STUDY OF IN-VITRO AND IN VIVO ANTIBACTERIAL EFFECTS OF SILVER NANOPARTICLES

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Abstract - *Silver nanoparticles (Ag-NPs) are the silver particles sized between 1-100nm in at least one of their dimensions. Their activities in biological systems make them potential tools in different aspects of medicine. This study is aimed to explore antibacterial properties of silver nanoparticles in vitro as well as in-vivo.*
In our experiments, we explored the antibacterial potential of silver nanoparticles in vitro as well as in vivo and found it to extend over gram-positive (Staphylococcus aureus, Streptococcus pneumoniae and Staphylococcus epidermidis) as well as gram-negative bacteria (Escherichia coli and Klebsiella pneumoniae) effectively. The Gram-positive bacteria were more sensitive to AgNPs than Gram-negative bacteria exhibited by 2.6 cm zone of inhibition in Staphylococcus aureus plates while zone of inhibition in Escherichia coli plates was found to be 1.53cm. Minimum concentration of silver nanoparticles to show its antibacterial properties was found to be 1.56mg/ml against Staphylococcus aureus whereas high concentration of silver nanoparticles 6.25mg/ml was required against Escherichia coli which evinced that there is significant difference in mechanism of Silver nanoparticles as antibacterial agent against gram positive and gram negative bacteria. Further, in-vivo assay was conducted to study the antimicrobial potential of silver nanoparticles inside living system. The assay demonstrated the efficacy of the nanoparticles in mice models through symptomatic and survivability assay.

Key Words: Silver nanoparticles (Ag-NPs), Antimicrobial activity, *in vivo* assay, in vitro assay, Minimum Inhibitory Concentration (MIC), Zone of inhibition, Survivability assay, Symptomatic assay

1. INTRODUCTION

As particles approach the size of nanoscale, the physical properties of the particles change significantly[1]. For example, the color and optical properties of gold nanoparticles differ significantly from its larger counterparts[1], [2]. This significant difference between a bulk and a Nano-sized particle is the basis for the introduction of nanotechnology. Further, the ability to control and tune the size and shapes of nanoparticles has focused development of nanoparticles with desired morphologies and influenced their properties and applicability.

Ag-NPs have received significant interest because of their promising applications in catalysis [3], plasmonics [4], optoelectronics [5], biological sensors [6], antimicrobial activities [7], DNA sequencing [6], Surface-Enhanced Raman Scattering (SERS) [6], climate change and contamination control [8], clean water technology [9], energy generation [10], information storage [11] and biomedical applications [12A]

The in-vitro antimicrobial activities of silver nanoparticles have been demonstrated by previous researchers. However, research focused on studying in-vivo efficacy were found to be wanting. This research aimed to narrow the gap and contribute to the understanding of the antibacterial potential of Ag-NPs in living mammalian system.

2. EXPERIMENTAL PROCEDURE

2.1 Test Organisms

Overnight culture of *Escherichia coli (*ATCC 25922*), Streptococcus pneumoniae (*ATCC 7491*), Klebsiella pneumoniae (*ATCC 13883*), Staphylococcus epidermidis (*ATCC 12228*) and Staphylococcus aureus* were used as the sample organisms to carry out the antimicrobial activity. The organisms belonging to genus *Staphylococcus* are gram-positive whereas *K. pneumoniae* and E.coli are gram-negative organisms. Hence, three Gram-positive and two Gram-negative bacteria were used for the assessment. Mice were obtained from Department of Plant Resources. The experiment was conducted partly in the laboratory of SANN International College, partly in Kalapas biotech and partly in Department of Plant Resources.

2.2 Chemicals and Media

Various chemicals and culture media were used during the work. Muller Hinton Agar, nutrient broth (NB), nutrient agar (NA), Potato dextrose agar (PDA) and YEPD (yeast extract peptone dextrose) were used for the culture of microorganisms. All of these chemicals were the product of Hi Media laboratories (Hyderabad, India).

