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RESEARCH ARTICLE

BIOGENIC SYNTHESIS OF SILVER NANOPARTICLES AND THEIR POTENTIAL BIOACTIVE APPLICATIONS AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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ABSTRACT

The development of a reliable, cost effective and environmentally-friendly technique for the biogenic synthesis of nanomaterials is an important aspect of current nanotechnology research. In the present attempt, we describe biogenic synthesis of silver nanoparticles (AgNPs) from 1mM $AgNO_3$ solution using *E. coli* strain. The bio-based silver nanoparticles in the reaction mixture appeared brown coloration. Nanoparticles were characterized using UV–Vis spectroscopy, X-ray diffraction (XRD) and Scanning electron microscopy (SEM) analysis. The nanoparticles showed maximum absorbance at 420 nm on ultraviolet-visible spectra. The average size of the nanoparticle was 26.25 ± 13.10 nm. The energy-dispersive spectroscopy (EDS) of the nanoparticles confirmed the presence of elemental silver. No other signal peaks of impurity were detected. Silver nanoparticles exhibited high antibacterial activity using *Staphylococcus aureus* as an indicator strain. Bacterial killing kinetics in the presence of fabricated silver nanoparticles exhibited higher killing rate against *Staphylococcus aureus* (94.8%). The correlation coefficient between silver nanoparticles and selected bacterial pathogens revealed that there is a strong negative correlation with (r = -0.83 to -0.99).

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INTRODUCTION

Nanotechnology is a new scientific field being developed since 1980s. Nano materials have a lot of different characters compared to the general materials with same components because of their small size effect, surface or interface effect, quanta tunnel effect, etc. With the development of new chemical or physical methods, the concern for environmental contaminations are also heightened as the chemical procedures involved in the synthesis of nanomaterials generate a large amount of hazardous byproducts. Thus, there is a need for clean, nontoxic and environment-friendly method of nanoparticle synthesis (Mukherjee *et al.*, 2001).

Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process (Jose *et al.*, 2005; Lok *et al.*, 2007). The most important application of silver and silver NPs is in medical industry such as topical ointments to prevent infection against burn and open wounds (Ip *et al.*, 2006). Silver is known as a powerful disinfecting agent for killing unicellular microorganism by inactivating enzymes having metabolic functions, in the microorganisms by oligo dynamic action (Kim *et al*., 1998). Moreover, various inorganic antimicrobial agents that use silver have been developed to date. Due to the outbreak of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance the pharmaceutical companies and the researchers are searching for new antibacterial agents (Rai *et al*., 2009). The development of new resistant strains of bacteria to current antibiotics has become a serious problem in public health; therefore, there is a strong incentive to develop new bactericides. Therefore, the present research has been focused to synthesize and assess the antibacterial activity of silver nanoparticles against both gram positive and gram negative bacteria.

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MATERIALS AND METHODS

Bacterial Strains

Gram positive (*Staphylococcus aureus, Bacillus cereus* and *Streptococcus epidermis*) and gram negative bacteria (*Klebsiella pneumoniae, Shigella flexneri, Salmonella typhimurium, Pseudomonas aeruginosa* and *Escherichia coli*) were procured from the Microbial Type Culture Collection, Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C.

Biogenic Preparation of silver nanoparticles
Preparation of Bacterial Culture

E. coli stock culture was maintained by sub culturing it at monthly intervals. Growth medium used was Luria Broth containing 1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl (Himedia (p) Ltd., Mumbai), and pH 7.5. 250 ml of Luria broth (LB) was prepared, sterilized and inoculated with fresh *E. coli* strain. The culture flask was incubated at 37°C for 24 hrs with shaking at 150 rpm.

Inoculum Preparation

After incubation, the *E. coli* culture was centrifuged at 5000 rpm for 10 minutes, and the biomass (pellet) was collected, and washed three times with sterile double distilled water. Finally, the wet biomass (pellet) was dried in the oven at 60°C. This biomass (pellet) was used as the starting material for the synthesis of silver nanoparticles.

