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Efficacy of Antibiotics Combined with Biosynthesized Silver Nanoparticles on some Pathogenic Bacteria

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Abstract: ***Biosynthesis of Silver Nanoparticles (AgNPs) has received great attention due to their numerous advantages like being rapid, simple and eco-friendly protocol. In the present study, weused cultures supernatant of pathogenic bacteria and fungi like Staphylococcus aureus 29737 ATCC, Staphylococcus epidermidis 12228 ATCC and Klebsiella pneumoniae 700603 ATCC,as well as cell free filtrate of Fusarium sp. and Aspergillus flavusfor rapid synthesis of silver Nanoparticles.UV- visible spectrometry study revealed the plasma resonance from420 to 440nm.Transmission electron microscope of AgNPs showed the mean average size of most particles was ˜ 11,29.9, 16.8, 23.4 and 23.4 nm for Staphylococcus aureus, Staphylococcus epidermidis and Klebsiella pneumoniae, Fusarium sp. and A. flavusrespectively. The Fourier Transmittance Infrared spectrum (FT-IR) confirmedthe reduction of the silver ions and forming stable and control shaped silver nanoparticles due to the protein component resulting from the enzyme nitrate reductase.Results of this study revealed that the synthesized silver nanoparticles have potential antibacterial activity and were also capable of enhancing the efficiencies of some commercial antibiotics.***

Keywords: Biosynthesis of silver nanoparticles, UV-Vis spectrophotometer, FTIR, TEM antibacterial, antibiotic enhancer

1. Introduction

Emerging infectious diseases and the increase in incidence of drug resistance among pathogenic bacteria and fungi have made the search for new antimicrobials inevitable. In the current situation, one of the most promising and novel therapeutic agents are the nanoparticles. The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials[1].

Nanotechnology finds its application in various areas of medicine ranging from diagnosis, therapeutic drug delivery to treatment of many diseases. Silver nanoparticles (AgNPs) are one of the promising products in the field of nanotechnology because of its application as antimicrobials, therapeutics, biomolecular detection and catalysis [2].The most widely used and known applications of silver and AgNPs are in the medical industry. These include topical ointments and creams containing silver to prevent the infection of burns and open wounds [3].

Microbial synthesis of nanoparticles is a green chemistry approach that interconnects nanotechnology and microbial biotechnology. Biosynthesis of gold, silver, gold–silver alloy, selenium, tellurium, platinum, palladium, silica, titania, zirconia, quantum dots, magnetite and uraninite nanoparticles by bacteria, actinomycetes, fungi, yeasts and viruses have been reported. However, despite the stability, biological nanoparticles are not monodispersed and the rate of synthesis is slow. To overcome these problems, several factors such as microbial cultivation methods and the extraction techniques have to be optimized and thecombinatorial approach such as photobiological methods may be used [4].

Fungi have only been used to produce a few types of nanoparticles, and research in this area has only gained attention in the last few years. The main advantages of using fungi over bacteria for NPs synthesis are: their large biomass enables easy handling during biosynthesis [5]. High metal tolerance and economic viability [4]. Bioaccumulation ability and larger protein secretions [6]. Ease of scale up andhigh wall binding ability [7].

The purpose of the present study involves the extracellular biosynthesis of AgNPs using pathogenic bacteria like *Staphylococcus aureus*, *Staphylococcusepidermidis* and *Klebsiella Pneumoniae* and pathogenic fungi like Fusarium *sp.* and *Aspergillus flavus*; Moreover evaluation of their antimicrobial activity against various human pathogens and their capability of enhancing some commercial antibiotics.

2. Materials and Methods

2.1. Preparation of bacterial and fungal cultures

Staphylococcus aureus 29737 ATCC,*Staphylococcus epidermidis* 12228 ATCC,*Klebsiella pneumoniae* 700603 ATCC,*Fusarium sp.* and *Aspergillus flavus*were provided from Microbiology Laboratory of Applied Studies Center of Medical Plants (National Organization for Drug Control and Research), bacterial cultures maintained in Mueller-Hinton medium and fungal cultures maintained in potato dextrose

agar as well as subcultured from time to time to regulate its viability during study period.

2.2. Biosynthesis of silver nanoparticles by Bacteria

Bacteria cultures were freshly inoculated on Mueller-Hinton broth and incubated for 24 hours at 37°C. The culture was centrifuged at 12,000 rpm for 5 minutes, and the supernatant was used for the synthesis of silver nanoparticles (AgNPs). Deionized water was used as solvent in the synthesis of AgNPs. The supernatant was added separately to the reaction vessel containing silver nitrate ($AgNO_3$) at concentrations of 1mM, 2 mM and 3 mM (1% v/v).The reaction between this supernatant and Ag+ ions was carried out in bright conditions for 5 minutes [8].

2.3. Biosynthesis of silver nanoparticlesby fungi

Erlenmeyer flasks (500ml) contains 200 ml of sterile potato dextrose broth were then inoculated with 5mm disks taken from *Fusarium* sp. and*Aspergillus flavus* (7days-old). The flasks were incubated in the dark at 25± 2^{O}C for 1 week. For silver nanoparticles (AgNPs) bioproduction, the culture was filtered (Whatman filter paper No.1) at the end of incubation period to separate the biomass from filtrate followed by extensive washing with deionized water to remove any medium component from biomass. Typically 20 g of fresh biomass was brought in contact with 100 ml of deionized water for 72 hrs at 28±2 ^{O}C in an Erlenmeyer flask. After incubation cell filtrates was obtained by passing through Whatman filter paper No.1 and 50 ml of *Fusarium* sp. and*Aspergillus flavus* cell free filtrates were completed to 100 ml of 1mM of $AgNO_3$ in 250 ml Erlenmeyer flasks. Positive controls containing cell free filtrate without silver nitrate and only 1mM silver nitrate as negative control were also maintained [9].

