrum antimicrobial activity.¹⁷ In general, the bacteriostatic effect of AgNPs was enhanced by their stability and the occurrence of biosurfactants in the cell-free filtrate used in the AgNPs formation experiment. In this study, AgNPs exhibited broad-spectrum antibacterial activity against both gram-positive and gram-negative bacteria. Our findings are consistent with those of Arokiyaraj et al.¹⁸ and Qaralleh et al.,¹³ Other researchers have demonstrated that AgNPs possess stronger antibacterial activity against gram-negative bacteria than gram-positive bacteria.¹⁹ The explanation for this may be referred to the nature of the cell wall.²⁰ Gram-positive bacteria have a thick layer of peptidoglycan in their cell wall, whereas Gram-negative bacteria have a thick layer of lipopolysaccharide followed by a thin layer of peptidoglycan.²¹ The nanosize of these particles enables them to have a large surface area in contact with the cell surface, resulting in a high probability of bacterial eradication.²² In contrast to that Ontong et al.²³ recently

reported that when *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans* were treated with AgNPs at a similar treatment period, the same pattern of potassium ions leaking, and morphological changes were observed. It appears as though the same mechanism of action affects both gram-positive and gram-negative bacteria regardless of their cell wall nature.

Although the precise antibacterial mechanism of action of AgNPs is unknown, several hypotheses have been advanced. According to one theory, AgNPs react with oxygen and thus disrupt the electron transport chain and decreased ATP levels in bacterial cells.²⁴ Additionally, AgNPs interfere with the plasma membrane and cause cell death. Another possibility was that AgNPs imposed their effect by inhibiting DNA unwinding.²⁵ Additionally, the antibacterial potential of AgNPs may be a result of oxidative stress caused by reactive oxygen species (ROS).⁷

The Synergistic Potential of the Synthesized AgNPs with Antibiotics

The synergistic potential of AgNPs with 16 standard antibiotics against two gram-positive

Table 6. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *Klebsiella pneumoniae*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	10±0.5	20±0.0	3.0
Clindamycin (CD)	7±0.0	10±1.5	1.0
Erythromycin (E)	6±0.0	6±0.0	0.0
Gentamicin (CN)	6±0.0	18±0.5	8.0
Chloramphenicol (C)	10±0.5	13±0.5	0.7
Amoxicillin (AMP)	6±0.0	15±0.5	5.3
Ampicillin (AML)	6±0.0	6±0.0	0.0
Ertapenem (ETP)	15±1.0	16±1.0	0.4
Azlocillin (AZL)	8±0.5	10±0.5	0.6
Aztreonam (ATM)	10±1.5	10±0.5	0.0
Vancomycin (VA)	6±0.0	17±1.0	7.0
Cefoxitin (FOX)	25±0.0	33±0.5	0.7
Ceftriaxone (CRO)	6±0.0	13±0.0	3.7
Ciprofloxacin (CIP)	13±1.0	30±0.0	4.3
Nitrofurantoin (F)	15±0.5	15±1.0	0.0
Colistin (CS)	15±0.5	17±0.5	1.3

Table 7. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *Enterobacter cloacae*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	10±0.5	20±0.0	3.0
Clindamycin (CD)	7±0.0	10±1.5	1.0
Erythromycin (E)	6±0.0	6±0.0	0.0
Gentamicin (CN)	6±0.0	18±0.5	8.0
Chloramphenicol (C)	10±0.5	13±0.5	0.7
Amoxicillin (AMP)	6±0.0	15±0.5	5.3
Ampicillin (AML)	6±0.0	6±0.0	0.0
Ertapenem (ETP)	15±1.0	16±1.0	0.4
Azlocillin (AZL)	8±0.5	10±0.5	0.6
Aztreonam (ATM)	10±1.5	10±0.5	0.0
Vancomycin (VA)	6±0.0	17±1.0	7.0
Cefoxitin (FOX)	25±0.0	33±0.5	0.7
Ceftriaxone (CRO)	6±0.0	13±0.0	3.7
Ciprofloxacin (CIP)	13±1.0	30±0.0	4.3
Nitrofurantoin (F)	15±0.5	15±1.0	0.0
Colistin (CS)	15±0.5	17±0.5	1.3

and five gram-negative bacteria was studied using the disk diffusion method.