Nanoparticle Preparation

The preparation of silver nanoparticles was followed the method described by Mouxing *et al.* (2006). In brief, 10 ml aqueous solution of 1M silver nitrate ($AgNO_3$) was treated with 1g biomass of *E. coli* culture in 250 ml Erlenmeyer flask (pH adjusted to 8.5). The whole mixture was put into a shaker at 40^{o}C (200 rpm) for 5 days. Control experiments were conducted with inoculated media, to check for the role of bacteria in the synthesis of nanoparticles.

Characterization of Nanoparticles
Visual Inspection

The reduction of metal ions was roughly monitored by visual inspection of the solution by the conversion of the colorless reaction mixture to a colored solution (Fang *et al.,* 2005).

UV-Vis Spectroscopy

According to Mie (1908), UV–Vis spectroscopy of silver colloids was performed in Shimadzu dual beam spectrophotometer (model UV-1650 PC) by taking 1 ml of the sample, compared with 1 ml of distilled water used as blank with a wavelength ranging from 200 – 1100 nm.

X- Ray Diffraction

According to the description of Rau (1962), the nanoparticles were air dried, powdered and used for XRD analysis. X-ray diffraction pattern was recorded in the scanning mode on an X' PERT-PRO analytical instrument operated at 40kV and a current of 30 mA with Cu Kα radiation (λ=1.54060 A^o) in Alagappa University, Karaikudi. The observed diffraction intensity was compared with the standard JCPDS files. The software gave the information about the crystal structure of the particles. The average size of the nanoparticle was determined using the Debye – Scherrer equation. The size of the silver nanoparticle was made from the line broading of the (111) reflection using the Debye-Scherrer formula (Huang and Tang, 2005). According to the formula,

$$D = K\lambda / \beta\cos\theta$$

Where D = Thickness of the nanocrystals, K = Constant, λ = Wavelength of x-rays, β = Width at half maxima of (111) reflection at Bragg's angle 2θ, θ = Bragg angle.

Scanning Electron Microscopy

Morphology and size of the synthesized silver nanoparticles were investigated with SEM (JSM 35 CF JEOL) Model S – 3000 H and Make – Hitachi instrument at CECRI, Karaikudi in a resolution 60 Å at 20.0 kV, magnification of 1.0 k. The scale was about 13.4 mm to 10 µm for biologically synthesized silver nano particles. The size of the particle was calculated by using the scale provided in the micrograph.

Energy Dispersive Spectroscopy

The elemental analysis of the silver nanoparticles was performed using the EDS analysis (JSM 35 CF JEOL) in a resolution of 60 Å, magnification of 5 k. The operating conditions were 15.0 kV accelerating voltage and 15 mm working distance under high vacuum mode. It was making Thermo Electron Corporation and attach with super dry II detector.

Evaluation of Antibacterial Activity

The antibacterial activity of the biologically synthesized silver nanoparticles was assessed against gram positive and gram negative bacterial strains by agar well diffusion technique. The overnight bacterial cultures grown in nutrient broth was spread evenly over Mueller Hinton agar (MHA) plates with sterile cotton swab. Wells of 6 mm diameter were cut on the plates using sterile cork borer and 100 µl of nanoparticles suspension was dispensed in each well. The plates were left overnight at 37^{o}C and results were recorded by measuring the diameter of inhibition zone (mm) using measuring scale.

Bacterial Killing Kinetics (Growth Killing Kinetics)

To examine the bacterial killing effect in the presence of silver nanoparticles, the nutrient agar plates were supplemented with various concentrations of silver nanoparticles (20, 40, 60, 80 and 100 µl). For this, 20 ml nutrient agar was poured in well rinsed, autoclaved petriplates. 0.1 ml of active bacterial culture was homogeneously spread on the agar plates. All plates were incubated in bacteriological incubator for 24hrs. After incubation, the number of colonies were grown on agar plate was counted.

