

RESEARCH ARTICLE

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Silver Nanoparticles (AgNPs) Biosynthesized by *Aspergillus flavus* KF946095; their Characterization and Antibacterial Activity

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Abstract

The antimicrobial agents of silver nanoparticles (AgNPs) have been applied a little while back in diverse therapeutic studies. In this analysis, AgNPs were biosynthesized using an ecologically welcomed and cost-effective simple of bio-reduction. An isolate of *Aspergillus flavus* KF946095 (*A. flavus*) was found to biosynthesize AgNPs; the size of AgNPs was (56nm) and detected by UV-Vis analysis at (400 nm). The reducing properties for biosynthesis of AgNPs are mainly due to the protein functional surface reactive groups detected by Fourier Transform Infrared spectroscopy (FTIR).Whereas, FTIR for AgNPs showed different peaks at 3994.5, 3201.6, 1801.4, 1643.2 and 1604.7 cm^{-1} that shared with the biosynthesize and stability of AgNPs as protein capping agents. Transmission Electron Microscope (TEM) confirmed the scattering of biosynthesized AgNPs within a sol with oval and round shapes. The antibiotic susceptibility test was studied for some pathogenic bacteria. *Staphylococcus aureus* DSM 1104 (*S. aureus*) appeared to be the more resistant strain; it resisted the action of 6 antibiotics out of 8 ones tested. MIC value of AgNPs was 20 $\mu\text{g}/\text{mL}$ and antibiotic ciprofloxacin was 30 $\mu\text{g}/\text{mL}$. Mixture of MIC values or double MIC values distinctively inhibited the multidrug resistant (MDR) *S.aureus*.

Keywords: Silver nanoparticles, *Aspergillus flavus*, FTIR, *Staphylococcus aureus*, antibacterial activity

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(Received: September 10, 2020; accepted: December 25, 2020)

Citation: El-Mekkawy RM, Almanaa TN, Yassin MA, Rabie G, Saleh N. Silver Nanoparticles (AgNPs) Biosynthesized by *Aspergillus flavus* KF946095; their Characterization and Antibacterial Activity. *J Pure Appl Microbiol.* 2021;15(1):105-113. doi:10.22207/JPAM.15.1.05

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INTRODUCTION

The existence of antibiotic resistant strains is increased recently and their inhibition by alternative agents are necessary (Goffeau, 2008; Enan et al., 2013; Osman et al., 2016; El-Gazzar., et al., 2020 Abdel-shafi, 2020; Enan et al., 2020). *S. aureus* is a Gram-positive, coccid-shaped, facultative anaerobic and it forms yellow colonies on routine agar medium and forms black colonies with halo zones after its growth on its specific medium Baird Parker agar. *S. aureus* is non-motile, non-spore former, ferments glucose and produces lactic acid; it shows both α - and β - blood hemolysis capability and is characterized by positive coagulase reaction (Colombari et al., 2007).

Previous studies (Osman et al., 2016; Enan et al., 2020) isolated and characterized MDR *S. aureus* from both foods and patients. Consequently, the biocontrol of the *S. aureus* pathogen is of interest for detection incoming effective protocols to inhibit such pathogen in *vitro*. In this regard, there is an inevitable and urgent medical need for developing other novel antimicrobial mechanisms with eco-friendly properties' (kim et al, 2008; Jo et al., 2009; Abdelshafi 2020; El-Sayed, 2020).

From the Elders, silver and its compounds have been considered to be Functional Antimicrobial active factors (Silver, 2003; El-Gazzar and rabie, 2018; Ngozi et al., 2019; El-Gazzar and Enan 2020; El-Gazzar and Ismail, 2020; Samson et al., 2020). Nano-silver has received particular attention as a possible antimicrobial agent, owing in particular to recent advances in metal nanoparticles research (Melaiye et al., 2005). Silver nanoparticles have been widely subjected due to their physicochemical properties (Lok, 2006) and are recently applied as anti-bacterial factors in food storage and health industries. The anti-microbial activity of Ag-Nps has now been well recognized and they are definite to possess anti-inflammatory, anti-viral, anti- platelet and anti-fungal efficiency (Panacek et al., 2009).

