INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 3, p. 250-261, 2018

RESEARCH PAPER OPEN ACCESS

Evaluation of antibacterial activity of plant based silver nanoparticles in Synergism with Antibiotics

Faisal Rasheed Anjum[1*], Sidra Anam[1], Sajjadur Rahman[1], Ashiq Ali[2], Ahsan Naveed[1], SadafNaz[1]

[1]*Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan*

[2]*Department of Pathology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan*

Key words: Silver nanoparticles, UV-vis spectrophotometer, Zeta potential, Minimal inhibitory concentration, Disk Diffusion Method.

http://dx.doi.org/10.12692/ijb/13.3.250-261 Article published on September 30, 2018

Abstract

Plant derived silver nanoparticles might be a possible replacement of antibiotics in order to treat multi drug resistant bacterial infections, hence could be the answer to antibiotic resistance. The current study deals with the biosynthesis of silver nanoparticles (AgNPs) and determination of their bactericidal activity against *Staphylococcus aureus* and *Streptococcus pneumoniae*. The reduction of silver nitrate was indicated by characteristic change of colour from light yellowish to deep brown and finally colloidal brown. Spectrophotometer analysis showed that maximum absorption was observed at 450 nm. Silver nanoparticles with crystalline structure having 19 nm domain size was observed. The surface charge of silver nanoparticles was found to be varying with pH of the environment. Minimal inhibitory concentration (MIC_{50} and MIC_{90}) against *Staphylococcus aureus* was 2.66 & 5.31 µg/100 µl, while MIC_{50} and MIC_{90} against *Streptococcus pneumoniae* were found to be 5.31 & 1.06x10^1/100 µl, respectively. Disc Diffusion Method has revealed that biosynthesized AgNPs when used in synergism with antibiotics have significantly larger zone of inhibition as compared to AgNPs alone. Briefly, this research has provided a simple and environment friendly method for production of Ag particles as compared to complex chemical method. Moreover, green synthesized particles formulation showed a significant antibacterial activity against both test strains, whereas combinations of AgNPs and antibiotics have a better bactericidal effect.

* **Corresponding Author:** Faisal RasheedAnjum✉drfaissaltarar@gmail.com

Introduction

Biosynthesis of nanoparticles is an emerging field of material science where botanical plants are being used for preparation of silver nanoparticles that provides cost effective, environment friendly and pharmaceutically compatible nanoparticles (Mazur, 2004; Vijayaraghavan *et al.*, 2012; Manikandan *et al.*, 2015).Nanoparticles have various applications in catalysis, optoelectronics, photonics, and in medicine due to antibacterial effect (Lok *et al.*, 2006; Savithramma *et al.*, 2011; Banerjee *et al.*, 2014). Extreme small nature, high stumpy viscidness kinetic and constancy have made them very useful as bactericidal substances in medicine industry (Qian *et al.*, 2015). Mainly silver and silver nanoparticles are applied in medical field such as ointments to prevent infections (Dwivedi and Gopal, 2010). Several studies have suggested the use of silver nanoparticles (AgNPs) in foodstuff and farming, but their applications in drug carrier and pharmaceutical have been restricted. Apart from these applications, they have also been utilized in shampoos, soaps, detergents, toothpastes and cosmetics (Janard hanan *et al.*, 2009).

Nanoparticles are synthesized by several methods i.e. chemical reduction, photo reduction, irradiation method but these are very harmful and lavish due to the involvement of very toxic chemicals i.e. sodium/potassium borohydrate, making their use very risky for eukaryotic cells (Lalitha *et al.*, 2013). Currently, nanoparticles are being used in pharmaceutical industry, but there is a constant concern of their toxicity towards human health and environmental safety. So, there is need to develop a safe and eco-friendly method that does not involve the use of toxic organic and inorganic chemical (Malik *et al.*, 2014; Abubakar*et al.*, 2014).

To combat against infections or microbes, multiple varieties of antibiotics have been used for therapeutic purpose over time. Antibiotics fight to eliminate the bacteria but bacteria have natural processes that enable them to resist the antibiotics. Antibiotic resistance is the ability of bacteria to resist effects of antibiotics used to treat them (Zaman *et al.*, 2017). Due to unprescribed and irrational use of antibiotics, bacteria are getting resistant to these antibiotics (Gilberg *et al.*, 2003). So, there is need to look forward for new antibacterial agents that could serve as an alternative to antibiotics in order to treat the multidrug resistant bacterial infections. To cope both these problems of toxicity from chemically prepared environment unfriendly nanoparticle as well as antibiotic resistance, a green method called bio reduction is used in the present study to synthesize safe and eco-friendly silver nanoparticles and to evaluate the antibacterial effect of nanoparticles with and without antibiotics against bacteria.