Staphylococcus aureus

The synergistic effect of AgNPs with 16

different standard antibiotics against *S. aureus* was evaluated using the disc diffusion method. As shown in Table 3, the most effective antibiotics tested against *S. aureus* were cefoxitin, tigecycline, chloramphenicol, and ciprofloxacin with inhibition

Table 8. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *Shigella sp*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	15±0.5	17±0.0	0.3
Clindamycin (CD)	6±0.0	6±0.0	0.0
Erythromycin (E)	6±0.0	6±0.0	0.0
Gentamicin (CN)	15±0.5	15±0.5	0.0
Chloramphenicol (C)	10±0.0	10±0.5	0.0
Amoxicillin (AMP)	6±0.0	6±0.0	0.0
Ampicillin (AML)	6±0.0	7±0.0	0.4
Ertapenem (ETP)	10±1.5	10±1.0	0.0
Azlocillin (AZL)	6±0.0	6±0.0	0.0
Aztreonam (ATM)	10±0.5	10±0.5	0.0
Vancomycin (VA)	6±0.0	6±0.0	0.0
Cefoxitin (FOX)	20±0.5	20±0.5	0.0
Ceftriaxone (CRO)	6±0.0	6±0.0	0.0
Ciprofloxacin (CIP)	17±0.5	20±1.0	0.4
Nitrofurantoin (F)	7±0.5	7±1.0	0.0
Colistin (CS)	6±0.0	8±0.5	0.8

Table 9. Inhibition Zones (mm) of the antibiotics alone and Inhibition Zones (mm) of Antibiotics combined with AgNPs against *P. aeruginosa*

Antibiotic	Inhibition zone (mm)		IFA
	Antibiotic	Antibiotic: Nanoparticles (10 µm:Concen.)	
Tigecycline (TGC)	17±1.5	23±0.0	0.8
Clindamycin (CD)	6±0.0	20±0.5	10.1
Erythromycin (E)	10±0.0	30±1.0	8.0
Gentamicin (CN)	10±1.5	15±0.5	1.3
Chloramphenicol (C)	10±0.5	15±0.5	1.3
Amoxicillin (AMP)	6±0.0	15±0.0	5.3
Ampicillin (AML)	6±0.0	15±0.5	5.3
Ertapenem (ETP)	10±0.5	17±1.5	1.9
Azlocillin (AZL)	6±0.0	10±0.0	1.8
Aztreonam (ATM)	6±0.0	15±0.0	5.3
Vancomycin (VA)	6±0.0	34±0.5	31.1
Cefoxitin (FOX)	17±0.5	20±0.5	0.4
Ceftriaxone (CRO)	6±0.0	10±0.0	1.8
Ciprofloxacin (CIP)	20±0.5	25±0.5	0.6
Nitrofurantoin (F)	7±0.5	14±1.0	3.0
Colistin (CS)	10±0.0	10±1.0	0.0

zones ranging from 22-20 mm. However, *S. aureus* was resistant to clindamycin, erythromycin, ampicillin, azlocillin, vancomycin, and ceftriaxone since no inhibition zones were observed.

The result of the combination of AgNPs with antibiotics (Table 3) showed that 88% of

the combinations tested exhibited a synergistic effect against *S. aureus*. The antibacterial activity of AgNPs combined with all antibiotics except ertapenem and colistin was remarkably increased compared to antibiotics tested individually. This can be easily indicated by the increase in the

inhibition zone. All these active antibiotics tested in combination with AgNPs showed an increase in folding area (IFA). Interestingly, the combination of AgNPs with ampicillin and erythromycin exhibited a potent increase in IFA, reaching 29.3-fold and 25.7-fold, respectively. In addition, a remarkable increase in IFA was noted for the combined AgNPs with ceftriaxone, vancomycin, azlocillin, and amoxicillin with IFA of 16.4, 13.7, 10.1, and 8.0-fold, respectively. This is particularly interesting because *S. aureus* is resistant to all of the antibiotics mentioned previously.

