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The combination effect of the biologically synthesized silver nanoparticles and several antibiotics toward multidrug resistant *Staphylococcus aureus*

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ABSTRACT

As a result of increasing resistance toward known antimicrobials and appearing of MDR isolates over world; and since is hard to develop new groups of antimicrobial agents with enough safety and effectiveness, for all that alternative methods were followed to get the desired goal, hence, alternative antimicrobial agents were developed to overcome this issue. Silver admitted as powerful antibacterial agent has marked effect on several species of microorganisms contrasted with other metals also sliver displays greater effect on bacterial cells in the time it show lower harmless to eukaryotic cells. For this reason it has been chosen in nanotechnology to prepare AgNPs and they showed same antimicrobial effect and less toxicity to human cells which allows it use in medical filed. Biosynthesis effective non toxic colloidal silver nanoparticles can use as alternative antimicrobial agent. The nanoparticles synthesized through the biosynthesis method by using *Eucalyptus* leaf extract with assist of Microwave radiation, the morphology and properties of the synthesized nanoparticles were characterized via several methods; UV-vis Spectroscopy, the Fourier Transform Infra-red Spectroscopy (FTIR), Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) finally the antimicrobial activity of the nanoparticles were tested by three methods (Disk Diffusion Method, Well Diffusion Method, MIC Method). Synthesis of small, globally, colloidal and low toxicity silver nanoparticles with high stability in liquids even after more than five weeks without aggregation in addition to facilitated the synthesis process with assist of Microwave depending on the biological method

Keywords: silver nanoparticles, Biosynthesis, *Eucalyptus*, MDR-MRSA, *S.aureus*. TEM, DLS and FTIR.

1. INTRODUCTION

As a result of increasing resistance toward known alternative antimicrobials and appearing of MDR isolates over world; and since it hard to develop new groups of antimicrobial agents with enough safety and effectiveness, for all that alternative methods were followed to get the desired goal, so, alternative antimicrobial agents were developed to overcome this issue (1). Silver admitted as powerful antibacterial agent has marked effect on several species of microorganisms contrasted with other metals also sliver displays greater effect on bacterial cells in the time it show lower harmless to eukaryotic cells. For this reason it chosen in nanotechnology to prepare AgNPs and they showed same antimicrobial effect and less toxicity to human cells which allows using it in medical filed (2).

This paper aims to biologically synthesize effective nanoparticles which can be used as alternative antimicrobial agent towards MRSA isolates.

2. MATERIALS AND METHODS

2.1 Eucalyptus leaf extract

Amount of 10 gm of healthy and fresh *Eucalyptus* leaves were washed by tap water and air drying in room temperature. Then leaves are tiny chopped and immerged in D.W in sterile flask, and applied in Microwave for (60sec) then filtered with whatman

filter paper (NO.1) to get free remains extract and kept in sterile container at 4C°. The phytochemical components of the extract solution were investigated through several tests.

2.2 Biological Syntheses of nanoparticles

About 88 ml of the AgNO3 Solution (1mM) was mixed with 12 ml of fresh leaf extract and allow reacting in Microwave for (60 sec) followed by incubation in dark for 4h at room temperature. Then the reaction was centrifuged at 13,000 rpm\min for 15 min and supernatant was discarded while the precipitate containing AgNPs resuspended with D.W and washed several times, finally AgNPs kept in dark at room temperature.

2.3 Characterizations of Synthesized Nanoparticles

The biological synthesized nanoparticles that visualized through color change of the reacted solution were characterized with several techniques as following: UV-visible Spectroscopy (UV-vis) to estimate the particles formation and size ranges; the Fourier Transform Infra-red Spectroscopy (FTIR) for determining the organic and non-organic components of the molecules. The morphology and size distribution of synthesized AgNPs was examined via Transmission Electron Microscopy (TEM) and also to investigate the antimicrobial effect of the AgNPs on the MDR-MRSA isolates. Also another correlative analysis was achieved to get the size, state of dispersion and characterization of nanoparticles in fluent solution using dynamic light scattering (DLS)

2.4 Antimicrobial Effects of Synthesized Nanoparticles

The antimicrobial effect of the synthesized nanoparticles was tested on five isolates multi drug resistant MRSA (MDR-MRSA) with three methods (Disk Diffusion, Well Diffusion, Overnight Incubation) methods.