The nanoparticles syntheses are characterized by an effective and a potent role in application as novel antimicrobial factors (El-Gazzar and Enan 2020). The newly nano-products have already applied against pathogenic bacteria (Kim et al., 2008; El-Gazzar and Ismail,

2020). So, the biosynthesis and functionalization of nanoparticles create the most potent antimicrobial products. Thus, this work was aimed to biosynthesize of AgNPs by *A. flavus* and investigates the antimicrobial effect of AgNps alone or in combination with antibiotics to inhibit MDR *S. aureus*.

MATERIALS AND METHODS

The fungal strain used for biosynthesis of AgNPs

The fungal strain used in this study for biosynthesis of AgNPs was *Aspergillus flavus* KF946095. It was isolated from soil contaminated with wastes from Photographic Industries (El-Sharkia Governorate, 80 km North Cairo, Egypt) (El-Gazzar, 2015). It was stored in glass beads and subcultured into potato dextrose broth (Oxoid) (Abdel-Salam et al., 2001).

Biosynthesis of AgNPs

A. flavus KF946095 was isolated, identified and characterized to possess the capability to biosynthesize AgNPs by its extracellular enzyme to reduce AgNO_3 solution at 1mM to AgNPs according to El-Gazzar and Rabie, (2018). The color of AgNO_3 at the beginning of the experiment was colorless and the developed AgNPs were visually monitored from the intensity of yellow-brown color. The concentration and physical properties of the developed AgNPs were determined (El-Gazzar et al., 2020).

Characterization of the bio synthesized AgNPs

The prepared post-biotransformation fluid of bio synthesized AgNPs was detected with (T80+UV Flash spectrophotometer PG Instrument LTD), (DeAlba-Montero et al., 2017). The diameter of particles determined by nanosizer (Malvern, UK) at agriculture research Center, Egypt (Halima and Archana, 2016). The morphology of AgNPs was subjected by Transmission electron microscope (TEM) (JEOL.JEM.1010) in National Research Center, Egypt. Silver nanoparticles size, shape and size distribution profile were determined (Jain et al., 2011). Fourier transform infrared spectroscopy (FTIR) (Thermo Nicolet model 6700 spectrum) in Micro-Analytical Center, Egypt, using KBr methods with 400–4000 cm^{-1} range (DeAlba-Montero et al., 2017).

Antibiotics susceptibility test of some pathogenic bacteria

Staphylococcus. aureus DSM 1104,

Streptococcus. pyogenes ATCC 19615, *Listeria. monocytogenes* LMG10470, *Escherichia. coli* LMG 8223, *Klebsiella. pneumonia* ATCC 43816 and *Pseudomonas. aeruginosa* LMG 8029 were subjected.. Stock bacterial cultures were routinely were sub-inoculated in brain heart infusion broth (BHIB) (Oxoid) (Abdel-shafi et al., 2016), then kept at 4°C for uses in this investigation.

The susceptibility of Gram positive bacteria; *S. aureus* DSM 1104, *St. pyogenes* ATCC 19615, *L. monocytogenes* LMG10470 and Gram negative bacteria; *E. coli* LMG 8223, *K. pneumonia* ATCC 43816 and *P. aeruginosa* LMG 8029 to 8 antibiotics were tested by standard disc diffusion technique (Enan et al., 1996; Enan e t al., 2014; Abdel-shafi et al.,2019). The cultures were grown in nutrient broth (Oxoid) for 12 h. Inocula were subjected at 10⁵ CFU/mL and then were plated onto Muller Hinton agar (Hi-Media, Mumbai, India). The following antibiotic discs with their concentrations determined in parenthesis were applied (All from Johnson& Johnson, Egypt. Branch, Heliopolis, Cairo, Egypt) viz. clindamycin (DA: 2 µg), ampicillin (AM: 10 µg), tetracycline (TE: 30 µg), ciprofloxacin (CIP: 5 µg), ofloxacin (OFX: 5 µg), amoxicillin (AMC: 30µg), penicillin G (P: 10 µg), methicillin (ME: 5 µg). These antibiotics were put within Muller Hinton agar dishes which