Materials and methods

Synthesis of Silver Nanoparticles

Fresh leaves of *Azadirachta indica* were collected, washed with distilled water several times to remove the dust particles. These washed leaves were incised into small pieces and dried at room temperature. 10 gram of the dried leaves were mixed with 100 ml of distilled water in 250 ml glass flask. Boiling was done at 80^{o}C for 20 minutes followed by filtration through What man No. 1 filter paper (pore size 25 µm), twice. For synthesis of silver nanoparticles, 45 ml of 1mM aqueous solution of $AgNO_3$ was added to 5 ml of freshly prepared neem leaves extract. This composite mixture was then heated discontinuously in microwave oven at 100 ^{o}C for 4 minutes. Now the reaction mixture was cooled and kept at the room temperature for the next 24 hours. Newly synthesized silver nanoparticles were stored at room temperature in amber colour bottle to avoid the oxidation of photo sensitized silver nanoparticles.

Characterization of AgNPs

The optical property of AgNPs was determined by T-60 UV-Vis spectrophotometer having a resolution of 1 nm. Firstly, cuvettes were filled with deionized water for spectrophotometric analysis and absorbance value observed was nullified. Immediately, after the addition of silver nitrate solution and leaf extract, 1ml of deionized water was mixed with 2 ml of nanoparticle solution for analysis. Five samples were

taken at a duration of 15 minutes, 30 minutes, 45 minutes, 60 minutes and 24 hours and spectrophotometer scanning was done to observe the absorption peak at different wavelengths from 250 nm to 650 nm for individual samples.

The observed values of absorption peak were taken on Y-axis and wavelength on X-axis and a graph was plotted. Structural analysis of silver nanoparticles was carried out through X-ray diffraction technique. Purified silver nanoparticles were obtained by repeated centrifugation of AgNPs solution at 5000 rpm for 20 min followed by resuspension in 10ml of deionized water. The dried AgNPs were then processed for XRD measurement by X-ray diffract meter. Scherer's formula given below was used to check the domain size.

$D = 0.94 \lambda / \beta \cos \theta$

The overall surface charge present on silver nanoparticles in the liquid medium was measured by computer controlled ZETA sizernanoseries, Malvern instrument nano Zs. A 20 ppm concentration of all the particles were prepared in deionized water to measure the particles size and surface charge distribution.

Determination of MIC and Antibacterial Activity

Broth micro dilution method was used to calculate the minimal inhibitory concentrations (MIC) of silver nanoparticles against pathogenic bacteria (Fig. 5). A duplicate of experiment was performed for both bacterial species.

The first two rows (a & b) of microdilution plate contains *S. aureus* and the next two rows (c & d) are of *S. Pneumonia* cultures. 100 µl of nutrient broth was added in microdilution plate up to well no 12. Now, 100 µl of silver nanoparticles were added into the 1st well and two-fold serial dilution was done up till well no 10. After that 10 µl of 0.5 McFarland overnight fresh cultures of *S. aureus* and *S. pneumonia* were added into the wells. Well no 11 and 12 were maintained as positive (containing antibiotic) and negative control (bacterial cultures only). Incubation was done at 37 °C for 24 hrs and presence of turbidity was observed.

Disk diffusion method was used in order to pattern the antibacterial effect of silver particles with and without antibiotic against the *S. aureus* and *S. Pneumonia* bacterial strains on Petri plates containing Muller-Hinton agar. A 0.5 McFarland standard inoculum was prepared by diluting the freshly grown overnight culture of both test strains with Normal Saline. A lawn culture of each standard bacterial inoculum was prepared on the Petri plates containing the Muller-Hinton agar medium. Prepared discs of AgNPs, antibiotics (Gentamycin 10 µg), and antibiotic disc impregnated AgNPs was applied on the Petri plates containing the test culture. After 24 hours incubation, zone of inhibition was measure around each disc and statistical analysis was done. Each assay was performed in the triplicate.

Statistical analysis

Histogram was plotted by using the mean zone of inhibition observed against four treatment groups through Tukey HSD all pair wise comparisons at alpha 5% via Minitab 18.1 software.

Results

Visual examination and UV-vis spectrophotometer analysis

After mixing 1 mM silver nitrate solution (Fig 1a) with leaf extract (Fig. 1b), a change in colour was observed from watery to light yellow to brown and ultimately colloidal brown (Fig. 1c).

This colour change was indicative of silver nanoparticles formation. UV- vis spectrophotometer analysis of newly synthesized AgNPs at different intervals showed that the maximum absorption was observed at 450 nm suggesting reduction of silver nitrate into silver nanoparticles (Fig. 2).

X-ray Diffraction and Zeta potential analysis

Fig. 3 is shows two slightly intense peaks in the whole spectra of 2θ that ranges from 10 nm to 70 nm. It is interpreted that these slight peaks might be due to

crystalline nature of silver nanoparticles. Using Scherrer's formula, silver nanoparticles were found to be approximately 19 nm in size. Zeta potential analysis was carried out to measure the surface charge on silver nanoparticles. ZETA potential measured by the electrokinetics (ZETA sizer Nanoseries, Malvern instrument Nano) with function of pH (Fig. 4). It was also observed that charge on nanoparticles varies with the pH of the medium comprising of nanoparticles. At pH of 7, zeta potential value was -25 nm indicating that at neutral pH, AgNPs show a greater degree of stability. The results interpreted from the zeta analysis by function of pH represented that with the decrease in pH, there is increase in surface charge. The more the pH, lower be the charge and more be stabilized nanoparticles.