2.4. Estimation and Characterization of silver nanoparticles

2.4.1. UV-Vis Spectrometry

The UV-visual spectra of the samples were recorded by spectrophotometer (Nicolet evolution 100, Cambridge) with digital data acquisition, wavelength range 220– 600 nm. The UV spectrophotometer present in biochemistry laboratory of Applied Studies Center of Medical Plants (National Organization for Drug Control and Research).

2.4.2. Fourier Transformer Infra Red (FT-IR)

Theinstrument used is AKX0901119012A0607, genesis series Nicolet IS-10 F, Infrared spectrophotometer (IR) in Egyptian Petroleum Research Institute (EPRI).

2.4.3. Dynamic Light Scattering

Technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution.When light hits small particles, the light scatters in all directions (Rayleigh scattering) as long as the particles are small compared to the wavelength (below 250 nm). The scattering intensity fluctuates over time. This fluctuation is due to the fact that the small molecules in solutions are undergoing Brownian motion, the larger the particle, the slower the Brownian motion will be [10]. The size and size distribution of particles in the colloids were measured using a Nano ZS zetasizer system Malvern Instruments in Egyptian Petroleum Research Institute (EPRI). Measurement parameters were as follows: a laser wavelength of 633 nm (He–Ne), a scattering angle of 173^{O}C(fixed—without changing possibility), a measurement temperature of 25^{O}C, a medium viscosity of 0.8872mPa.s and a medium refractive index of 1.330, and material refractive index of 1.59. Before DLS measurement, the colloid was passed through a 0.2 µm polyvinylidene fluoride (PVDF) membrane. The sample was loaded into quartz microcuvette.

2.4.4. Transmission Electron Microscopy (TEM)

TEM was performed using JEOL-JEM 2100 Electron microscope operating at 200KV in Egyptian Petroleum Research Institute (EPRI).TEM studies were done at magnification 1.5 X and resolution up to 0.143 nm. Samples were prepared by placing a drop of solution with the silver nanoparticles (AgNPs) on carbon-coated TEM grids. The films on the TEM grids were allowed to dry at room temperature before analysis.

2.4.5. Determination of antibacterial activity by well diffusion method

The AgNPs synthesized from bacteria and fungi was tested for its antibacterial activity against pathogenic bacteria such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,*Escherichiacoli* and *Pseudomonas aeruginosa* by standard well diffusion method in Mueller-Hinton Agar (MHA) plates. Pure cultures of bacterial pathogens were grown in nutrient broth at 37^{O}C for 18-24 hours. Wells were made on the Mueller-Hinton agar plates using a gel puncture with diameter 4mm. The plates were inoculated by swabbing the bacterial pathogens to create a confluent lawn of bacterial growth. Then 100 µl of the biosynthesized AgNPs solution were, poured on to corresponding well using a micropipette. As control 100 µl of 1 mM $AgNO_3$ solution were poured on to control well. After incubation at 37^{O}C for 24 hours, diameter of zone of inhibition in millimeter around each well was measured[11].The assay was repeated in triplicate.

2.5. Synergistic antibacterial activity combined with antibiotics

Antibacterial enhancing activity of biosynthesized silver nanoparticles in combination with some important commercial antibiotics was also evaluated as per disk diffusion method. In this method, the standard antibiotic discs with concentrations 10 µg ampicillin, 30 µg chloramphenicol, 30 µg tetracycline, 30 µg cefotaxime and piperacillin/tazobactam 110 µg(Oxoid manufacture) were impregnated with different biosynthesized silver nanoparticles (10 µl AgNPs) by*S.aureus*, *S.epidermidis* and *K.pneumoniae*and placed onto Mueller-Hinton agar inoculated with test organisms (*S.aureus*, *S.epidermidis*, *K.pneumoniae*, *E.coli* and*P.aeruginosa*). These plates were then incubated at 37^{O}C for 24 hours. The formation of zone inhibition (if any) was observed and measured in millimeters and compared with Control (antibiotic discs without silver nanoparticles). In case of absence zone of inhibitionthe standard diameterof antibiotic itself was 6mm.

3. Results and Discussion

3.1. UV-Vis Spectrometry :

The formation and stability of the reduced AgNPs in thecolloidal solution was monitored by using UV–Vis spectralanalysis. It is one of the importanttechniques to ascertain the formation of metal nanoparticles, provided surface plasmon resonance exists for the metal. The reaction of silver nitrate solution with the culture supernatant *K. pneumoniae*, *S. epidermidis*and *S. aureus* started within a few minutes and the color of solution turned to yellowish brown, indicating the formation of AgNPs. The observation indicated that the reduction of the Ag+ ions took place extracellularly (Figure 1 A-F).