were inoculated by the tested bacteria; plates were then inverted and incubated at 37°C for 24 h (Enan et al., 2018). Results were indicated by determination inhibition zones diameters (IZDs) by millimeters as described by CLSI (2008). The indicator of Multiplied antibiotics impedance was determined according to Gu et al., (2003); Enan et al.,(2014); Abdel-shafi et al., (2019).

Antibacterial assay of AgNPs

Brain Heart infusion agar plates (DifcoTM, Maryland, USA) were prepared and seeded with log phase cells (10⁵ CFU/mL) of *S. aureus* DSM 1104 as it was the most resistant organism to the antibiotics tested, then the sterile singly AgNPs were applied to discs (6mm diameter) which were added onto the above plates compared with controls discs that saturated with sterile distilled water. All samples were incubated at 37°C for 24–48 h. IZDs was determined (Aziz et al., 2014; El-Gazzar and Enan, 2020).

Determination of minimum inhibitory concentration (MIC) of AgNPs

The antibacterial activity of AgNPs was studied by different concentration of AgNPs discs and placed onto Muller Hinton agar (DifcoTM, Maryland, USA) that seeded previously with activity growing cells of *S. aureus* (10⁵ CFU/mL). MIC was visually measured trough the minimum

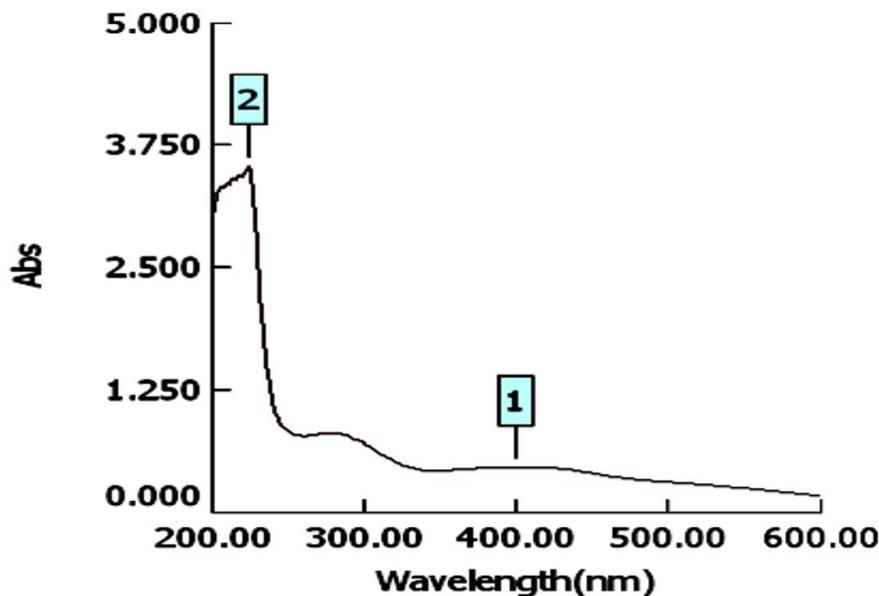


Fig. 1. The UV-Visible spectrum showing the absorption peak of of AgNPs at 400 nm

concentricity of AgNPs which suppress bacterial development (Abdelshafi et al., 2019 a, b, c, d).

The efficiency of Ciprofloxacin and AgNPs incorporation against bacteria

The antibiotic Ciprofloxacin that inhibited the *S. aureus* DSM 1104 was combined with MIC concentration of AgNPs. Sterile filter paper discs were impregnated either by the antibiotic only or by the AgNPs only and also by combinations them and tested for their antimicrobial activity against *S. aureus* as described above. Mixtures of AgNPs with Ciprofloxacin were prepared as follows: (30 µg/mL Ciprofloxacin + 20 µg/mL AgNPs) and (60 µg/mL Ciprofloxacin + 40 µg/mL AgNPs (Osman et al., 2016)

RESULTS

The preliminary investigation showed that the *A. flavus* KF946095 changed the color of medium into yellowish brown indicating on conversion of AgNO₃ into AgNPs as revealed from the necessary analysis used in further experiments.