These chemicals along with other accessories chemicals like CTAB (cetrimonium bromide), tri-sodium citrate, NaBH4 (sodium borohydride), NaOH (sodium hydroxide), Ascorbic acid, AgNO3 (silver nitrate), PVP (polyvinylpyrrolidone and deionized water were also procured from Eureka International Pvt.Ltd at Tripureshwor, Kathmandu.

2.3 Synthesis of Silver nanoparticles (AgNps)

Silver nanoparticles were synthesized by the chemical reduction of the nitrate salt of silver (AgNO3) by using a method proposed by (Pal, Tak and Song, 2015) [13] with few modifications.
Firstly, the seed solution was prepared by dissolving 0.5 ml of 10 mM $NaBH_4$ in 0.5 ml of $AgNO_3$ (0.01M) and 20 ml of 0.001M sodium citrate. The reaction mixture was continuously stirred for 5 minutes and settled to age for 1.5 hours. [13].

Particle growth solution was made by mixing 5ml of 0.01 M AgNO3 to the mixture of 10ml of 0.1M of ascorbic acid, 146ml of 0.1M CTAB and 5ml of silver seed solution. To the growth solution, 1ml of 1M sodium hydroxide solution was added. It was followed by a change in colour from light yellow to brown, red and green within few minutes. The final mixture was successively left to age at 21˚C for 12 hours, 35˚C for 5 minutes and 21˚C for 24 hours. The colour of the aged solution changed from green to red. The solution was purified by frequent centrifugation at different speeds. The surfactants and the small particles were separated by centrifugation at 2100xg for 10 minutes. The resultant precipitate was suspended in water and then centrifuged at 755xg for 10 minutes followed by suspension of precipitate in water.

2.4 Material Characterization

The nanoparticle solution subjected for the spectroscopy was placed in a 1cm cuvette. The spectroscopy was conducted by diluting the small aliquot of samples into the deionized water since concentrated samples tend to show the peak absorbance beyond the range. Similarly, deionized water was used as the blank. The absorbances of nanoparticles were noted at different wavelengths. The characteristic λ_{max} of nanoparticles were found at a varying wavelength. The absorbance was taken in the chemistry laboratory of ASCOL (Amrit Science College) at Lainchaur, Kathmandu.

2.5 In-Vitro Antibacterial Assessment

Agar well diffusion [14]and micro broth dilution[15] were the two techniques used under in-vitro. The former was used to determine the zone of inhibition against the various gram-positive and gram-negative organisms under the given concentration of nanoparticles and the latter was used to find the minimum inhibitory concentration (MIC) of nanoparticles against the bacteria samples.

2.5.1. Agar Well Diffusion

For each bacterial sample, 5 sterile MHA (Muller Hinton Agar) plates were prepared. Two of the plates were used for positive and sterility control and remaining were used for the test. All the bacteria were grown on NB at 37˚C up to a turbidity of 0.5 Mac Farland standards (1.5×10^8CFU/ml). The organisms were swabbed onto the plate by using sterilized cotton swab sticks under the laminar hood. Finally, wells of 6mm were made using cork-borer and 70ul of nanoparticle was inoculated into each well. A total of 3 wells were made in a petri-dish thus inoculating 210µl of nanoparticle sample. The plates were left for 1 hour (for diffusion) and finally incubated at 37˚C for 24 hours. This process was repeated for all the different organisms combining with the different shapes of nanoparticles.

2.5.2 Determination of MIC (Minimum Inhibitory Concentration)

The MIC of different organisms were determined by microbroth dilution method following Nature's protocol [15] with some modifications.

The stock of the silver nanoparticles taken was 0.1 mg in 1 ml. The half-fold serial dilutions were performed and pipetted into the broth such that the concentration of nanoparticles in the broth became 50 µg/ml, 25 µg/ ml, 12.5 µg/ml, 6.25 µg/ml,3.125 µg/ml, 1.56µg/ml and 0.78 µg/ml respectively. 100 µl of nanoparticle solutions were added each to 100 µl of bacteria-inoculated broth. Sterility control was made with no bacterial inoculation and growth control was made with no nanoparticles. Resazurin was used as an indicator. The microtiter plate was incubated at 35˚C and solutions from sterility and growth controls were plated in NA media and incubated at 37˚C.