Staphylococcus Epidermidis

As shown in Table (4), out of 16 antibiotics tested, 10 antibiotics showed no inhibitory activity against *S. epidermidis*. The most effective antibiotics tested against *Staphylococcus epidermidis* were ciprofloxacin and ceftiofloxacin with inhibition zones of 22 and 20 mm, respectively. Moderate inhibitory effects were reported for the antibiotics gentamicin, ertapenem, and colistin with inhibition zones ranging from 15-10 mm.

Whilst the most resistant antibiotics tested against *S. epidermidis* were tigecycline, aztreonam, amoxicillin, ampicillin, and nitrofurantoin, a remarkable increase in the inhibition zones was observed when AgNPs was combined with these antibiotics. The maximum IFA for the combined AgNPs with the standard antibiotics against *S. epidermidis* was reported for the antibiotics tigecycline and aztreonam, with an IFA of 10.1 and 7.0-fold, respectively. Rank second is the combination of AgNPs with amoxicillin, ampicillin, and nitrofurantoin that showed IFA of 5.3, 5.3, and 4.9-fold, respectively. All other combinations tested exhibited no more than 1.9 IFA.

Escherichia Coli

As shown in Table (5), *Escherichia coli* was considered resistant to clindamycin, erythromycin, amoxicillin, ampicillin, azlocillin, vancomycin, and ceftriaxone since no inhibition zones were observed. The most effective antibiotics tested against *E. coli* were ciprofloxacin with an inhibition of 20 mm. Moderate inhibitory effects were reported for the antibiotics tigecycline, gentamicin, nitrofurantoin, colistin, ertapenem, and ceftiofloxacin against *E. coli* with inhibition zones ranging from 17-11 mm.

The maximum synergistic effect was observed against aztreonam and azlocillin-resistant *E. coli* when these antibiotics were combined with AgNPs (IFA of 5.3 and 3.7, respectively). An IFA of 1.8-fold was noted when AgNPs were combined with clindamycin, erythromycin, vancomycin, and ceftriaxone. However, amoxicillin and ampicillin showed negative synergistic effects (0.0 increasing IFA). All other combinations tested showed a slight synergistic effect with AgNPs (IFA of no more than 1.4-fold).

Klebsiella Pneumoniae

As shown in table 6, *K. pneumoniae* was resistant to erythromycin, gentamicin, amoxicillin, ampicillin, vancomycin, and ceftriaxone. The most effective antibiotic tested against *K. pneumoniae* was ceftiofloxacin with an inhibition zone of 25 mm.

Gentamicin and vancomycin-resistant *K. pneumoniae* showed an increase in IFA of 8.0 and 7.0-fold, respectively, when these antibiotics were combined with AgNPs. The IFA of the combined AgNPs with amoxicillin, ciprofloxacin, ceftriaxone, and tigecycline were 5.3, 4.3, 3.7, and 3.0-fold, respectively. The activity of the non-effective antibiotics erythromycin and ampicillin were unchanged when it was synergized with AgNPs. All other combinations exhibited an IFA of less than 1.3-fold.

Enterobacter Cloacae

As shown in table 7, *E. cloacae* was resistant to erythromycin, amoxicillin, azlocillin, aztreonam, vancomycin, and ceftriaxone since no inhibition zones were observed. *E. cloacae* was sensitive to tigecycline, ceftiofloxacin, ciprofloxacin, and ertapenem.

E. cloacae resistant to erythromycin and vancomycin showed a maximum increase in IFA when these antibiotics were synergized with AgNPs (IFA of 10.0 and 9.0, respectively). The susceptibility of *E. cloacae* was unchanged when amoxicillin was combined with AgNPs. The IFA of the combined AgNPs with nitrofurantoin and ceftriaxone were 6.4 and 5.3, respectively. All other combinations exhibited an IFA of less than 1.4-fold.

Shigella sp.

As shown in Table 8, all combinations tested against *Shigella* sp showed no increase

in folding area and the inhibition zone produced by the antibiotics is similar to the inhibition zone produced by the combination of antibiotics with AgNPs. The exception to this was the slight synergistic effect of colistin, ciprofloxacin, ampicillin, and tigecycline with IFA of 0.8, 0.4, 0.4, and 0.3-fold, respectively.