For characterization whether the yellowish brown fluids contain AgNPs or no; this suspension was subjected to spectral scan UV-visible which had maximum absorption at 400 nm corresponding to surface Plasmon resonance indicating the formation of AgNPs (Fig. 1). The diameter of nano-product showed an average particle size at 56 nm that based on intensity

distribution (Fig. 2). Moreover, TEM measurements showed well distribution of AgNPs without any agglomeration at 6.49 nm with oval and round-outline (Fig. 3). The results of FTIR for AgNPs included different peaks at 1643.2 cm⁻¹, 1604.7 cm⁻¹ and 1801.4 cm⁻¹ (Fig. 4). The band at 1643.2 cm⁻¹ and 1604.7 cm⁻¹ classified into the amide bands. Whereas, the band at 1643.2 cm⁻¹ arises due to carbonyl stretch, unsaturated aldehyde, ketone and alkene group. While the band at 1604.7 cm⁻¹ occurs because of N H stretching vibrations in the protein's amide linkages. At 1801.4 cm⁻¹ is assigned to c=c stretch alkenyl stretch from the proteins in the solution which is a useful tool for quantifying secondary structure in metal nanoparticles protein interaction. In addition, it shows the presence of 3201.6 and 3994.5 cm⁻¹ bands. The band 3201.6 cm⁻¹ is characteristic of amide II band also alchole / phenol O=H stretch. At 3994.5 cm⁻¹, the band reflected the N=H stretch at the amide.

The results were extended to study the antibiotic sensitivity test of different pathogenic bacteria as given in Materials and Methods. Results are given in Table (1). MAR index of the indicator bacteria tested could be arranged in the following descending manner: 75%; 62.5%; 37.5%; 25% for *S. aureus*; *Listeria monocytogenus*; *Pseudomonas aeruginosa* and *Escherichia coli*, respectively (Table, 1). Since the antibiotic ciprofloxacin was the more effective antibiotic and *S.aureus* bacterium

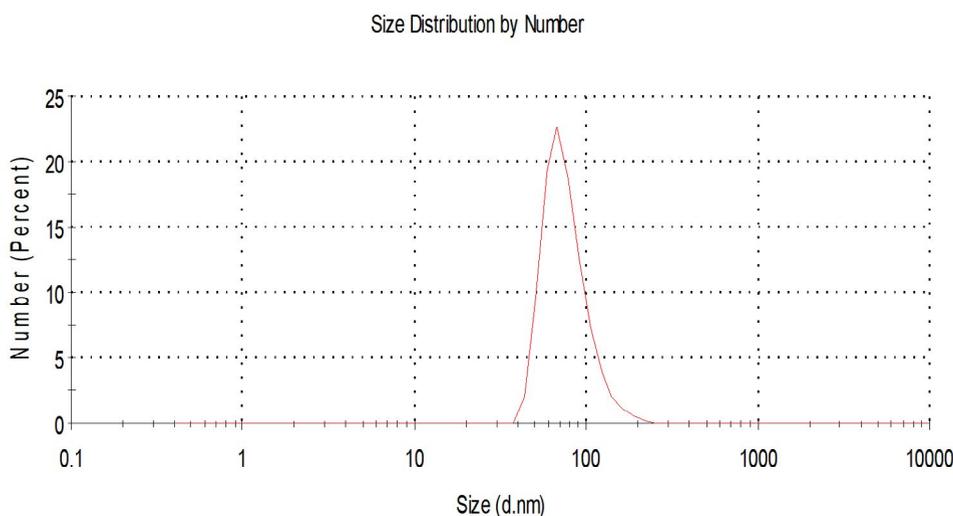


Fig.2. Histogram shows particle size distribution analyzed by Dynamic light scattering system (DLS) Zeta sizes; an average particle size based on intensity distribution for AgNPs at 56 nm.

was possessed the finger MAR index (Multidrug resistant), they were selected for further study.