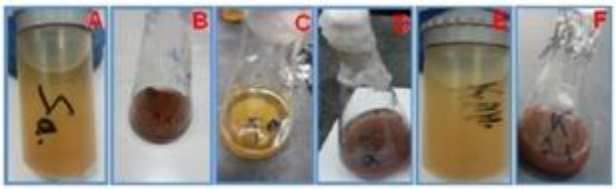

Figure 1: The conversion of silver nitrate to nano silver by*Staphylococcus aureus* (A- Culture, B- AgNPs synthesis);*Staphylococcus epidermidis*(C- Culture, D-AgNPs synthesis) and *Klebsiella pneumoniae*(E- Culture, F-AgNPs synthesis) using culture supernatant

Also aqueous silver nitrate ions were reduced during exposure to the *Fusarium* sp.and *Aspergillus flavus* cell free filtrate. The color of the reaction mixture changed from pale yellow to yellowish brown which indicates the formation of silver nanoparticles. Silver nanoparticles showed yellowish brown color solution after 72 hrs of incubation and the color increase by time (Figure 2 A-F).

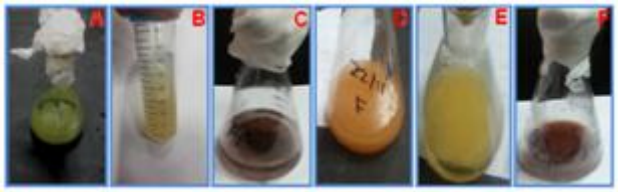

Figure 2: The conversion of silver nitrate to nano silver by *Aspergillus flavus* (A- in potato dextrose broth, B- cell free filtrate, C-cell free filtrate after addition $AgNO_3$) and *Fusarium* sp. (D- in potato dextrose broth, E- cell free filtrate F- cell free filtrate after addition $AgNO_3$)

Also the color change from pale yellow to dark brown colorobserved by[12]when cell free extract of *K.pneumoniae* challenged with 1mM and 2mM $AgNO_3$ and the color intensity increased with increasing incubation period. Beside that control without silver ions showed no change in color of the cell filtrates when incubated under the same conditions which agree totally with our results. Also the brown color of silver nanoparticles solution synthesized by *E.coli* observed by [13], and they explained that cells in the mid of exponential phase or starting range of stationary phase of culture (maximum reproduction of microorganism) produce maximum activity of enzymes involved in aggregation and reduction of silver ions to produce silver nanoparticles. In that concern, [14] showed that the synthesis of maximum cadmium sulfide during the stationary phase. Their results are suggesting that the formation silver nanoparticle has maximum activity at stationary phase of *Bacillus licheniformis*.

TheUV–Vis spectra recorded from449nm for culture supernatant of *S.aureus* when it subjected on 1mMof $AgNO_3$and broad peaks was noted around 422nm and 420nm when culture supernatant of *K. pneumoniae*and *S. epidermidis*was subjected to 1mM of $AgNO_3$ subsequently as in (Figure 3A, B and C). **Jeevan *et al.*** [15] observed a characteristic strong broad peak located between 420 and 430 nm that confirmed the biosynthesis of AgNPs by *Pseudomonas aeruginosa* that agree with our results. It has been hypothesized that nitrate reductase enzyme shuttle electron from nitrate to the metal group and its role in biosynthesis of silver nanoparticles.Also UV-Vis spectra of *Fusarium sp.* and *Aspergillus flavus* cell free filtrates showed maximum absorption peaks (SPR) at 419.60nm and 421 nm subsequently (Figure 1D and 1E).

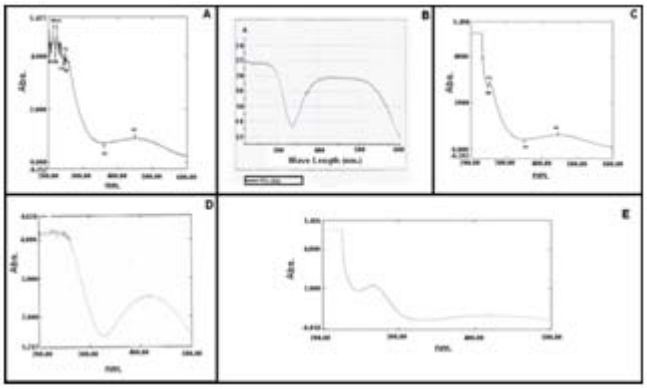

Figure 3: Synthesis of AgNPs by: -A)culture supernatant of *Staphylococcus aureus*;B) culture supernatant of *Klebsiella pneumoniae*;C)culture supernatant of *Staphylococcus epidermidis*;D)cell free filtrate of *Aspergillus flavus* and E)cell free filtrateof *Fusarium* sp.by using 1mM of $AgNO_3$

3.2. Fourier Transformer Infra Red (FT-IR)

FT-IR spectrum analysis of silver nanoparticles synthesized by *S. aureus* showed intense absorption bands at 3441.75, 2923.99, 1630.94, 1384.35, 1034.41 and 539.41 cm^{-1}as in (Figure 4A). Silver nanoparticles synthesized by *S.epidermidis* showed intense absorption bands at 3290.76, 2925.31,1629, 1539.31, 1384.8, 1236.3, 1077.8 and 549 cm^{-1}as in (Figure 4B). Also,silver nanoparticles synthesized by *K.pneumoniae* showed intense absorption bands at 3420, 2926.35, 1626.8, 1386.6, 1317.5 and 670 cm^{-1} as in Figure (4C). The band around 3420, 3290 and 3441 cm^{-1} attributes almost entirely to the N–H stretch vibrations of the peptide linkages or hydroxyl group of carboxylic acid. Further, the signature of stretching vibrations of methylene groups of proteins was noticed at bands number (2923, 2925 and 2926 cm^{-1}). The presence of amide carbonyl stretching referred to the bands number (1630.94, 1629 and 1626.8 cm^{-1}). Thus FT-IR spectrum signatures showed that protein component resulting from nitrate reductase enzyme present in the sample is responsible for reduction of silver ions. It is well known that protein is involved in binding and stabilizing of silver nanoparticles. Also, **Jayalakashmi and Dinakaran**