3. RESULTS AND DISCUSSION

3.1 Silver nanoparticles synthesis

The biological method was selected to synthesizes silver nanoparticles and preferred on the physical and chemical methods since it used safe non-hazardous agent for the reduction of Ag molecule which make it as environment friendly method, for the same it consider safe and less cost than other methods (3).

Silver nitrate aqueous solution was used to provide the Ag^+ ions and the biochemical molecules exist in the aqueous *Eucalyptus* leaf extract (ELE) (enzymes, proteins, phenols, flavanoids) used as a reducing and capping agents for the synthesis of silver nanoparticles (4).The proteins exist in the extract plays a role in the reducing process and controlling the final shape of synthesized nanoparticles as (5) suggested due to the reaction of carboxyl groups and hydroxyl groups of the proteins with the Ag^+ ions; this mechanism provides a good income of tiny AgNPs with colloidal properties.

Instead of boiling method this study used microwave method to prepare the leaf extract which shorten the time needed for preparing the leaf extract to 1min in place of 10 min with the boiling method, Also the incubation time in dark, that needed to complete the reaction, became shorten which is 4h instead of 24h (6).

Exchanging the color from the pale yellow before radiation to dark yellow after the radiation was indication of the initiate formation of AgNPs and after 4h of dark incubation a dark brown color mixture seen that verified the formation of the AgNPs. After centrifugation the dark gray color precipitate that reveals synthesized nanoparticles was appeared. It was collected and kept for further characterization of the synthesized AgNPs (7).

3.2 Silver nanoparticles characterization

In order to confirm the properties of bio-synthesized silver nanoparticles in this study numerous techniques were used to confirm the results as following:

3.2.1 UV-visible spectroscopy

According to the fact that different molecules can absorb ultra-violet and visible light at different wave lengths, this assay estimate the intensity of absorption or optical absorption of the solution in order to get the particles formation peak (Figure 1).

The assay of UV-vis spectroscopy data appeared an appearing of surface Plasmon resonance peak (SPR) at wave length of (400-418nm) range that agrees with tiny metallic AgNPs creating. The narrow range was indicating the similarity of particles sizes and absence of particles aggregation. Since that AgNPs “absorb radiation intensity” at wavelength of 400nm because of the transition of electrons (8).

3.2.2 Fourier Transform Infra-red Spectroscopy

Fourier Transform Infra-red Spectroscopy (FTIR) a fine technique for determining the organic and non organic components of the molecules since they absorbed light in infrared zone which respond to the chemical bonds exist in the molecule. As shown in figure (4-2), absorption bands at the 1637 and 1390 revealed the presence of (O-H), 3432 to (C=O), 3000 to (COOH), 3112 to (C-H), 1506 to (C=N), 1435 to (C-N), 1277 to (N-N), 1004 to (C-H), 661 to (C-N) finally the metallic nanoparticles synthesized were marked with the strong peak at 750 cm^{-1}.

3.2.3 Transmission electron microscopy

The morphology and size distribution of synthesized AgNPs was examined via Transmission electron microscopy (TEM) to clear the proprieties of the synthesized particles; and the *S.aureus* bacterial cells that treated with AgNPs was examined too in order to examine the effect of the synthesized nanoparticles on cells comparing with normal bacterial cells.

The TEM picture of AgNPs arose tiny ball shape nanoparticles with size range of (15-20) nm free of aggregation and surrounded by variant biological extract content which may prevent stabilizing functional groups of silver nanoparticles from agglomeration as shown in figure 3.