The experiments were investigated the possibility to inhibit the *S.aureus* strain that was resistant to 8 antibiotics tested by different concentrations of AgNPs Table (2). AgNPs inhibited

the MDR *S. aureus* strain. The inhibition was dependant on concentration. Inhibitory activity against *S. aureus* was increased with higher concentrations of AgNPs. Minimum inhibitory concentration (MIC) of AgNPs was 20µg/mL. MIC of ciprofloxacin was 30µg/ disc.

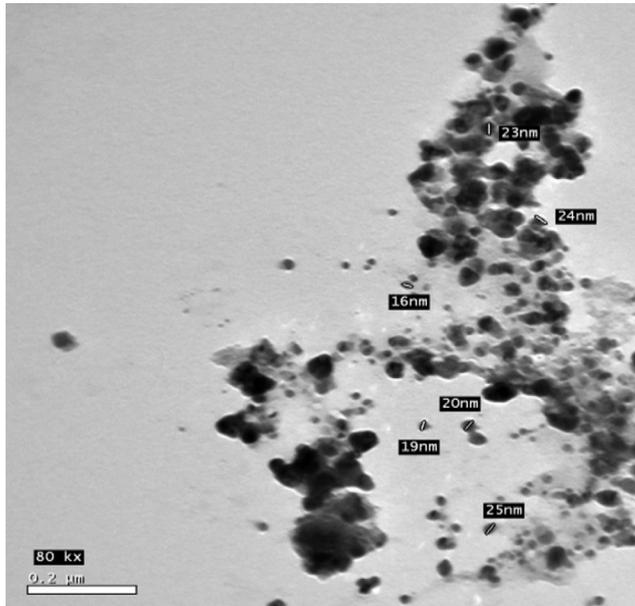


Fig. 3. The shape and size of AgNPs under Transmission Electron Microscope (TEM).

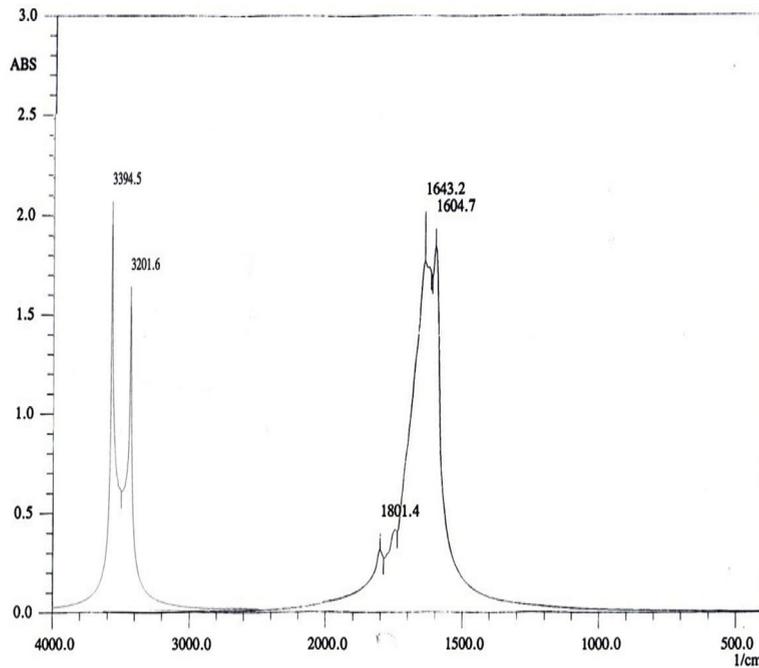


Fig. 4. FTIR measurements for biosynthesized AgNPs.