2.6 In vivo antibacterial activity

Mice model was used to explore the *in-vivo* activities of nanoparticles. The experiment was carried out in the Department of Plant Resources, Thapathali following standard rules and regulations. Due consideration was taken as described by Reilly *et al*. (1991) [16] and Kim *et al.* (2014) [17].

For this, the mice were grouped into three categories based on the specimen injected. The route of injection for every rat was intra-peritoneal. Three rats were placed in each category. The bacterial injection (K. penumoniae) was given only once whereas the saline and nanoparticle were injected for 5 days regularly with 0.5 ml of dose per day via 1 ml syringe. The first group (Group A) of mice were injected with nothing. The second group (Group B) of mice were injected with bacteria (Klebsiella pneumoniae) and further injected with normal saline (0.5ml).

Table -1: *In vivo* experiments for antimicrobial assay

Group of mice	Specimen injected	Dose administered to each mouse
A	none	0.5 ml
B	*K.pneumoniae* (Bacteria)	0.5 ml
C	Bacteria and Nanoparticle (After 48 hours) of bacteria injection).	0.5 ml

The third group (Group C) of mice were injected with the bacteria and then each of them was administered with 0.5ml of mg/ml nanoparticles after one hour. Nanoparticles were injected every 24 hours for five days. The specimen injected and dose administered to each group of mice is shown in table 1.

3. Results and Discussions

3.1 Physical characteristics

The silver nanoparticles solution synthesized in the laboratory is shown in the figure 1. Red colored solution of silver nanoparticles was obtained.

3.2 UV/Visible Spectrophotometry

The final report of spectrophotometry is shown on figure 2.

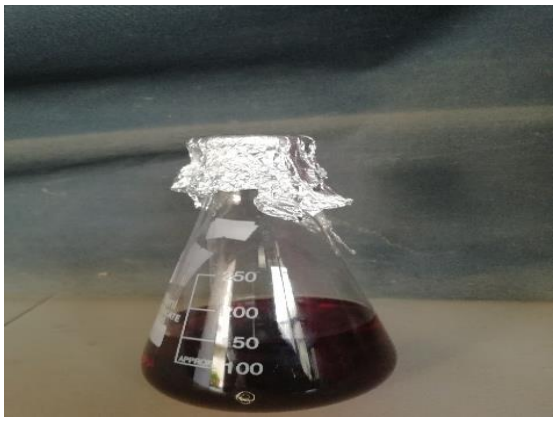

Fig -1: silver nanoparticle solution synthesized in laboratory

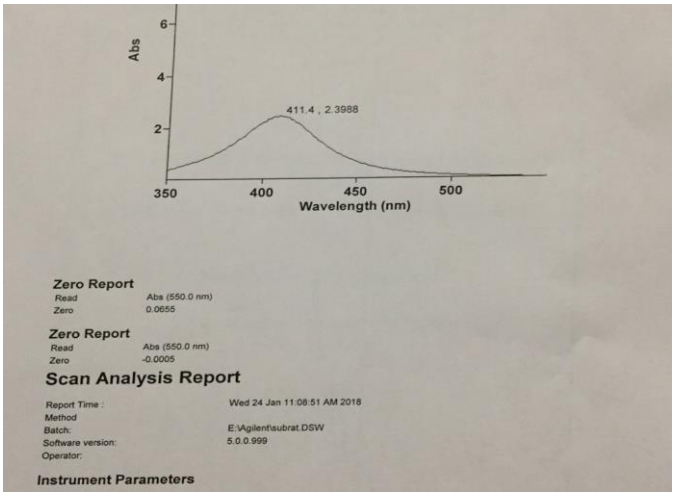

Fig -2: The absorbance spectrum of nanoparticles

The absorbance for the nanoparticle obtained showed its peak value at a wavelength of at 411 nm (λ_{max}) which falls in the visible spectrum.

