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Silver Nanoparticles: Biosynthesis Using *Pseudomonas Aeruginosa* ATCC 27853, Its Characterization and Study of Its Antibacterial Activity Against ESBL Producers

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KEYWORDS
Silver nano-particles, *Pseudomonas aeruginosa*, ESBL, Agar well diffusion, MIC

ABSTRACT:

Introduction There is a need of developing alternative compounds to overcome the multidrug resistance in ESBL producers. Biosynthesized silver nanoparticles (AgNPs) play an important role as the new antimicrobial compounds. AgNPs can control the growth of different organisms by releasing silver ions.

Objectives: To provide a method for the biosynthesis and characterization of AgNPs from *Pseudomonas aeruginosa* ATCC 27853 and to study its antibacterial activity against ESBL producing gram negative bacilli.

Methods: AgNPs were synthesized from *Pseudomonas aeruginosa* ATCC 27853. Characterization was done by UV spectroscopy, FTIR and SEM. Agar well diffusion method was employed to study the antibacterial activity of AgNPs and MIC was calculated by standard broth micro- dilution method.

Results: 575 ESBL producers have been isolated from different clinical specimens. Biosynthesis of AgNPs was done under controlled physicochemical parameters. In UV spectrophotometer, the maximum absorption of UV rays was seen at 422 nm. FTIR identified the role of biological molecules in the reduction of silver nitrate to silver nanoparticles. In SEM analysis, spherical shaped AgNPs showed the mean size of 26.187 ± 9.109 nm. In agar well diffusion method, antibacterial activity of AgNPs was seen at 10 and 20 µg/ml of AgNPs and MIC was in a range of 2.30 µg/ml - 3.28 µg/ml of AgNPs.

Conclusions: Green synthesis of AgNPs provides easiest method to modify the important properties of silver in the form of silver nanoparticles having a good antibacterial activity which have promising applications in various branches of medicine.

1. Introduction

Nanotechnology refers to the modification and development of important properties of metal to nanoparticles. The nano size of material results in specific physicochemical characteristics different than those of the bulk materials or larger particles. This effect is mainly credited to high surface area to volume ratio, which results in increased reactivity; so, the nanoscale materials are more advantageous than their bulk materials. Among all the metallic nanoparticles, silver nanoparticles (AgNPs) inherit many properties from silver.[1] AgNPs effectively control growth of over 650 species of bacterial, fungi and algae by releasing silver ions.[2]

AgNPs show antibacterial activity more against Gram negative bacteria.[3] There is a rapidly developing resistance in GNB against various antibiotics.[4] An important mechanism of antibiotic resistance in these bacteria is the production of Extended Spectrum beta

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lactamases (ESBLs).[5] Due to the increase in multidrug resistance in ESBL producers, there is necessity to develop alternative compound to overcome this problem of MDR-ESBL bacteria. Nanotechnology is an advanced alternative to develop new antimicrobial compounds.[6]

Synthesis of nanoparticles can be carried out by different methods: physical, chemical and biological methods. Biological synthesis of nanoparticles is simple, environment friendly, cost effective and pollution free approach. Biosynthesized AgNPs are more biocompatible and exhibit higher antibacterial effect when compared with chemically synthesized AgNPs.[7] In biosynthesis of AgNPs, an important consideration is the selection of an appropriate bacteria which produces the nanoparticles with a desired particle size range and shape.[8]

Pseudomonas aeruginosa potentially reduces $AgNO_3$ to AgNPs.[9] The stability of *Pseudomonas* nanoparticles is most among all the biosynthesized nanoparticles.[8] Therefore, this study aims to develop a method for the biosynthesis and characterization of AgNPs from *Pseudomonas aeruginosa* ATCC 27853 and to study its antibacterial activity against ESBL producing GNB.

2. Methods

An experimental study was carried out in the department of Microbiology, BV(DU) MCH, Sangli, Maharashtra, India from December 2020 to May 2022.