The experiments were further extended to check whether mixtures of both the antibiotic ciprofloxacin (the more effective antibiotic) and AgNPs will give greater inhibitory activity against the MDR *S. aureus* or no. Mixtures of MIC values

of both the antibiotic ciprofloxacin and AgNPs showed obvious and distinctive inhibition of MDR *S. aureus* more than that obtained by either ciprofloxacin or AgNPs alone. Double amounts of MIC values showed distinctive inhibition of MDR

Table 1. Antibiotic sensitivity test of the studied bacteria based on the diameter of inhibition zone (mm)

Bacterial strains	DA	AM	TE	CIP	OFX	AMC	P	ME	MAR index
Gram positive									
<i>Staphylococcus aureus</i> DSM 1104	0	0	3	3	0	0	0	0	6 (75%)
<i>Streptococcus pyogenes</i> ATCC 19615	0.9	0	3	2.1	0	1.2	1.7	0.9	2 (25%)
<i>Listeria monocytogenes</i> LMG10470	2.9	0	0	3	0	1	0	0	5 (62.5%)
Gram negative									
<i>E.coli</i> LMG 8223	0.9	0	1	2.1	0	1.6	0	0.9	3 (37.5%)
<i>Pseudomonas aeruginosa</i> LMG 8029	0.8	0	0	2.1	0	0	0	1.7	5 (62.5%)
<i>Klebsiella pneumoniae</i> ATCC 43816	0	2.9	0	0	0	1.1	1.7	1.1	3 (37.5%)
No. of strains (%)	2(6) 33.3%	4(6) 66.67%	2(6) 33.3%	1(6) 16.67%	6(6) 100%	2(6) 33.3%	4(6) 66.67%	2(6) 33.3%	

Multiple antibiotic resistance index was calculated by using the following formula: MAR Index = Number of antibiotics to which the isolate was resistant/Total number of antibiotics tested. Clindamycin (DA: 2 µg), Ampicillin (AM: 10 µg), Tetracycline (TE: 30 µg), Ciprofloxacin (CIP: 30 µg), Ofloxacin (OFX: 5 µg), Amoxicillin (AMC: 30µg), Penicillin G (P: 10 µg), Methaicillin (ME: 5 µg)

Table 2. Inhibitory activity of different concentrations of AgNPs and Ciprofloxacin against *S. aureus*

The used aged	Concen. of (µg/mL)	IZD(mm)
AgNPs	10	0.00
	20	12.00±0.00
	30	15.20±0.01
	40	18.81±0.61
	50	34±0.11
	60	45±0.00
	70	46±0.45
	80	47±0.00
	90	48±0.42
	100	50±0.00
Ciprofloxacin	5	0.00
	10	0.00
	20	0.00
	30	3.00±0.00

All values reflect the mean values of 3 replicates and standard. * Minimum inhibitory concentration (MIC) of AgNPs and Ciprofloxacin.

S. aureus also; indicating on synergism between ciprofloxacin and AgNPs (Table,3)

DISCUSSION

The detection of a newly research for suppressing and blocking the various impedance

Table 3. Inhibition of *S.aureus* by combinations of both ciprofloxacin and AgNPs

The used aged	IZDs (mm)
Ciprofloxacin 30µg/mL(MIC)	3.00±0.00
Ciprofloxacin 60µg/mL	9.5±0.31
AgNPs 20µg/mL (MIC)	12.00±0.00
AgNPs 40µg/mL	18.81±0.01
Ciprofloxacin 30µg/mL+20µg AgNPs (MIC values)	28.00±0.11
Ciprofloxacin 60 µg/mL+ 40µg/mL AgNPs	62.00±0.00

All values reflect the mean values of 3 replicates and standard.

microscopic organisms to antibiotic is urgent (Enan *et al.*, 2013; Enan *et al.*, 2014; Abdel-Shafi *et al.*, 2020). Nanotechnology introduce effectiveness ways in inhibition of pathogenic multidrug resistant bacteria (Gu *et al.*, 2003; El-Gazzar and Ismail 2020).The emergence of nanotechnology in the last decade offers recent biotechnology in development of drugs based nanoparticles (El-Gazzar and Enan, 2020). From the obtained data, the absorbance of AgNPs detected at 400 nm, this is similar to latter published results in this respect (El-Gazzar and Rabie, 2018; El-Gazzar and Enan, 2020).