The TEM image of bacterial cells treated with synthesized AgNPs comparing with the picture of normal cell showed certain effects in the shape of the cell with losing the spherical shape of the normal cells and appeared the existence of AgNPs inside the effected cells as figure 4 shown.

3.2.4 Dynamic Light scattering method

Since the TEM and FTIR analysis was obtained under dry conditions with dry sample so another correlative analysis was achieved to get the size, state of dispersion and characterization of nanoparticles in fluent solution using Dynamic Light scattering (DLS) (9). The size obtained from a dynamic light scattering measurement is the hydrodynamic diameter of a sphere which has the same average diffusion coefficient as the molecule being measured (10).

The DLS analysis of synthesized AgNPs results was appeared that the particles size was about 35 nm in average what slightly larger than the nanoparticles size determine with the TEM analysis which can explained by that particles in a soluble condition appear with 50% increasing of agglomeration comparing with dry condition as reported by (9) (Figure 5).

3.3 Antibacterial activity of synthesized AgNPs

In consideration of silver role in medicine application especially for its antimicrobial activity against number of G-positive and G-negative bacteria).; the antimicrobial activity of synthesized nanoparticles was measured by colony forming unit method (CFU) against five isolates of MDR *S.aureus*.

Three methods was employed to determine the synthesized AgNPs antimicrobial activity against *S.aureus* isolates; disk diffusion method, well diffusion method and overnight incubation method.

Results showed that best method for determining the antimicrobial activity of the nanoparticles was the overnight incubation of synthesized AgNPs with the bacteria which gave best results of activity since it resulted in inhibition with a low concentration (0.3 µg\ml) while the other two methods disk and well diffusions methods were showed a constant low antimicrobial activity of AgNPs (8, 10) mm inhibition zone respectively even with high concentration (100 µg\ml).

This variation of the results can describe with fact that nanoparticles have a unique feature of Brownian movement which make it able to transfer with a random quick motion in the solution, indeed, when the particles incubated with the broth bacterial culture will be in permanent contact freely with the cells which provides better reaction between nanoparticles and the bacterial cells facilitated the adhesion of the particles to the surface of the cells exist in the broth culture and increased the ability of it to penetrate the bacterial cells and particles antimicrobial affect than when applied on solid medium where it be restricted to each other and to its applied place which decreases its activity (12 and 13).

3.3.1 Antimicrobial activity of AgNPs in combination with antibiotics

Antimicrobial activity of synthesized AgNPs in combination with three chosen antibiotics was examined for the development of the capacity of these antibiotics to treat bacterial infections therefore antibiotics; Amikacin, Amoxicillin and Gentamicin was chosen for this test after the bacterial isolates show high resistance to these traditional antibiotics in previous susceptibility test.

Results show that Amikacin gave best results in both disk and well diffusion methods comparing with Gentamicin and Amoxicillin (Table 4-1A and Table 4-2A), since AgNPs have ß-lactam action on the bacterial cells and the Amikacin is one of the amino-glycosides antibiotics. So, mixing them has better action on the bacteria than using each of them alone (Tables 1-3) and (Table 4-6); as 14 referred that when using amino-glycosides antibiotic in combination with ß-lactam antibiotic will increase the activity of both antibiotics and shorten the killing time for bacteria.

In another hand, the combination of AgNPs with Amoxicillin which is one of the ß-lactam antibiotics show a moderate activity as table (6) shown. That may be returned to fact that they both have ß-lactam action which means they both act on the same target what reduced the combination activity.