The nanoparticles synthesized showed a characteristic peak for silver nanoparticles which was comparable to the reference journal (Pal, Tak and Song, 2015) which confirmed that the silver nanoparticles had been synthesized and its shape can be estimated to be rod shaped. Further the size can be estimated to be around 14nm on the basis of the plasmon resonance shifts.

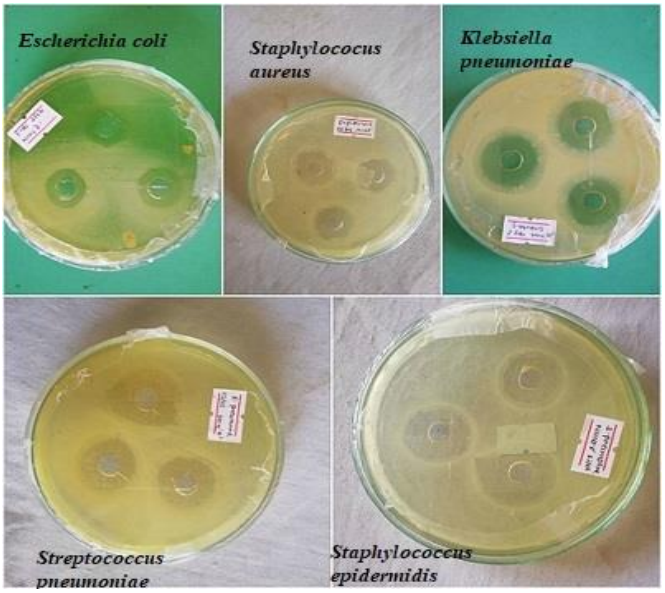

Fig -3: Zones of inhibition observed in bacteria samples Agar well diffusion assay of the silver nanoparticles showed the results as shown in table -2.

3.3 Antimicrobial activity

3.3.1 Well Diffusion Assay

Table -2: Mean values of the zone of inhibition and MIC values of Silver nanoparticles on different bacteria

Test organisms	Mean zone of inhibition (cm)	MIC values (µg/ml)
Staphylococus aureus	2.60 ± 0.1	1.56
Klebsiella pneumoniae	1.96 ±0.05	3.12
Escherichia coli	1.53 ±0.05	6.25
Streptococcus pneumoniae	2.30 ±0.05	3.12
Staphylococcus epidermidis	2.40 ±0.08	3.12

The agar well diffusion assay showed that the silver nanoparticles showed highest antimicrobial activity against Staphylococcus aureus and least against E. coli.

This result can be explained because E. coli is a Gram-negative bacterium and it is less susceptible to antimicrobials than Gram-positive bacteria. The Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than those Gram-positive, including dyes,

detergents, free fatty acids, antibiotics and chemotherapeutics agents [11].

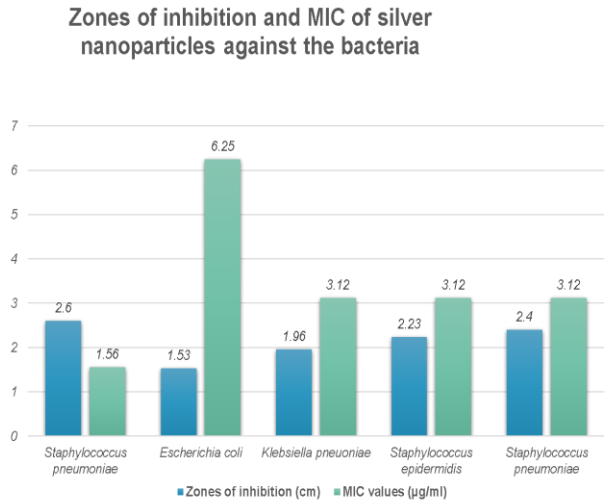

Fig -4: column graph comparing MIC and mean zone of inhibition for different bacteria

Fig -3 clearly illustrates the zones of inhibition for different bacteria while fig-4 shows the comparative study of mean zones of inhibition and MIC of different bacteria in column graph form.