In addition, the size of such nanoparticles was 56nm. Latter published results showed the ability of fungal strains to biosynthesize nanoparticles (El-Gazzar and Ismail, 2020). The obtained single peak indicated that the quality of the biosynthesized nanoparticles is well suitable (Nakkala *et al.*, 2017).Also, AgNPs was scattered with oval and round shapes. Moreover, the results confirmed the presence of different molecules which are responsible on synthesis with stabilization of AgNPs. This is in confirming with previous results in this respect (El-Gazzar *et al.*, 2020).

FTIR measurements of the prepared nanoparticles were carried out to identify the possible biomolecules presented in nanoparticles solution, for determination the stabilizing mechanism for AgNPs. The FTIR results of AgNPs detected the presence of adverse biomolecules; this is coupled with latter published work (El-Gazzar *et al.*, 2020).Thus, the existence of diverse biomolecules with nanoparticles increases its stability and ability in metal degradation (Dhoble and Kulkarni, 2016; El-Gazzar and Rabie, 2018).

The results employed herein approved that the *S. aureus* was multidrug resistant bacteria, such strain showed by many authors also to resist the action of different antibiotics authors also to resist the action of different antibiotics (Osman *et al.*, 2016). Inhibition of such MDR *S. aureus* by AgNPs is of interest.

Antibacterial potential of biologically synthesized silver nanoparticles was assessed. AgNPs were found to be extremely potent with inhibition of MSR *S. aureus* observed at 20 µg/ml concentration against all test organisms. In addition, to investigate if any synergism existed

between the ciprofloxacin antibiotic and AgNPs in their antimicrobial action, mixtures containing MIC values or their doubles of them were tested. These combinations showed distinctive inhibition of MDR *S. aureus* than that obtained by either Ciprofloxacin or AgNPs.

Silver was characterized by its higher efficiency versa microorganisms. Although the exact underlying mechanisms are yet to be fully elucidated, other literature has indicated that AgNPs interested by the permeability of cell membrane and in turn interferes with the bacterial respiratory chain. In addition, their connection with reactive oxygen species generation (e.g., hydrogen peroxide) and resultant oxidative stress and cell damage (Aziz *et al.*, 2014; Loo *et al.*, 2015).

The findings of the present study have yet again highlighted the significance of the combination between antibiotics and AgNPs as potential antimicrobial agents (Prasad *et al.*, 2016). The extract needs to be analyzed further for isolation and identification of active principles. Biogenic silver nanoparticles are not only extremely effective antimicrobial agents, as deduced from this study, but also economically. Although the AgNPs and antibiotic displayed significant antibacterial activity, a mixture containing both in much lower concentration that have the same efficiency, through a synergistic potency (Abdel-shafi *et al.*, 2019). Further work will be necessary to study the toxicity of AgNPs against living tissues and to study the mode of action of AgNPs alone and in combinations with antibiotics.

CONCLUSION

A. flavus KF946095 showed ability to biosynthesize AgNPs that were characterized by possible instrumental analysis herein. One strain of *S. aureus* DSM 1104 showed to resist the action of many antibiotics; it was inhibited by different AgNPs. The antibiotic ciprofloxacin and AgNPs in combinations showed distinctive inhibition of MDR *S. aureus* DSM 1104.

ACKNOWLEDGMENTS

Authors would like to thank Prof. Dr Gamal Enan Dean of Faculty of science, Zagazig University, Zagazig, Egypt for his revising the work and the isolates of bacteria.

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.

FUNDING

None.

DATA AVAILABILITY

All datasets analyzed in the study are included in the manuscript and presented as tables and figures.

ETHICS STATEMENT

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

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