[16]showed intense bands by FT-IR of silver nanoparticles synthesized by marine *E. coli* (3410 and 2940 cm^{-1})referring to primary and secondary amines, the presence of amide carbonyl stretching referred to the bands (1629, 1637 and 1654 cm^{-1}). They presumed the protein as reducing and capping agent forming stable and control shaped silver nanoparticles that agreed with our results. It also confirmed by [17] the protein component of nitrate reductase enzyme is responsible for formation of silver nanoparticles associated with subtle variations in biological components of the culture supernatant of *P. aeruginosa* since nitratereduction is the phenotypic biochemical characteristic of this culture and the capping is possibly due to the rhamnolipids present in the culture supernatant.

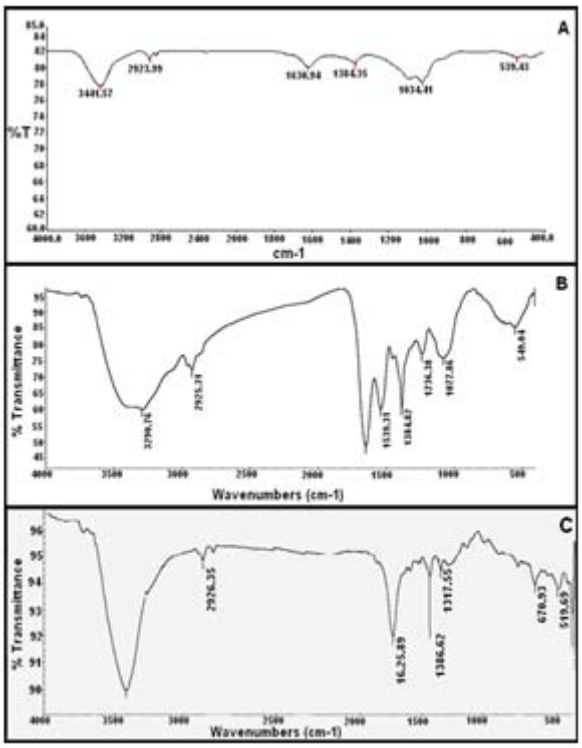

Figure 4: FTIR recorded from spectra of AgNPs synthesized by culture supernatant of A) *Staphylococcus aureus*; B) *Klebsiella pneumoniae*and C) *Staphylococcus epidermidis*

The FTIRspectrum of *A. flavus* cell free filtrate after adding silver nitrate is included in (Figure 5A). Data revealed two bands (1643.34 and 1543 cm^{-1}) that corresponds to the bending vibrations of the amide I and amide II bands of the proteins respectively; while their corresponding stretching vibrations were seen at (3440 and 2926 cm^{-1}) respectively and 1247 cm^{-1} C–O stretching vibration of carboxylic acids or glycogen. These results revealed the involvement of C=O, amide I and amide II in reduction of silver ion and capping of silver nanoparticles. AgNPs synthesized by cell free filtrate of *Fusarium* sp.,(Figure 5B) showed band at 3445 cm^{-1} is attributed to O-H and N-H stretching and the band at 2088.5 cm^{-1} is assigned to the stretching vibration of amide II. Whereas bending vibration of amid I and amide II were observed at (1636 and 1461 cm^{-1}) respectively, the band at 1161 cm^{-1} is stretching vibration of carboxylic acids or glycogen. Beside that[18] purified nitrate reductase from *Fusarium oxysporum* and a peptide, phytochelatin, for the in vitro synthesis of silver nanoparticles in the presence of a co-factor, NADPH and the nanoparticles were stabilized by the capping peptide, phytochelation.

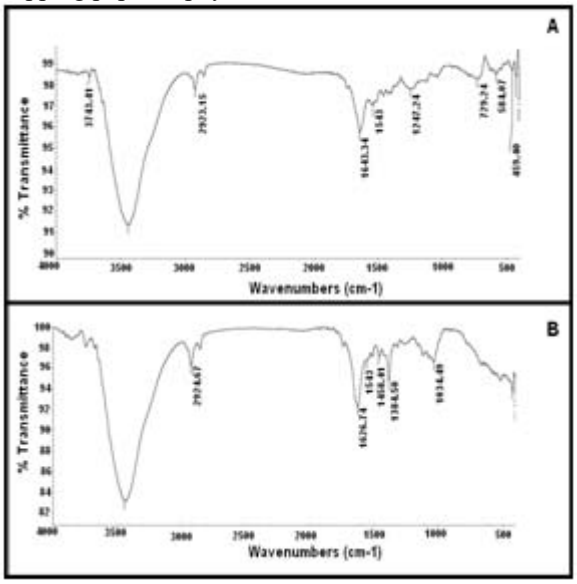

Figure 5: FTIR recorded from spectra of AgNPs synthesized by Cell free filtrate of A)*Aspergillus flavus*and B)*Fusarium* sp.