Table 1. Results of various concentrations of AgNPs against *S. aureus* and *S. pneumoniae.*

Well No.	1	2	3	4	5	6	7	8	9	10	11 (PC)	12 (NC)
AgNPs µg/100ul	$8.5x10^1$	$4.25x10^1$	$2.13x10^1$	$1.06x10^1$	5.31	2.66	1.33	$6.6x10^{-1}$	$3.3x10^{-1}$	$3.3x10^{-1}$	antibiotic	deionized water
. aureus	X	X	x	X	X	X**	x*	√	√	√	x	√
	X	x	x	X	X	X**	x*	√	√	√	x	√
S. pneumonia	X	x	x	X	X**	x*	√	√	√	√	x	√
	X	x	x	x	X**	x*	√	√	√	√	x	√

PC Positive control, NC Negative control

X= bacterial growth absent, √= bacterial growth present

*Minimal inhibitory concentration 50

** Minimal inhibitory concentration 90.

Minimal inhibitory concentration and synergistic effect of silver nanoparticles

Minimal inhibitory concentration was observed against both bacterial strains (*S. aureus*and *S. pneumoniae*) through standard broth dilution method. Fig. 5 illustrates the results of antibacterial activity of silver nanoparticles against various concentrations of AgNPs.

In this fig, the white turbidity against silver nanoparticles was observed at well no 7 (1st and 2ndrow) in case of *S. aureus* and well no. 6 (3rd and 4th row) against *S. pneumoniae*. Accordingly, MIC_{50} and MIC_{90} values were calculated for *S. aureus* and *S. pneumoniae.*

Table 1 suggests that MIC_{90} and MIC_{50}value of AgNPs against *S. aureus* was observed in well no 6 and 7 respectively, while MIC_{90} and MIC_{50} in case of *S. pneumonia* was found to be at well no. 5 and 6. Well no. 11 and 12 were maintained as control positive and control negative.

Table 2.MIC_{50} and MIC_{90} values against *S. aureus* and *S. pneumonia*.

Bacterial species	Minimal inhibiyory concentration (µg/100 µl)	
	MIC 50	MIC 50
Staphalococcus aureus	2.66	5.31
Streptococcus pneumoniae	5.31	$1.06x10^1$

The MIC_{90} andMIC_{50} of AgNPs against *S. aureus and S. pneumoniae was* found to be (5.31 & 2.66) and ($1.06x10^1$&5.31) µg per 100 µl (Table 2). Synergistic effect of nanosilver with antibiotics was evaluated by standard Kirby Bauer Disk Diffusion Method (Fig. 6A& B). The mean diameter of inhibition zone calculated around all the disks containing different contents (AgNPs, Gentamycin, AgNPs+Gentamycin,

$AgNO_3$ and Deionized water) are represented by plotting a histogram (Fig.7). It has been observed that silver nanoparticles with and without antibiotics showed significant antibacterial activity. Also antibacterial activity of AgNPs when used in combination with antibiotics was increased many folds (Table 3).

Table 3.% increase in inhibition zone by AgNPsin synergism with antibiotics.

Bacterial strain	Inhibition Zone(mm)		Fold increase % [(b-a)/a]
	AgNPs (a)	AgNPs + Antibiotic (b)	
S. aureus	14.33	21.83	52
S. pneumoniae	14.5	27	86

% age fold increase in bacterial inhibition zone was calculated using the formula [(b-a)/a x100)].

Discussion

Synthesis of silver nanoparticles

In the present investigation, the medical worth of the silver nanoparticle synthesized from the plant extracts is evaluated. A simple and cheaper method for green preparation of silver nanoparticles by means of *A. indica* leaf extract at room temperature is used. Immediately after adding silver nitrate to leaf extract, change in colour of the reaction mixture from initial pale to brown and ultimately colloidal brown was observed. Shankar *et al.* (2004) reported that silver nanoparticles exhibit yellowish to brown colour in the aqueous solution due to the presence of surface plasmon resonance. Results of AgNPs synthesis were comparable to nanoparticles synthesized by other researchers (Sarah Ibrahim *et al.*, 2014; Banerjee *et al.*, 2014).