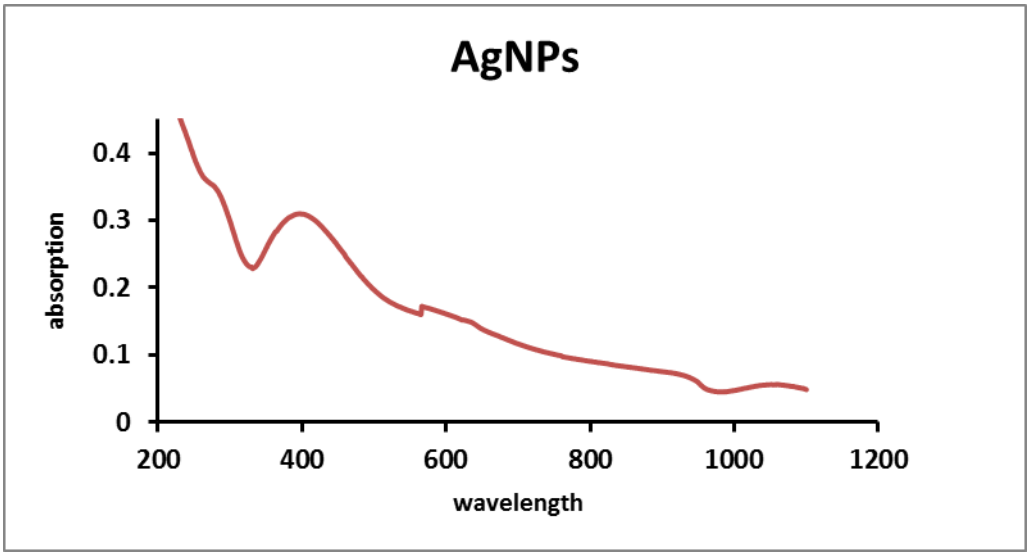

Figure 1: The surface-Plasmon absorbance spectrum of Ag NPs formed.

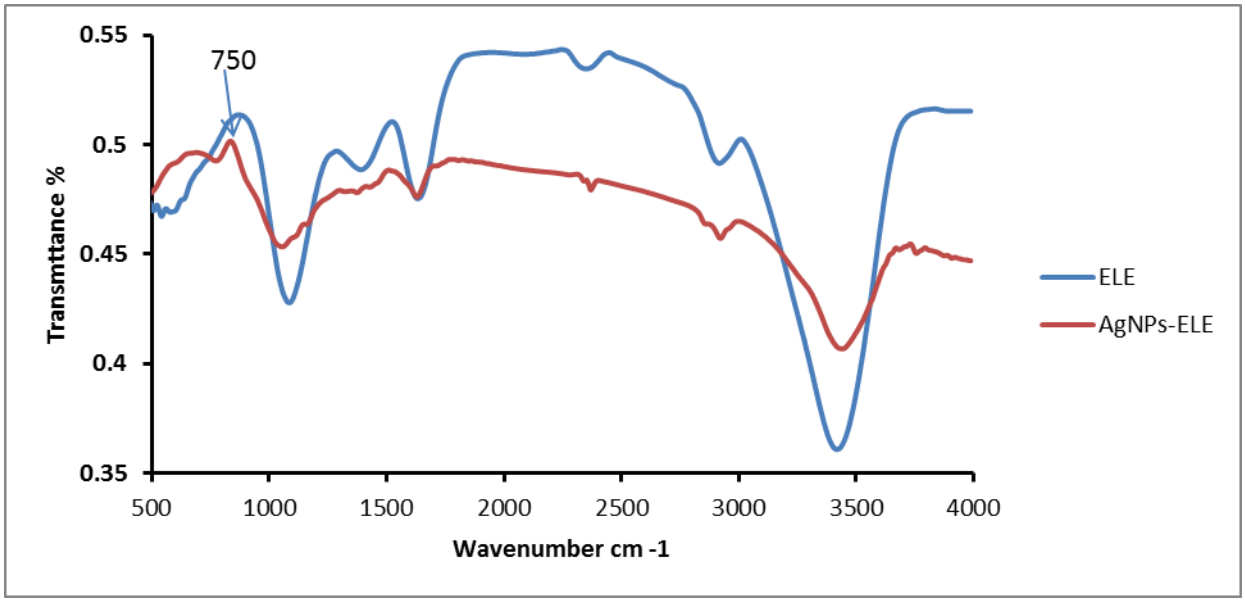

Figure 2: The FTIR results of AgNPs

Figure 3: The TEM image of synthesized AgNPs.