3.3. Dynamic Light scattering

The curves of size distribution demonstrate that diameter of AgNPs synthesized by *S. aureus* is around 58.76 nm (Figure 6A), by *K. pneumoniae*is around 78.82nm (Figure 6B) and by *S. epidermidis* isaround 91nm (Figure 6C). However The curves of size distribution demonstrate that diameter of AgNPs synthesized by *Fusarium* sp. is around 122.4 nm, and by *Aspergillus flavus* is around 78.82nm (Figure4D and 4E). Measurements parameters were as follows: dispersant RI = 1,330; viscosity = 0,8872cP; temperature = 25^{O}C.Measurements uncertainties were calculated as the standard deviation as in figures (7A, B, C, D and E).

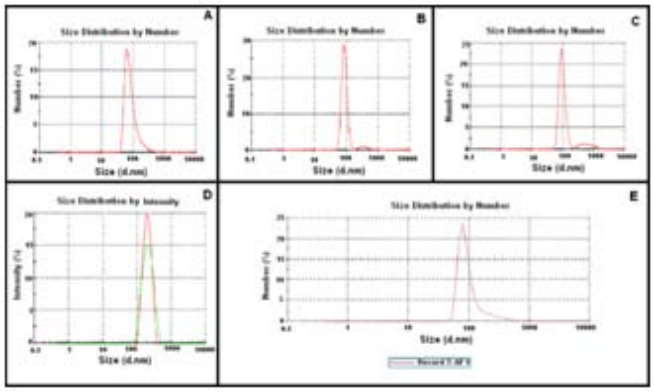

Figure 6: Size distribution curve of AgNPs by DLS method repeated three times for the sample A) *Staphylococcus aureus*;B)*Klebsiella pneumoniae*; C)*Staphylococcus epidermidis*; D) *Fusarium* sp. and E)*Aspergillus flavus*

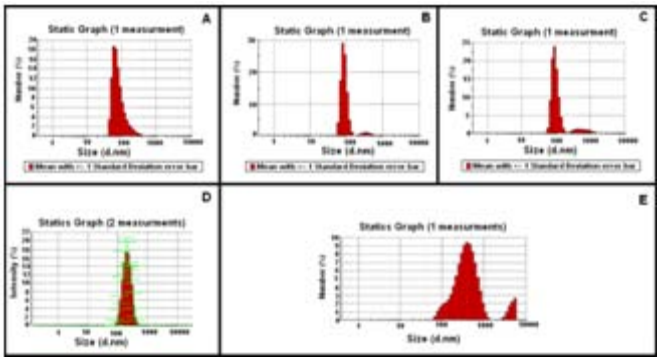

Figure 7: Static graph for size distribution of AgNPs by DLS method for the sample A) *Staphylococcus aureus* B)*Klebsiella pneumoniae*; C)*Staphylococcus epidermidis*; D) *Fusarium* sp. and E)*Aspergillus flavus*

The radius of silver nanoparticle (AgNPs) by S. aureus is 29.36 nm for 18.8% mean number of the sample. The radius of silver nanoparticle (AgNPs) by *K.pneumoniae* is 39.41nm for 29% the mean number of the sample. The radius of silver nanoparticle (AgNPs) by *S. epidermidis* is 45.5 nm for 23.9% the mean number of the sample. The radius of silver nanoparticle by *Fusarium* sp. is 29.36 nm for 17.9% mean number of the sample. The radius of silver nanoparticle by *Aspergillus flavus* is 39.41nm for 23.7 % the mean number of the sample.

Dynamic Light Scattering (DLS) depend on the light interaction with particle, large particles can screen the smaller one as [19]explained that Sample polydispersity can distort the results, and we could not see the real populations of particles because big particles presented in the sample can screen smaller ones.

3.4. Transmission Electron Microscope(TEM):

TEM is powerful method to determine the size of nanoparticles. TEM analysis showed that most particles synthesized by *S. aureus* had a size of ~11nm with spherical shape. Most particles synthesized by *K. pneumoniae* had a size of mean average of ~ 16.88nm with spherical shape. AgNPs synthesized by *S. epidermidis* had a size of mean average ~ 29.9 nm with spherical shape (Figure 8 A, B and C).TEM images for AgNPsof *Fusarium* sp.(Figure 8 D) showed spherical shape to some ellipsoidal with little assembling in the size range between 7.9 and 48.43 nm. The mean average size is ~ 23.4 nm. On other hand, TEM images of *Aspergillus flavus* AgNPs (Figure 8 E) showed AgNPs of varied shaped. It contains spherical, irregular, rod and few triangle shapes and also aggregates of nanoparticles. The size of AgNPs ranged between 13.11 and 48.90 nm with mean average size is ~ 23.4 nm which is in agreement with the results of [20] showed spherical, irregular and few triangles shaped silver nanoparticles and also aggregates of nanoparticles to form rosette shape were observed and the nanoparticles were 23.88 in size.