3.3.2 Microbroth dilution: MIC

Indicator resazurin reduced in the presence of living bacteria. Color changed from purple to pink or to colorless in the presence of live bacteria while in the absence of living organism no color change was observed. The lowest concentration at which color change occurred was taken as MIC.

Mean MIC of the nanoparticles against each bacterium obtained from microtiter plate assay using broth dilution method is shown as in table -2.

The least MIC of nanoparticles was observed against Staphylococcus aureus, which coincided with the result for highest inhibition zone in well plate assay. The values of MIC for E. coli are higher than those for S. aureus. This result can be explained by the fact that E. coli is a Gram-negative bacterium and it is less susceptible to antimicrobials than Gram-positive bacteria. The Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than those Gram-positive, including dyes, detergents, free fatty acids, antibiotics and chemotherapeutics agents. [18]

3.3.3 *In vivo* antimicrobial activity

1) Survivability assay

No mice from the control group died. From group B(mice infected with Klebsiella pneumoniae and injected with normal saline), one mouse died after one day and the two other died after 3 days. The death might have been a result of cross-contamination of *K. pneumoniae* bacteria along with other bacteria and the mice's differences in immunity should have been the reason behind the differences in their times of death. No mouse from group C died which indicates that nanoparticles injected after the infection with bacteria probably played some roles in reducing the bacterial load and thus eliminating the pathogenicity.

2) Symptomatic assay

While no symptoms could be observed in uninfected mice, infected mice showed typical symptoms of pneumoniae like frequent sneezing, the rise of body temperature to 38.4° C (after infection) from 36.5° C(before infection) in one mouse and 38.2° C from 36° C in the other mouse after infection. The symptoms were not evident in the mice treated with nanoparticles after bacterial inoculation. Table -3 clearly summarizes the symptoms observed in mice.

The choice of *Klebsiella pneumoniae* for *in vivo* antimicrobial assay was because of its high pathogenicity and quick onset of symptoms in mice after infection. The results from survivability and symptomatic assay showed that injection of silver nanoparticles did provide certain survival advantage to infected mice and proved the effectiveness of the silver nanoparticles *in vivo*.

Table -3: Symptomatic assay of the mice under observation

Group	Bacterial infection (10^5 colonies of *Klebsiella pneumoniae*)	AgNPs injection	Symptoms
A	No	No	No symptoms
B	IP administration of 0.1 ml suspension of	No	1.Rise of body temperature 2.Frequent sneezing 3.Rapid breathing
C	IP administration of 0.1 ml suspension of	0.1 ml of 1mg/ml AgNPs every 12 hrs for 5 days)	No symptoms

4. CONCLUSIONS AND RECOMMENDATIONS

The potential benefits of nanotechnology in biomedical applications have become widely accepted. Nanotechnology is among the most promising sectors for the invention of new applications in medicine. Our experiment was successful in establishing that silver nanoparticles exhibit

broad and effective antimicrobial activity in vitro as well as *in vivo.*

Multi-drug resistance is a growing problem in the treatment of infectious diseases. The widespread use of broad-spectrum antibiotics has produced antibiotic resistance for many human bacterial pathogens. Further research focused on the antimicrobial activity of silver nanoparticles against multidrug resistant bacteria is likely to result in favorable outcomes. This research has opened new horizons in the field of nanomedicine, especially on using silver nanoparticles as therapeutic agent inside living system. There is a lot of scope for the silver nanoparticles in medicine with a need for a lot of future research to be conducted. The genetic mechanism controlling the interaction of a living system with silver nanoparticles can be further studied through gene expression analysis as well as other mechanisms which may help on providing tools to manipulate the interaction to achieve maximum benefits.

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