Growth kinetics or killing rates and bacterial concentrations were determined by measuring the colony forming unit in the nutrient agar plates. Percentage of bacterial growth inhibition was calculated as per the equation of Shahi *et al.* (1997).

BGI % = (BC – BT) X 100 / BC

Where

BGI =Bacterial Growth Inhibition
BC = Number of Bacterial colonies in the control
BT = Number of Bacterial colonies in the treatment set

Statistical Analysis

The results obtained in the present experiment were subjected to statistical analysis such as Mean, Standard Deviation, Percent Frequency Distribution and Correlation co-efficient.

RESULTS AND DISCUSSION

Characterization of Nanoparticles
Visual Inspection of Silver Nanoparticles

The appearance of pale yellow to brown coloration in the reaction mixture clearly indicated the formation of the biogenic synthesis of silver (Fig. 1). Ahmad *et al.* (2003) reported that the appearance of yellowish brown color in the reaction vessels suggested the formation of Ag-NPs. The appearance of the yellow color indicated the formation of silver nanoparticles in the reaction mixture, as it is well known that silver nanoparticles exhibits striking colors (light yellow to brown) due to excitation of surface Plasmon vibrations in the particles (Kapoor, 1998).

UV-Vis Spectroscopy of Silver Nanoparticles

The UV-Vis spectra of the biologically fabricated silver nanoparticles showed a strong surface Plasmon resonance was centered at 430 nm (Fig. 2) confirmed the nanocrystalline character of the particles (Schneider *et al.*, 1994) and the low degree of their polydispersity. Sileikaite *et al.* (2006) reported that the UV-vis spectra of the silver colloid ranging from 300 nm – 700 nm. Observations of this strong but broad surface plasmon peak have been well documented for various metal nanoparticles (Me-NPs), with sizes ranging from 2 to

100 nm (Henglein, 1993; Sastry *et al*., 1997; Sastry *et al*., 1998).