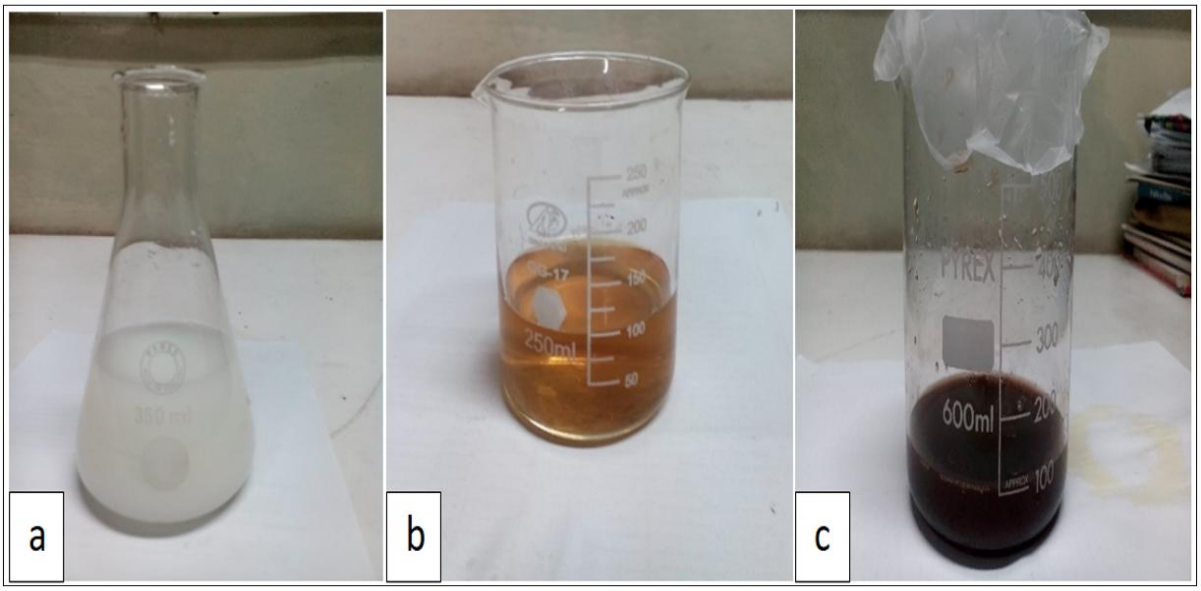

Fig. 1.(a) 1 mM aqueous solution of silver nitrate ($AgNO_3$) (b) leaf extract obtained from fresh neem leaves after filtration through What man no. 1 filter paper (c) Colloidal brown colored reaction mixture of $AgNO_3$ + leaf extract after heating in water bath at 80 ^{O}C indicating the newly synthesized silver nanoparticles.

The results obtained by biosynthesis of silver nanoparticles were also quite close to that one obtained by the Goswami *et al.* (2015). In another study, *pongamia pinnata* was used as a plant source for the preparation of silver nanoparticles synthesis suggesting that AgNPs prepared by green method were found to be effective and efficient in relations of reaction time and were also stable (Qian *et al.*, 2015).

Characterization of AgNPs:

Further confirmation was carried by spectrophotometer. The absorption peak in UV-vis spectrophotometer of 450nm confirmed that silver has been reduced by the plant extract also confirmed by Manikandan *et al.* (2017). The domain size confirmed by X-ray diffraction analyses shows that AgNPs were approximately 19 nm in diameter. Moreover, it was seen that nanoparticles prepared by green method have a potential charge of more than -10 mV suggesting that synthesized nanoparticles are stable. Sheehy *et al.* (2014) performed the same kind of technique to check out the size distribution and stability of nanoparticles and quite similar results were found.

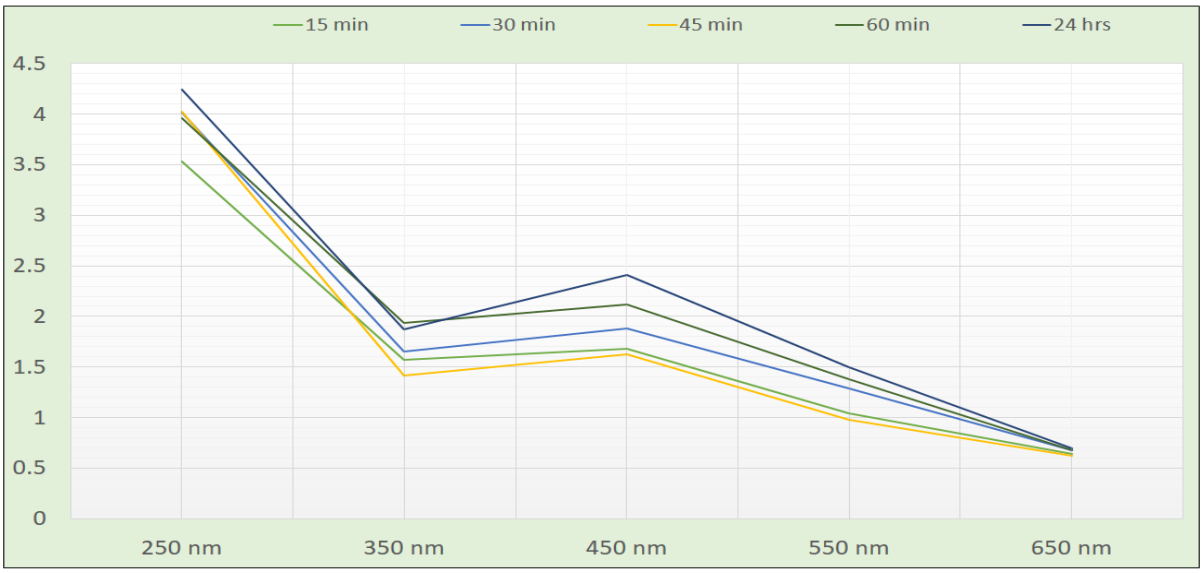

Fig. 2. A line graph plotted against wavelength vs absorption peaks obtained by scanning the newly synthesized nanoparticles at different duration of time in UV-vis spectrophotometer. Analysis of AgNPs showed that the maximum absorption was observed at 450 nm suggesting reduction of silver nitrate into AgNPs.