Pseudomonas Aeruginosa

In general, all antibiotics tested in combination with AgNPs showed a synergistic effect against *P. aeruginosa* except colistin. Remarkably, the combination of AgNPs with vancomycin, clindamycin, and erythromycin leads to an increase in the fold area of inhibition by up to 31.1, 10.1, and 8.0-fold, respectively. However, *P. aeruginosa* was resistant to vancomycin and clindamycin (Table 9). The IFA of the combined AgNPs with amoxicillin, ampicillin, aztreonam, and nitrofurantoin were 5.3, 5.3, 5.3, and 3.0, respectively.

According to previous reports, AgNPs in combination with standard antibiotics has synergistic effects against pathogenic bacteria.²⁶ Synergistic effects were reported when silver nanoparticles were combined with β -lactams, glycopeptides, aminoglycosides, and sulphonamides antibiotics.²⁷ A synergistic effect has been found between AgNPs and vancomycin against *E. coli* (a 10.1-fold increase). The Vancomycin, Cefotaxime, Ampicillin, Kanamycin, Amikacin, Cefepime resistant strains of *S. epidermidis*, *E. coli*, and *K. pneumonia* were susceptible to these antibiotics when they were combined with AgNPs²⁸ showed that AgNPs possess a clear synergistic effect against *E. coli* when they are combined with azithromycin, cefotaxime, cefuroxime, fosfomycin, and chloramphenicol. Ciprofloxacin in combination with AgNPs contributed to a 40% increase in the zone of inhibition compared to the inhibition zone of ciprofloxacin alone against *E. coli*.²⁹ An increase in Folding Area (IFA) was observed when AgNPs were combined with ampicillin, streptomycin, and vancomycin against *E. coli*, *P. aeruginosa*, and *S. aureus*.³⁰ The synergistic effect was reported when AgNPs were combined with penicillin G, amoxicillin, clindamycin, erythromycin, and vancomycin against *E. coli* and *S. aureus*.³¹

In this study, both gram-positive and gram-negative bacteria were susceptible to the

combination of AgNPs with standard antibiotics. *S. aureus* was the most susceptible strain among gram-positive bacteria, while *P. aeruginosa* and *E. cloacae* were the most susceptible strains among gram-negative bacteria. Birla et al.,³⁰ found that gram-negative bacteria (*E. coli* and *P. aeruginosa*) are more susceptible to the combination of AgNPs and antibiotics than gram-positive bacteria (*S. aureus*). However, other studies have shown that gram-positive bacteria are more susceptible to this combination than gram-negative bacteria. The IFA for the combined AgNPs with penicillin G, amoxicillin, and vancomycin against the gram-positive strain *S. aureus* was higher than the IFA of the gram-negative strain *E. coli*.³¹ On the other hand, several mechanisms for the synergistic effects of AgNPs and antibiotics have been proposed. The AgNPs may act as a carrier and deliver the antibiotics to the target site. Also, the synergist activity may result from the double force of binding AgNPs and antibiotics. In this case, the AgNPs may increase the permeability of the plasma membrane and facilitate the entry of antibiotics³² proposed a four-step pathway to understanding the mechanism underlying AgNP's synergy with antibiotics: tetracycline, kanamycin, neomycin, and Enoxacin. AgNPs form a complex containing antibiotics, which increases their interaction with target cells. This event will lead to an increase in the concentration of Ag + ions in the vicinity of the target cell and eventually to its death. Thirumurugan and colleagues³³ showed that the pharmacodynamic interaction between AgNPs and antibiotics ends with an enhanced level of reactive oxygen species (ROS), followed by microbial membrane damage and K⁺ ion leakage and inhibition of biofilm formation, resulting in the killing of the target microbe.³³

CONCLUSION

The results of this study showed that biogenic synthesized AgNPs improved the inhibitory effect of several antibiotics against beta-lactamase producing isolates, suggesting that using these two medicines together can reverse beta-lactam resistance in beta-lactamase producing isolates. It may be a potential treating strategy against beta-lactamase producing bacteria. However, more investigations are

required to confirm the synergistic effect using more reliable tests such as the checkerboard assay. Also, studies of the mode of antibacterial action, cytotoxicity, and blood compatibility are necessary.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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