Fig. 1 Silver nanoparticles formed in the biological method

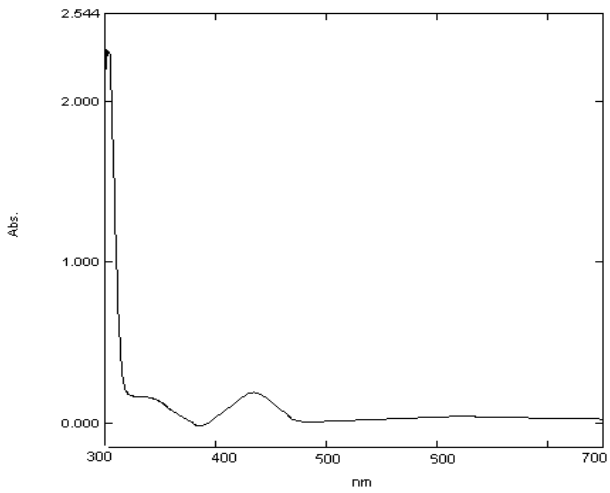

Fig. 2 UV-Vis spectrum of biologically synthesized silver nanoparticles

X-Ray Diffraction of Silver Nanoparticles

An XRD pattern obtained for the silver nanoparticles showed a number of Bragg reflections corresponding to (111), (200), (220), (311) and (222) sets of lattice planes, which may be indexed based on the face centered cubic (FCC) structure of silver nanoparticles. The XRD pattern thus clearly showed that the silver nanoparticles are crystalline in nature. The diffraction peak at a 2θ value of 38.17° from the (111) lattice plane and the three additional broad bands are observed at 44.31° (2θ), 64.50°(2θ) and 77.05°(2θ) correspond to the (200), (220) and (311) planes of biologically synthesized silver nanoparticles respectively (Fig. 3). Mouxing *et al*. (2006) reported that the number of Bragg reflections corresponding to (111), (200), (220) and (311) sets of lattice planes are observed, which may be indexed based on the fcc structure of silver. Morones *et al*. (2005) proved that faceting of the particles as well as the direct interaction of the (111) facets with the bacterial surface. Sen *et al*. (2003) reported that the bulk silver have diffraction lines (111), (200), (220) and (311) at $2\theta = 38.14^{o}$, 44.27^{o}, 64.5^{o} and 77.3^{o} respectively. Furthermore, the average diameter of the biologically synthesized silver nanoparticle is 20.8 nm by the Scherer equation.

Table 1 Size of Silver Nanoparticles prepared by Biological Method

Sl. No.	Particle size of (nm)
1	30
2	40
3	10
4	50
5	20
6	30
7	10
8	20
Total	210
Mean (Average)	26.25
Standard deviation	13.10

Scanning Electron Microscopy of Silver Nanoparticles

The reduction of metal ions are due to the capture of these particles by *E. coli* was verified by SEM. The micrograph showed that the fabricated particles have a spherical nature (Fig. 4) and the average size (Mean ± SD) of the biologically synthesized silver nanoparticles was calculated as 26.25 ± 13.10 nm (Table 1). The corresponding percentage frequency distribution of the obtained nanoparticles size is given in Table 2. The spherical shape silver nanoparticles formed with a diameter ranging from 30 to 40 nm in *Boswellia* (Ankanna *et al*., 2010). Warisnoicharoen *et al*. (2011) synthesized silver nanoparticles with a diameter of about 12 nm.

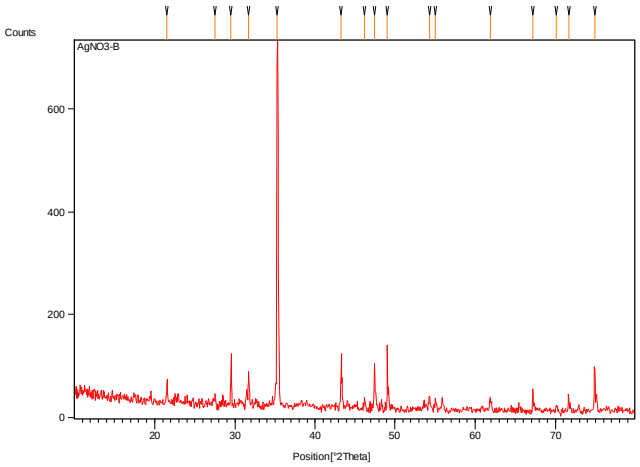

Fig. 3 X-ray diffraction spectrum of silver nanoparticles prepared by biological method

Energy Dispersive Spectroscopy of Silver Nanoparticles

EDS micrograph explained the surface atomic distribution and chemical composition of biologically synthesized silver nanoparticles. In this spectrum, stronger signals from the silver atoms (31.15% in mass) in the silver nanoparticles and the weaker signals from N, Na, O and Cl atoms were observed (Fig. 5). Mouxing *et al*. (2006) reported that the ED spectrum recorded in the spot – profile mode. Strong signals from the silver atoms are observed about 31.23% in mass.

Savithramma *et al*. (2011) reported that the ED spectrum is clear that *Boswellia ovalifoliolata* and *Shorea tumbuggaia* have showed weight percent of 39.88% and 33.52% for silver nanoparticles respectively.