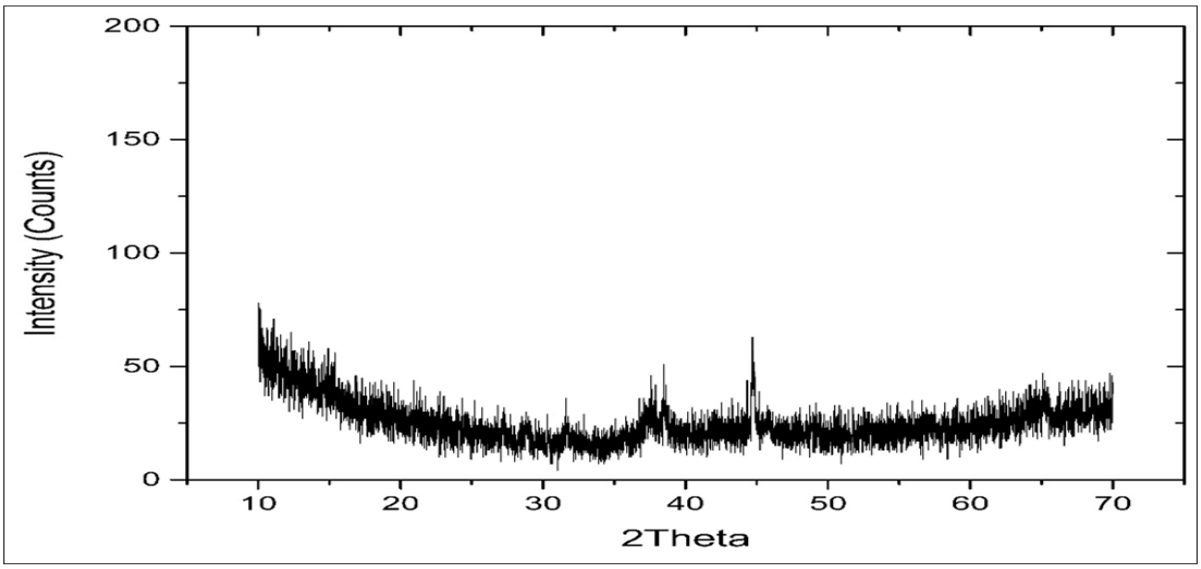

Fig. 3.XRD spectrum of synthesized silver nanoparticles with four distinct diffraction peaks.

Measurement of Minimal inhibitory concentration:

Antibacterial activity against *S. aureus* and *S. pneumoniae* was evaluated through broth dilution method according to the standards and minimal inhibitory concentration was observed against both of the bacterial strains. Some of the researchers used a

modified disc diffusion method to check out the antibacterial effect of silver nanoparticles but micro dilution was preferred in this study due to the lack of diffusion ability of silver nanoparticles aliquoted onto the filter paper discs into the media plates containing the bacterial inoculum (Sheehy *et al.*, 2014).

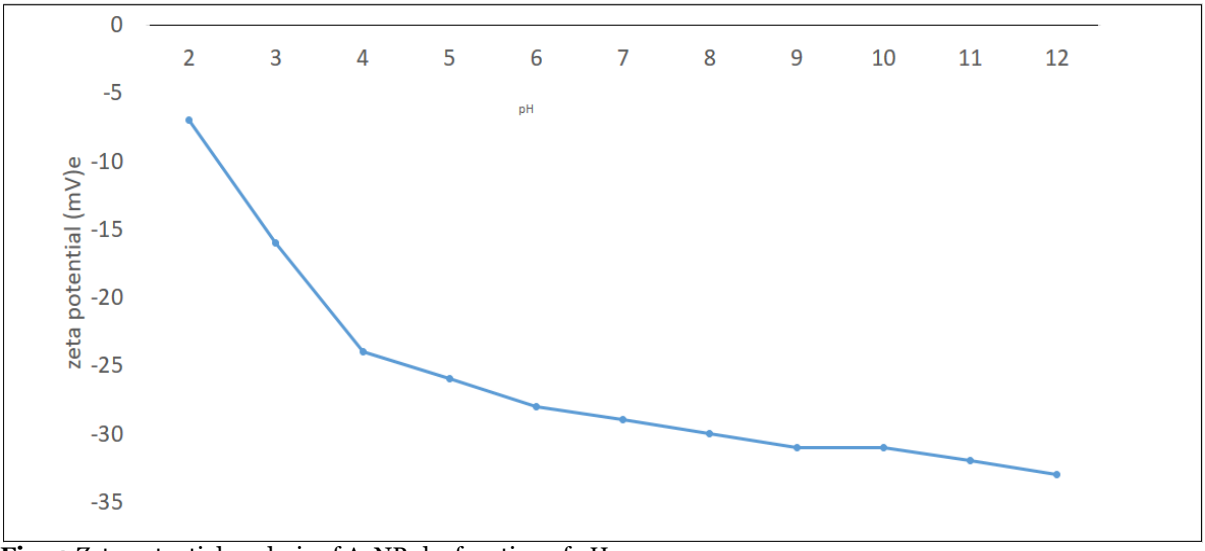

Fig. 4. Zeta potential analysis of AgNPs by function of pH.