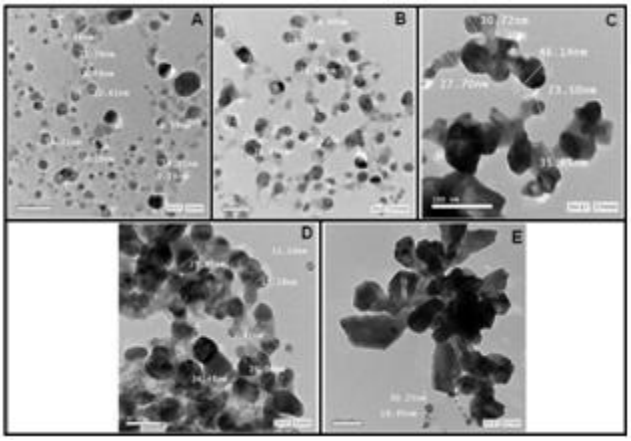

Figure 8: TEM image of developed AgNPs by A)*Staphylococcus aureus*;B)*Klebsiella pneumoniae*; C)*Staphylococcus epidermidis*; D) *Fusarium* sp. and E)*Aspergillus flavus*

In our results, it's clear that the size of silver nanoparticles synthesized in range between 5 and 45 nm,also spherical silver nanoparticles synthesized by bacteria as *Aeromonas sp.* SH10, *E. cloacae*, *K. pneumoniae*, *E. coli*, *Bacilluslicheniformis* and *Morganella sp* with size rangedbetween6.4 and 52.5 nm, fungi as*Fusarium oxysporum* in the range of 5~50nm andcyanobacteria as *Spirulinaplatensis* and *Nostoc* sp. in the range of 5~40nm that agree with our results [4][21][22].

3.5. Antibacterial activity of AgNPs synthesized by Pathogenic Bacteria and Fungi:

The antibacterial activity of biosynthesized silvernanoparticles was performed against both gram positive bacteria (Staphylococcus *aureus*,*Staphylococcus epidermidis*) and gram negative bacteria(*Klebsiellapneumoniae*, *E.coli and Pseudomonas aeruginosa*)by well diffusion method.The AgNPs synthesized by pathogenic bacteria and fungi showed excellent antibacterial activity againstall tested bacterial strains at volume of 100µl/well compared to silver nitrate solution as in Table (1).**Theivasanthi and Alagar** [23]agree with our study by showing that silver nanoparticles have antibacterial activities more than silver.

In the present investigation, after 24 hours of incubation, AgNPs synthesized by bacterial supernatant of *Klebsiella pneumonia* give highest inhibition zone (19 mm) followed by AgNPs synthesized by bacterial supernatant of *S. epidermidis* against *S. aureus* (16 mm). Beside that AgNPs synthesized by bacterial supernatant of *Klebsiella pneumonia* give highest inhibition zone (20.3 and 16.3mm) followed by AgNPs synthesized by bacterial supernatant of *S. aureus* (19.6 and17.3 mm) against *S. epidermidis* and *E. coli* subsequently.

Table 1: Antibacterial activity of AgNPs prepared by pathogenic bacteria and Fungi against different pathogenic bacteria (Inhibition zone by mm)

Tested organisms	$AgNO_3$ as control	AgNPs by *S. aureus*	AgNPs by *S. epidermidis*	AgNPs by *K. pneumoniae*	AgNPs by*Fusarium* sp.	AgNPs by *A. flavus*
S. aureus	11	15.3	16	19	13.5	15
S. epidermidis	11	19.6	15.5	20.3	15.5	15
P. aeruginosa	11	17	17	16	11	11
K. pneumoniae	10.6	16.3	16	16	14.6	15
E. coli	10	17.3	13.6	16.3	15.5	13

Concerning other gram negative bacteria like *Pseudomonas aeruginosa, Klebsiella pneumoniae*, AgNPs synthesized by bacterial supernatant of *S. aureus* and *S. epidermidis* give the highest inhibition zone (17 and 16mm) compared to other biosynthesized silver nanoparticles. On other hand AgNPs synthesized by cell free filtrate of *Fusarium*sp. and *A. flavus*show lowest inhibition zone against gram positive and negative bacteria compared to AgNPs synthesized by bacteria supernatant (Table 1).The mechanism of the bactericidal effect of silver nanoparticles is not very well-known. It is believed that cellular proteins become inactive after treatment with silver nanoparticles. Silver nanoparticles after penetration into the bacteria have in-activated their enzymes, generating hydrogen peroxide and caused bacterial cell death.Heavy metals are toxic and react with proteins, therefore they bind protein molecules; as a result cellular metabolism is inhibited causing death of microorganism.It is known that silver sources such as silver nitrate and silver sulfadiazine release Ag^+ only but high activity of silver nanoparticles is attributed to the release of Ag^0 and Ag^+ clusters when they dissolve [24].It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, which affects bacterial cell viability.Silver ions (particularly Ag^+) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or can react with sulfur-containing proteins, leading to the inhibition of enzyme functions. Inthe present study the diameter of Ag nanoparticles is 11, 16.8 and 29.9 nm.The general understanding is that Ag nanoparticle of typically less than 20 nm diameters get attached to sulfur-containing proteins of bacterial cell membranes leading to greater permeability of the membrane, which causes the death of the bacteria [25].

3.6. Synergistic antibacterial activity combined with antibiotic

Due to overuse of antibiotics and growing problem of antibiotic resistance, nanoparticles are being researched as an alternative antibacterial agent, the inhibitory activity of the silver nanoparticles was evaluated against and their potency was assessed qualitatively by the presence of inhibition zone. Different pathogenic bacteria exhibit different susceptibilities to nanoparticles. The result obtained on the efficacy of silver nanoparticles (synthesized by *Staphylococcus aureus*, *Klebsiella pneumoniae*and *Staphylococcus epidermidis*) in combination with antibiotics revealed overall increase of zone inhibition size which indicated the increase in antibacterial potential of all antibiotics against the test bacterial strains as in tables (2,3 and4).