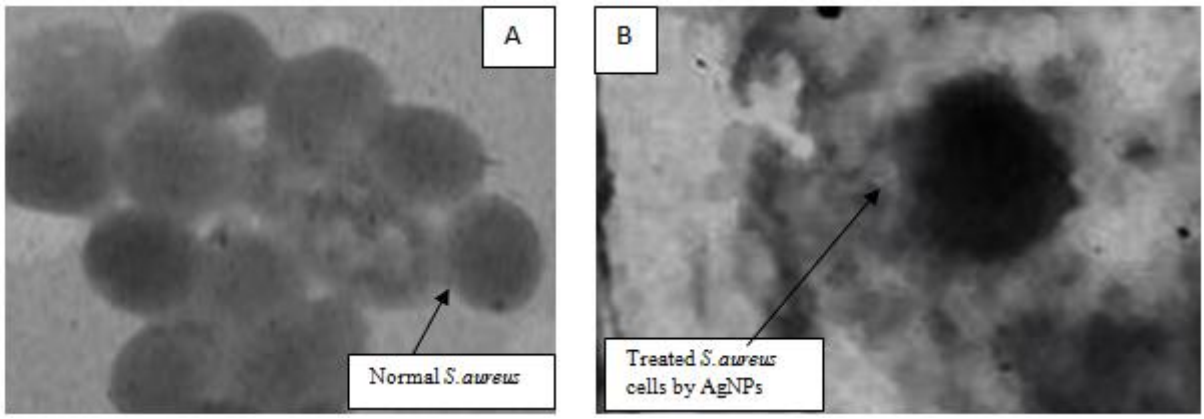

Figure 4: The TEM picture shown difference between A. normal *S.aureus* and B. *S.aureus* treated by AgNPs.

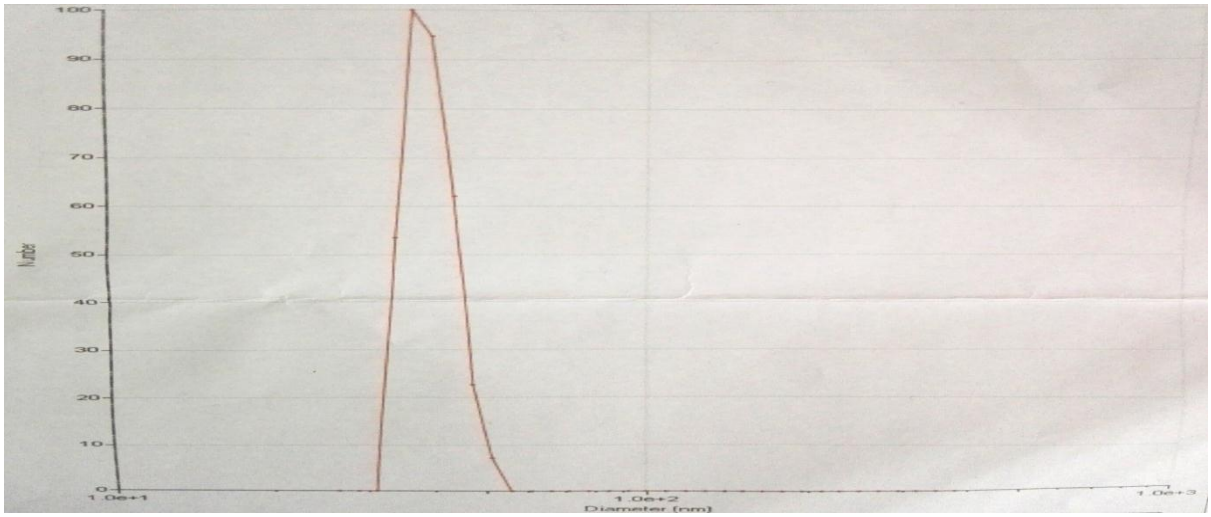

Figure 5: The DLS graph

Table 1: Discs method results for Amikacin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
AK	30 25 20	0
AgNPs	20 25 30 60 100 120 180	8
AgNPs + AK	20+30 25+30 30+30 60+30 100+30 120+30 180+30	12 13