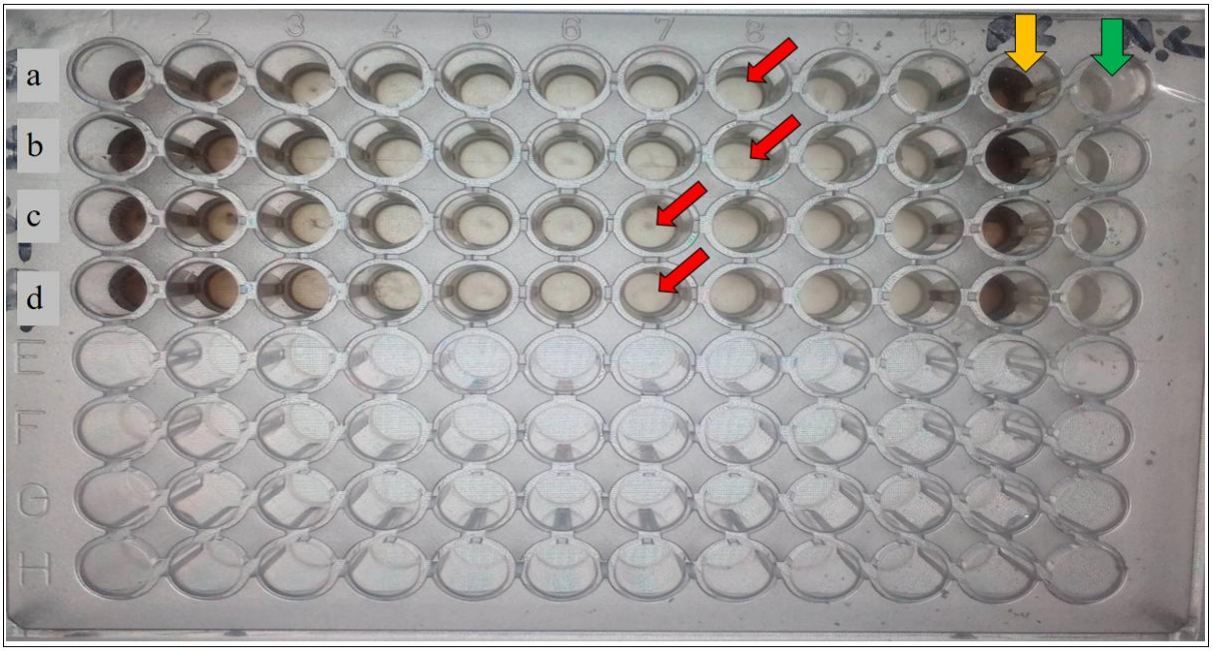

Fig. 5. Microdilution Plate showing inhibition of bacterial growth at different conc. of AgNPsRed arrows in the first two rows (a & b) of microdilution plate indicates the turbidity (viable *S. aureus*) at well no. 8 while red arrows in 3rd and 4th row (c & d) indicates turbidity of *S. pneumonia* at well no. 7. Well no. 11 (yellow arrow) and 12 (green arrow) were maintained as positive and negative control.

The MIC_{90} and MIC_{50} of AgNPs against *S. aureus* and *S. pneumoniae was* found to be (5.31 & 2.66) and ($1.06x10^{1}$&5.31) µg per 100 µl. MICs observed were comparable to the one reported by Sathish kumar *et al.* (2009) who applied nanoparticles against on *E. coli.* Saxena *et al.* (2012) demonstrated that AgNPs bactericidal property was observed at a concentration of 45μg/mL against E. coli.

The results of antibacterial activity obtained were found to be consistent with another study conducted by Mirzajani and Khataee (2010). Many diverse mechanisms are involved in bactericidal properties of silver nanoparticles (fig.8). The MIC of biogenic AgNPs against test strains showed that AgNPs have less effective response on Gram positive bacteria as compared to Gram negative (Sathish kumar *et al.* 2009).