Table 2: Synergistic effect of different antibiotics with or without AgNPs synthesized by *Staphylococcus epidermidis* against some pathogenic bacteria

Types of Antibiotics	Name of the Antibiotics	Pathogenic Bacteria	Zone of Inhibition (mm)		Increased ZoneSize(mm)
			Ab*	Ab+AgNPs	
Cephalosporins 3rd generation	Cefataxime	*S.aureus*	22	23	1
		E.coli	28	30	2
		S. epidermidis	14	18	4
		K.pneumoniae	Nil	7	1
		P.aeruginosa	Nil	7	1
Sulphonamides	Chloramphenicol	*S.aureus*	25	31.6	6.6
		E.coli	25	28	3
		S. epidermidis	25	26	1
		K.pneumoniae	Nil	1	4
		P.aeruginosa	Nil	7	1
	Tetracycline	*S.aureus*	36	38.5	2.5
		E.coli	22	26	4
		S. epidermidis	7	14	7
		K.pneumoniae	Nil	10	4
		P.aeruginosa	Nil	7	1
Penicillin	Ampicillin	*S.aureus*	37.5	39	1.5
		E.coli	18	21	3
		S. epidermidis	13	15	2
		K.pneumoniae	Nil	8	2
		P.aeruginosa	Nil	7	1
Penicillin Combination	Piperacillin/ Tazobbactum	*S.aureus*	34	37	3
		E.coli	22	27	5
		S. epidermidis	15	16	1
		K.pneumoniae	21	26	5
		P.aeruginosa	10	11	1

Table 3: Synergistic effect of different antibiotics with or without AgNPs synthesized by *Staphylococcus aureus* against some pathogenic bacteria

Types of Antibiotics	*Name of the Antibiotics*	*Pathogenic Bacteria*	*Zone of Inhibition (mm)*		*Increased ZoneSize(mm)*
			Ab*	Ab+AgNPs	
Cephalosporins 3rd generation	Cefataxime	*S.aureus*	22	24	2
		E.coli	28	30	2
		S. epidermidis	14	20	6
		K.pneumoniae	Nil	7	1
		P.aeruginosa	Nil	7	1
Sulphonamides	Chloramphenicol	*S.aureus*	25	35	10
		E.coli	25	28	3
		S. epidermidis	25	26	1
		K.pneumoniae	Nil	10	4
		P.aeruginosa	Nil	8	2
	Tetracycline	*S.aureus*	36	39	3
		E.coli	22	26	4
		S. epidermidis	7	14	7
		K.pneumoniae	Nil	9	3
		P.aeruginosa	Nil	7	1
Penicillin	Ampicillin	*S.aureus*	37.5	39	1.5
		E.coli	18	21	3
		S. epidermidis	13	17	4
		K.pneumoniae	Nil	7	1
		P.aeruginosa	Nil	7	1
Penicillin Combination	Piperacillin/ Tazobbactum	*S.aureus*	34	39	5
		E.coli	22	27	5
		S. epidermidis	15	16	1
		K.pneumoniae	21	25	4
		P.aeruginosa	10	11	1

Table 4: Synergistic effect of different antibiotics with or without AgNPs synthesized by *Klebsiella pneumoniae* against some pathogenic bacteria

Types of Antibiotics	Name of the Antibiotics	Pathogenic Bacteria	Zone of Inhibition (mm)		Increased ZoneSize(mm)
			Ab*	Ab+AgNPs	
Cephalosporins 3rd generation	Cefataxime	*S.aureus*	22	23	1
		E.coli	28	31	3
		S. epidermidis	14	17	3
		K.pneumoniae	Nil	7	1
		P.aeruginosa	Nil	Nil	Nil
Sulphonamides	Chloramphenicol	*S.aureus*	25	28.5	2.5
		E.coli	25	26	1
		S. epidermidis	25	28	3
		K.pneumoniae	Nil	10	4
		P.aeruginosa	Nil	8	2
	Tetracycline	*S.aureus*	36	37	1
		E.coli	22	27	5
		S. epidermidis	7	12	5
		K.pneumoniae	Nil	8	2
		P.aeruginosa	Nil	9	3
Penicillin	Ampicillin	*S.aureus*	37.5	39	1.5
		E.coli	18	21	3
		S. epidermidis	13	14	1
		K.pneumoniae	Nil	7.5	1.5
		P.aeruginosa	Nil	Nil	Nil
Penicillin Combination	Piperacillin/ Tazobbactum	*S.aureus*	34	37	3
		E.coli	22	30	8
		S. epidermidis	15	16	1
		K.pneumoniae	21	27	6
		P.aeruginosa	10	11	1

It was also observed that there was no zone inhibition in the control as some antibiotics alone in case of *E.coli*, *K. pneumoniae*and *P.aeruginosa*.The highest increase zone size was observed for chloramphenicol (6.6, 10 and 2.5 mm) then piperacillin/tazobactum against *S.aureus* (3, 5and 3mm) for AgNPs by *S. epidedrmidis*, *S.aureus* and *K.pneumoniae*(Tables 2 , 3 and 4).On other hand tetracycline showed highest size increase against *S. epidermidis* (7, 7 and 5mm). Also chloramphenicol with AgNPs showed high increase zone size with gram negative bacteria with *K.pneumoniae.*The diameter of inhibition zones for antibiotics in combination with silver nanoparticles showed significant increase in zone size in all cases.