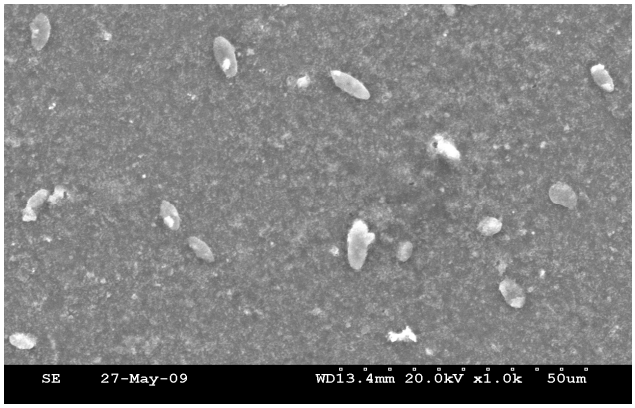

Fig. 4 Scanning electron micrograph of biologically synthesized silver nanoparticles

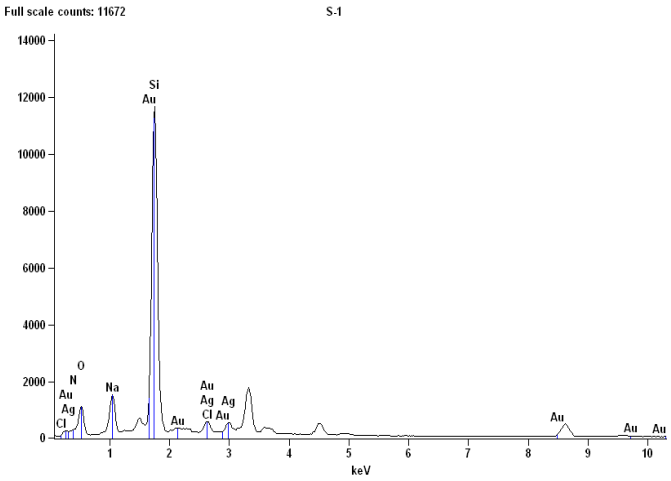

Fig. 5 Energy dispersive spectrum of biologically synthesized silver nanoparticles