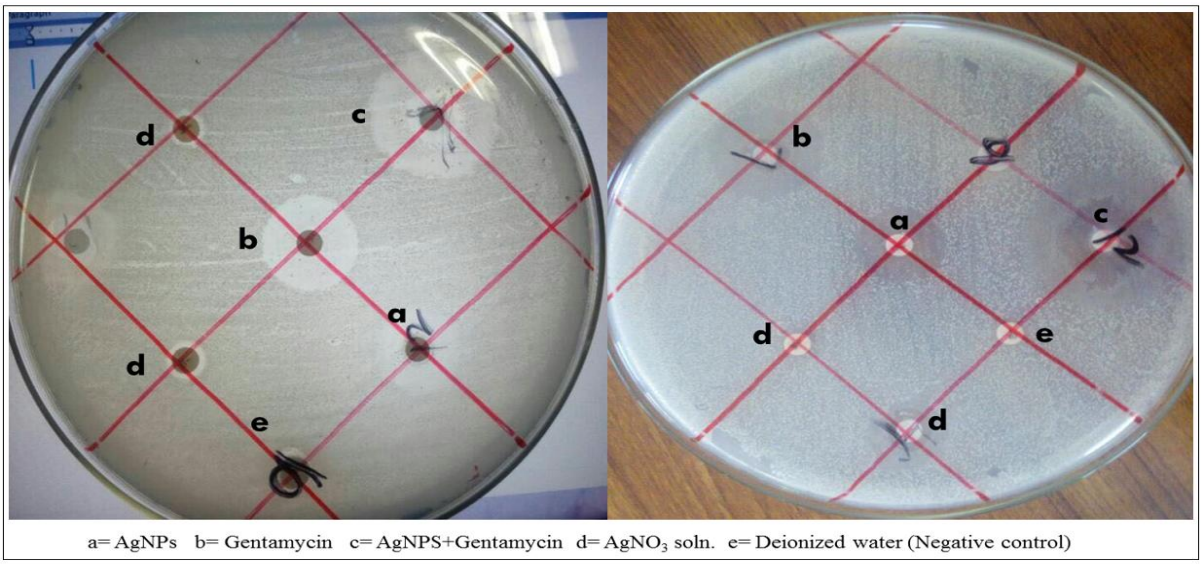

Fig.6 (A & B). Kirby Bauer Disk Diffusion Method to check out the antibacterial and synergistic effect of AgNPs against *S. aureus* (A) and *S. pneumonia* (B). Disks containing combination of antibiotic and nanoparticles (c) showed a greater zone of inhibition as compare to silver nanoparticles (a) and antibiotic (b) alone.

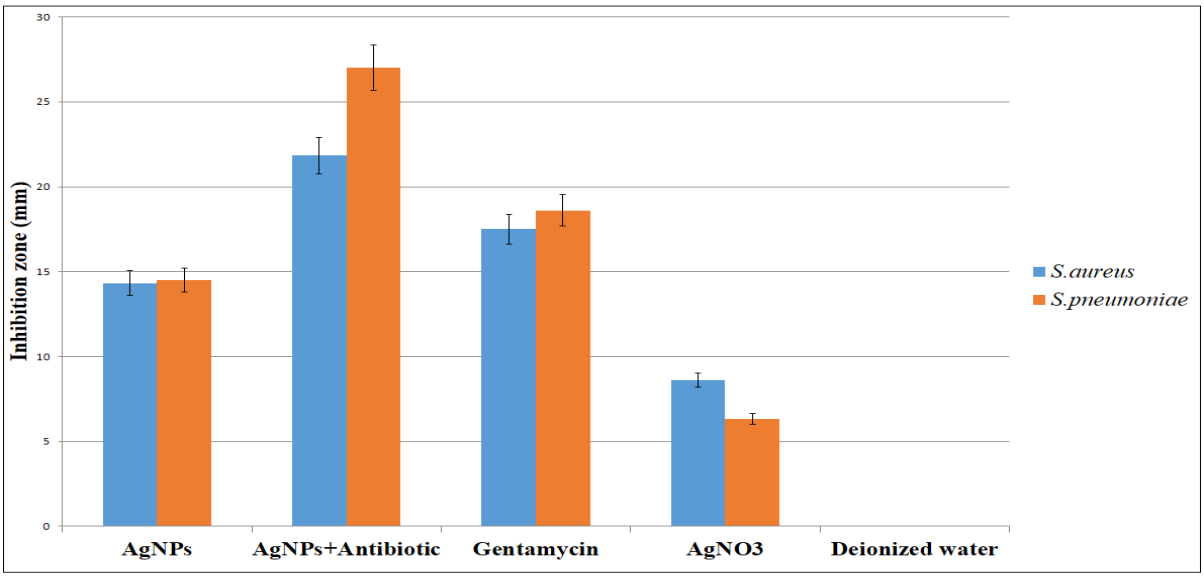

Fig. 7. Histogram was plotted by using the mean zone of inhibition observed against four treatment groups through Tukey HSD All-Pair wise Comparisons at alpha 5%. Group 5 (Deionized water) was used as a negative control. In case of *S. aureus*, two treatment groups (AgNPs and Antibiotic) have a relative non-significant effect while significant synergistic effect was observed for AgNPs + antibiotic. For *S. pneumoniae*, all the means were found to be significantly different from one another.