Table 2: Discs method results for Gentamicin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
	30	
GEN	25	0
	20	
	20	
	25	
	30	
AgNPs	60	
	100	
	120	
	180	8
	360	
	20+30	
	25+30	
	30+30	
	60+30	
AgNPs + GEN	100+30	
	120+30	10
	180+30	15

Table 3: Discs method results for Amoxicillin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
	30	
AMX	25	0
	20	
	20	
	25	
	30	
AgNPs	60	
	100	
	120	8
	180	
	20+30	
	25+30	
AgNPs + AMX	30+30	
	60+30	
	100+30	
	120+30	10
	180+30	12

Table 4: Well diffusion method results for Amikacin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
	30	0
AK	25	
	20	
	20	9
	25	
	30	
AgNPs	60	
	100	10
	120	
	180	
	20+30	14
	25+30	
	30+30	15
AgNPs + AK	60+30	14
	100+30	
	120+30	14.5
	180+30	15

Table 5: Wells diffusion method results for Gentamicin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
	30	
GEN	25	0
	20	
	20	9
	25	
	30	
AgNPs	60	
	100	
	120	
	180	10
	20+30	
	25+30	
	30+30	
AgNPs + GEN	60+30	
	100+30	
	120+30	11
	180+30	

Table 6: Well diffusion method results for Amoxicillin antibiotic (mg) +AgNPs

Treating agents	Con. mg\ml	Inhibition zone (mm)
	30	
AMX	25	0
	20	
	20	
	25	9
	30	
AgNPs	60	
	100	
	120	
	180	10
	20+30	
	25+30	
	30+30	
AgNPs+AMX	60+30	
	100+30	
	120+30	13.5
	180+30	15

3.3.2 Minimum inhibition concentrations of the AgNPs

The experiment was set with three repeats for five MDR *S.aureus* isolates first was incubated for 4h and the second for 24h. The results showed that the incubation for 4h was not useful for the inhibition of the bacteria even with the high concentration of the nanoparticles, the reason behind this was that the AgNPs effect on the bacterial cells is similar to the action of the ß-lactam antibiotics since it working on the cell wall so it need more time to affect the bacterial growth (3). While the effect of the incubation for overnight was beneficial even with the low concentration of synthesized particles as table 7 shown; and that was useful for the usage of the particles in medical fields since Cyto-toxicity of AgNPs was observed at the concentration of 10 ppm (7). This investigation demonstrated that silver nanoparticles with a concentration of 2 ppm and 4 ppm were not toxic for human healthy cells, while it inhibits bacterial growth (3).

Table 7: The AgNPs MIC (µg\ml)

AgNPs con. µg\ml	4h incubation	overnight incubation	results
0.2		53 colonies	Sub-MIC
0.3		22 colonies	MIC
0.6		3 colonies	MBC
20	Normal growth		
25			
30			Killing concentration
60		No growth	
100			
120			
180			

From the table above it can be seen that MIC of the nanoparticles was 0.3µg\ml after overnight incubation and MBC was 0.6 µg\ml while when the treated culture incubated for just 4h no detectable effect can notice on the bacterial growth which is agreement with what recorded by (3) that *S.aureus* DNA fail to keep replicated after treating with AgNPs for 6h at least because of the bulky Peptidoglycan coat in the Gram-positive bacteria cell wall that provide high protection for the cell versus toxins and other chemical material.

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