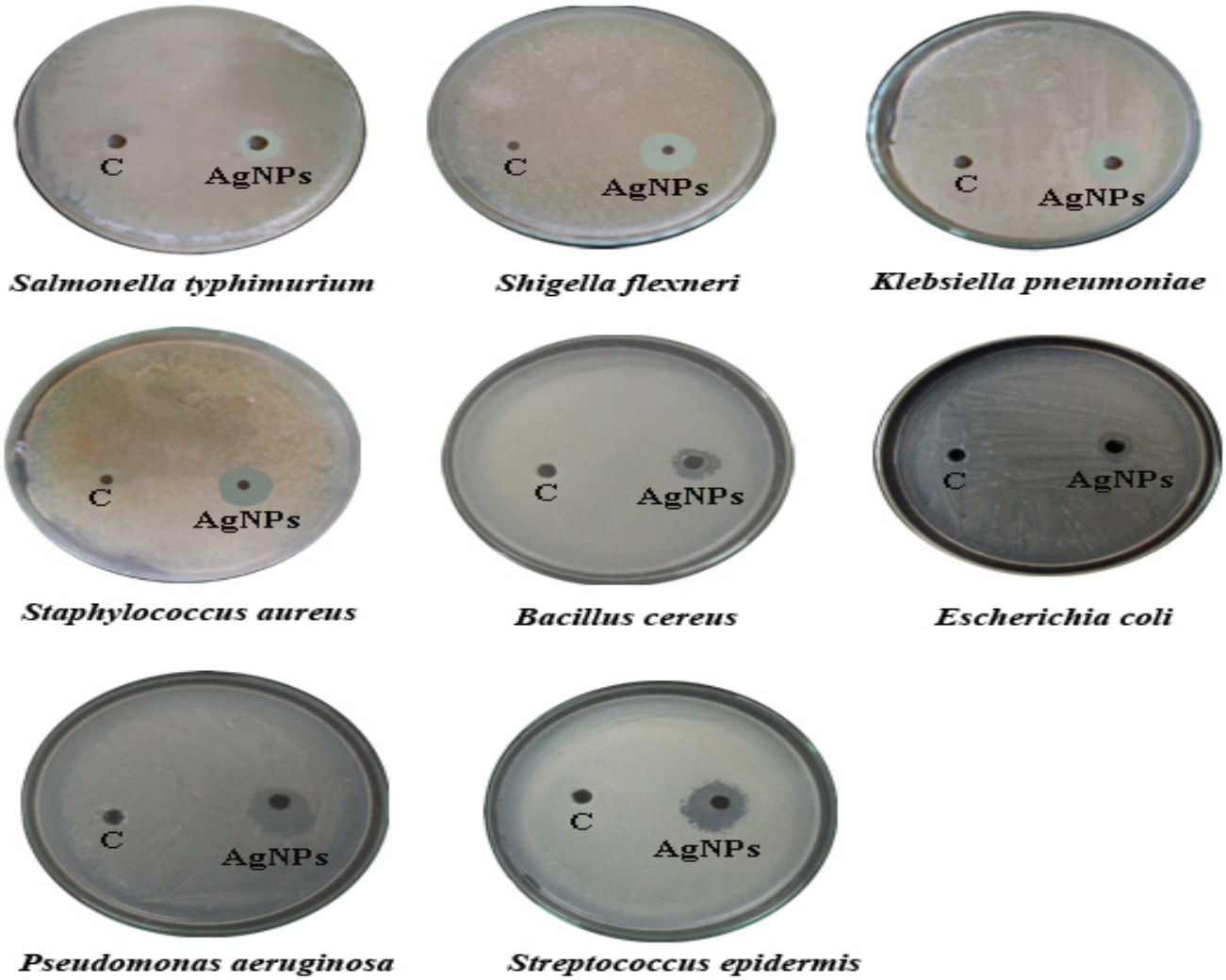

Fig. 6 Antibacterial activity of biologically synthesized silver nanoparticles against selected pathogens

Antibacterial Activity of Biologically Fabricated Silver Nanoparticles against Gram Positive and gram Negative Bacteria

The antibacterial activity of biologically synthesized silver nanoparticles has been investigated against both gram positive and gram negative bacteria. Biologically synthesized silver nanoparticles exhibited excellent antibacterial activity against all tested strains (Fig. 6). The zone of inhibition in diameter (mm) of biologically synthesized silver nanoparticle against bacterial pathogens gave an inhibition zone in the range of 10 -15 mm (Table 3). Silver nanoparticles obtained very strong inhibitory action against *Staphylococcus aureus* followed by *Shigella flexneri*. Gupta *et al.* (2008) reported that the observed inhibiting action of nano silver (Ag) is due to the release of Ag^+ ions come in contact with bacterial cells and kill them. The results showed that the Ag^+ containing nanoparticles kept effective antibacterial activities. Silver nanoparticles interactions with bacteria are dependent on the size and shape of the nanoparticles (Pal *et al.*, 2007). The greatest surface area of silver nanoparticles the greatest the antibacterial activity (Thiel *et al*., 2007). Colloidal silver showed a strong bactericidal effect against *E. coli, S. aureus*, *Klebsiella* and *Shigella* and these results were in agreement with those reported by Bryaskova *et al*. (2009) and Assar and Hamuoda (2010).

Table 2 Frequency Distribution of Biologically Synthesized Silver Nanoparticles Size (nm) Observed Under Scanning Electron Microscope

Silver Nano-particle size (nm)	Tally Marks	Frequency	Relative Frequency	Percent Relative Frequency (%)
10	II	2	2/8 = 0.25	0.25 x 100 = 25
20	II	2	2/8 = 0.25	0.25 x 100 = 25
30	II	2	2/8 = 0.25	0.25 x 100 = 25
40	I	1	1/8 = 0.125	0.125 x100=12.5
50	I	1	1/8 = 0.125	0.125 x100=12.5
Total	8	8	8/8 = 1.0	1.0 x 100 = 100

Table 3 Zone of Inhibition in Diameter (mm) of biologically synthesiszed Silver Nanoparticles against Selected bacterial Pathogens

Bacterial pathogens	Zone of inhibition (mm)
Salmonella typhimurium	12
Shigella flexneri	15
Klebsiella pneumoniae	13
Staphylococcus aureus	16
Bacillus cereus	11
Escherichia coli	9
Pseudomonas aeruginosa	13
Streptococcus epidermis	14

The bacterial killing kinetics of biologically synthesized silver nanoparticles against pathogens used in the present attempt is given in Table 4.