Also Antibacterial activity of silver nanoparticles produced from two fungi (*Fusarium* sp. and *A.flavus*) were carried out using AgNPs alone and in combination of different commercially available antibiotics against five pathogenic bacteria using the disc diffusion method (Tables 5 and 6).

Table 5: Synergistic effect of different antibiotics with or without AgNPs synthesized by *Fusarium* sp.against some pathogenic bacteria

Types of Antibiotics	Name of the Antibiotics	Pathogenic Bacteria	Zone of Inhibition (mm)		Increased ZoneSize(mm)
			Ab*	Ab+AgNPs	
Cephalosporins 3rd generation	Cefataxime	*S.aureus*	22	24.6	2.6
		E.coli	28	30	2
		S. epidermidis	14	14	Nil
		K.pneumoniae	Nil	11	5
		P.aeruginosa	Nil	9	3
Sulphonamides	Chloramphenicol	*S.aureus*	25	30.3	5.3
		E.coli	25	26	1
		S. epidermidis	25	25	Nil
		K.pneumoniae	Nil	11	5
		P.aeruginosa	Nil	11	5
	Tetracycline	*S.aureus*	36	37.5	1.5
		E.coli	22	27	5
		S. epidermidis	7	12	5
		K.pneumoniae	Nil	10	4
		P.aeruginosa	Nil	10	4
Penicillin	Ampicillin	*S.aureus*	37.5	39.5	1.8
		E.coli	18	20	2
		S. epidermidis	13	20	7
		K.pneumoniae	Nil	9.5	3.5
		P.aeruginosa	Nil	11	5
Penicillin Combination	Piperacillin/ Tazobbactum	*S.aureus*	34	37.5	3.5
		E.coli	22	12	-5
		S. epidermidis	15	14	-1
		K.pneumoniae	21	24.5	2.5
		P.aeruginosa	10	11	1

Table 6: Synergistic effect of different antibiotics with or without AgNPs synthesized by *Aspergillus flavus*against some pathogenic bacteria

Types of Antibiotics	Name of the Antibiotics	Pathogenic Bacteria	Zone of Inhibition (mm)		Increased ZoneSize(mm)
			Ab*	Ab+ AgNPs	
Cephalospor 3rd generationins	Cefataxime	*S. aureus*	22	24.5	1.5
		E. coli	28	29	1
		S. epidermidis	14	20	6
		K. pneumoniae	Nil	7	1
		P. aeruginosa	Nil	9	3
Sulphonamides	Chloramphenicol	*S. aureus*	25	29	4
		E. coli	25	26	1
		S. epidermidis	25	25	Nil
		K. pneumoniae	Nil	10	4
		P. aeruginosa	Nil	11	5
	Tetracycline	*S. aureus*	36	39.6	3.6
		E. coli	22	29	7
		S. epidermidis	7	12	5
		K. pneumoniae	Nil	7	1
		P. aeruginosa	Nil	10	4
Penicillin	Ampicillin	*S. aureus*	37.5	41.5	3
		E. coli	18	19	1
		S. epidermidis	13	15	2
		K. pneumoniae	10	7	3
		P. aeruginosa	Nil	11	5
PenicillinCombination	Piperacillin/Tazobbactum	*S. aureus*	34	37.6	3.6
		E. coli	22	12	-10
		S. epidermidis	15	15	Nil
		K. pneumoniae	21	25	4
		P. aeruginosa	10	11	1

Silver nanoparticles of cell free filtrate of *A. flavus* showed bactericidal activities in terms of inhibition zone (0-7mm).The highest increase zone size of AgNPs of cell free filtrate of *A. flavus* + tetracycline was in case of *E. coli*, *S. epidermidis* and *P. aeruginosa*, respectively. Also, the highest increase zone size of AgNPs of cell free filtrate of *A. flavus* + with chloramphenicol was in case of *S. aureus*, *K. pneumonia* and *P. aeruginosa*, respectively. Generally, tetracycline showed the highest increase in zone size with most different AgNPs against *S. epidermidis*, *K. pneumoniae* and in some cases with *P. aeruginosa*, choramphenicol with most different AgNPs showed the highest increase zone size against *S. aureus*.

Silver nanoparticles of cell free filtrate of *Fusarium* sp. showed mild bactericidal activities in terms of inhibition zone (8-12 mm). Also combination of ampicillin with silver nanoparticles synthesized by cell free filtrate of *Fusarium* sp. gave high increase zone size against *S. epidermidis*, *K.pneumoniae.* On other hand, the combination of AgNPs from *Fusarium* sp. + Piperacillin and tazobactum in case of *S. epidermidis* and *E. coli* gained high antagonistic effect.

Shaverdi *et al.* [26] studied the effect of silver nanoparticles alone and in combination with antibiotics on the bacteria. They synthesized the silver nanoparticle using *Klebsiellapneumoniae* and in combination with antibiotics on the *S.aureus* and *E.coli* bacteria. They observed a significant increase in antibacterial activity of antibiotics in presence of silver nanoparticles and there was highest synergistic effect of nanoparticles with erythromycin against *S.aureus*.Also,**Fayaz *et al.*** [27] observed that antibacterial activities of ampicillin and chloramphenicol were increased in the presence of Ag nanoparticles.

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