This is because of the difference in structure of cell wall of both types of bacteria. Gram negative bacteria have thin layer of peptidoglycan, although they have additional layer of lipopolysaccharides with covalent

linkage but this layer lacks rigidity (Madigan and Martinko, 2005). The positive charge on AgNPs attracts negative charge of lipopolysaccharide and makes the permeability possible. Also, these AgNPs act on Gram negative bacteria by metal depletion (Sui *et al.*, 2006). While the Gram positive bacteria having thick layer of peptidoglycan layer make penetration of AgNPs difficult. Other studies have confirmed that nanoparticles damage the DNA by interacting with phosphorous and sulphur containing compounds (Gibbins and Warner, 2005). It is believed that DNA loses its replication ability and proteins become denatured after treatment of bacterial cell with nanoparticles (Kumar *et al.*, 2008). After penetration inside the cell, these nanoparticles result in production of reactive oxygen species (ROS) that effect on cell viability (Shockman and Barre, 1983; Raffi *et al.*, 2008; Haider and Kang 2015).

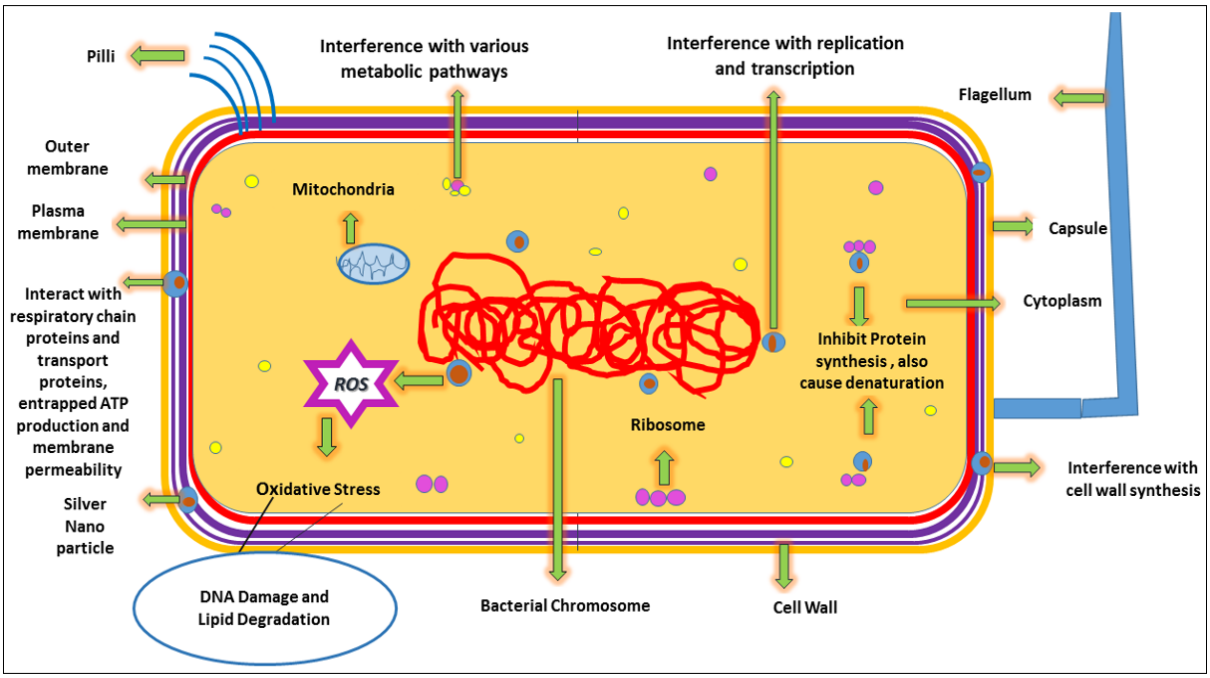

Fig. 8. Mechanism of Antibacterial action of AgNPs on bacteria.

Synergistic effect of AgNPs and Antibiotics

AgNPs with and without antibiotics showed significant antibacterial activity.It was also observed that synergistic effect of nanoparticles with antibiotic was significantly greater than AgNPs and antibiotic, individually. Gentamycin inhibits the bacterial growth by interacting with peptidoglycan layer and causes cell wall lysis. It might be possible that increase in fold of antibacterial activity of nanosilver + Gentamycin complex may be due to bonding of nanosilver through chelation with the antibiotic which contain many active groups i.e. amido groups (Batarseh, 2004). Gentamycin present in the nanosilver + antibiotic complex causes cell wall lysis of bacteria and allow the silver particles to penetrate inside the cell to inhibit bacterial replication, consequently causing more serious damage to bacteria and results in formation of larger inhibition zone. The nanoparticles prepared by green method showed excellent antimicrobial activity against pathogenic bacterial strains of *S. aureus* and *S. pneumoniae.*

Conclusion

In conclusion, we have observed that leaf extract mediated synthesis of silver nanoparticles has many advantages over the other conventional and chemical methods such as, ease of process, cost effectiveness, environmental friendly nature of nanoparticles.

It was observed that Silver nanoparticles produced from the biological plant source have pronounced antibacterial activity. Moreover, synergistic effect of nanosilver with gentamycin has shown a potential

antibacterial activity that might be helpful in development of new antibacterial agents to treat bacterial diseases. A few possible mechanisms of antibacterial action have also been proposed. AgNPs might be a possible replacement of antibiotics in order to treat multi drug resistant bacterial infections, hence could be the answer to antibiotic resistance.

Acknowledgement

The author would like to acknowledge all the supporting members and contributors.

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