The result shows the silver nanoparticles had a better activity against *Staphylococcus aureus* (94.8%). The decrease in number of viable cells with increasing amounts of silver nanoparticle in solution can be fitted with correlation coefficient. The correlation coefficient between silver nanoparticle and selected bacterial pathogens revealed that there is a strong negative correlation of silver nanoparticles against selected bacterial pathogens used in the experiment ($r = -0.83$ to -0.99). Warisnoicharoen *et al*. (2011) found that silver nanoparticles have an ability to interfere with metabolic pathways. In the present experiment, colloidal silver showed highly potent antibacterial effect towards gram positive and gram negative bacteria. This condition may be due to its accumulation in the membrane of the organisms.

Table 4 Bacterial killing kinetics in the presence of biologically synthesized silver nanoparticles

Conc. of nano-particles (µl)	*S. typhimurium*	*S. flexneri*	*K. pneumoniae*	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. epidermis*
0	275	262	230	215	285	290	252	218
20	224 (18.5%)	165 (37.0%)	178 (22.6%)	135 (37.2%)	230 (19.3%)	216 (25.5%)	160 (36.5%)	168 (22.9%)
40	168 (38.9%)	134 (48.8%)	120 (47.8%)	108 (49.7%)	172 (39.7%)	170 (41.4%)	124 (50.8%)	124 (43.1%)
60	120 (56.3%)	89 (66.0%)	74 (67.8%)	64 (70.2%)	124 (56.5%)	118 (59.31%)	84 (66.7%)	70 (67.9%)
80	54 (80.3%)	48 (81.6%)	50 (78.2%)	42 (80.4%)	58 (59.3%)	54 (81.38%)	42 (83.3%)	48 (78.0%)
100	21 (92.3%)	17 (93.5%)	15 (93.4%)	12 (94.8%)	26 (90.9%)	20 (93.1%)	15 (94.1%)	14 (93.6%)

Bacterial Killing Kinetics (Growth Killing Kinetics) in the Presence of Biologically Synthesized Silver Nanoparticles

The viable bacteria were monitored by counting the number of colony forming units from the appropriate dilution on nutrient agar plates. The survival rate was determined by calculating colony forming units per milliliters (mL) of the culture.

A membrane with such morphology exhibits a significant increase in permeability, resulting in death of the cell. Mean while, studies have demonstrated that silver ions interact with sulfhydryl (-SH) groups of proteins as well as the bases of DNA leading either to the inhibition of respiratory process (Bragg and Rainnie, 1974) or DNA unwinding (Batarseh, 2004). Inhibition of cell division and damage to bacterial cell envelops was also recorded (Richards *et al*., 1984) and interaction with hydrogen bonding processes has been also demonstrated to occur (Russell and Hugo, 1994). However, the mechanism depends on both the concentration of silver ions present and the sensitivity of the bacterial species to silver. This green chemistry approach towards the synthesis of silver nanoparticles has many advantages such as environmental-friendly, cost effective and easily scaled up to large scale synthesis. It may be suitable for the formulation of

new types of bactericidal materials and may be solve the problem of the emergence and spread of *in vitro* antibiotic resistance. The data presented here are novel in that they prove that silver nanoparticles are effective bactericidal agents. Therefore, silver nanoparticles can be recommended as an effective broad spectrum bactericidal agent.

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