ESBL producing gram negative bacilli from Enterobacteriaceae family were isolated from different clinical specimens received in the department of Microbiology, BV(DU) MCH, Sangli. ESBL detection was done according to CLSI guidelines. ESBL producing gram negative bacilli from Enterobacteriaceae family showing intrinsic resistant to cephalosporins were excluded from the study.[10]

2.1 Biosynthesis of silver nanoparticles (AgNPs):

The culture of *Pseudomonas aeruginosa* ATCC 27853 was incubated at 37° c for 72 hrs in nutrient broth (Himedia, India). The culture supernatant was obtained by centrifugation at 6000 rpm for 15 min.[8,11] 10 ml of culture supernatant was mixed with the 90 ml of $AgNO_3$ (1:9 ratio) solution[12,13] at a concentration of 0.4 g/L and optimized culture parameters i.e., pH- 9, incubation temperature- 70 ^{0}C and incubation time- 96 hours were used.[8,11]

The bio reduction of the Ag ions in the solution was monitored by change in colour during the incubation period of 96 hours. The absorption spectrum of this solution was recorded using UV visible spectrophotometer.

To separate and purify AgNPs from the reaction mixture, the mixture was centrifuged at 12,000-14,000 rpm for 10 min, followed by triple washing of the precipitate with sterile distilled water. The purified nanoparticles were air dried and obtained in powder form.[11,14]

2.2 Characterization of AgNPs:

The powder form of AgNPs was used for further characterization methods like FTIR by scanning the spectrum in the range 400-4000 cm^{-1} and to reveal the shape and the size of AgNPs scanning electron microscopic analysis was applied.[15]

2.3 Study of antibacterial activity of AgNPs by agar well diffusion assay: [16,17]

The antibacterial activity of biosynthesized AgNPs against ESBL producers was investigated by agar well diffusion method on Muller Hinton agar. Culture suspensions of bacterial strains were spread on MHA plates and 6 mm size of diameter well was made on these plates. The AgNPs solution of different concentrations (10, 20, 30, 40 and 50 µg/ml) were prepared and 20 µl of each concentration was poured into each well.

0.5% $AgNO_3$ and sterile distilled water were used as positive and negative control respectively. The petri plates were incubated for 24 hours at 37 ° C and zone of inhibition was measured.

2.4 MIC detection of AgNPs: [16]

MIC of AgNPs for isolated ESBL producers was determined using the standard broth micro- dilution method in 96 wells microtiter plates. Culture broth of each isolate was prepared in Luria Bertani broth and O.D of the cells were adjusted to 0.4 at 600 nm. The stock solution of AgNPs (5.12 mg/ ml) was prepared with sterile distilled water. 2-fold serial dilutions were prepared until column 11 to make concentration gradient, 512-0.5 µg/ml. Finally, 100 µl of test bacterial culture was added into each well of microtiter plate and column 12 was kept as control (no AgNPs). The plates were

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sealed and kept for overnight incubation for 24 hours at 37 º C. To indicate the presence of metabolically active bacterial cells, the presence of colour was observed after 30 minutes of addition of 10 µl/well of Triphenyl tetrazolium chloride (TTC) solution (Himedia, India**)**. The concentration at which no change in colour was noted as MIC.[18] This experiment was done in triplicates and the average of three values was considered as MIC.

3. Results

A total of 575 ESBL producing *E.coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca and Proteus mirabilis* have been isolated from urine, respiratory specimens, pus, blood and other sterile body fluids including CSF, Pleural fluid, peritoneal fluid and pericardial fluid (Table 1)

Table 1
Microbiological profile of ESBL producers based on clinical specimens

Clinical specimen	*E.coli* (Number)	*Klebsiella pneumoniae* (Number)	*Klebsiella oxytoca* (Number)	*Proteus mirabilis* (Number)	Number of ESBL producers (%)
Urine	130	66	17	22	235 (40.86 %)
Respiratory specimens	66	63	-	4	133 (23.13 %)
Pus	66	67	-	2	135 (23.47 %)
Blood	15	31	1	-	47 (08.17 %)
Other body fluids	18	6	-	1	25 (04.34 %)
Total	295 (51.30 %)	233 (40.52 %)	18 (3.13 %)	29 (5.04 %)	**575**

After the incubation of 96 hours at optimum conditions mentioned above, the colour of the mixture of culture supernatant of *Pseudomonas aeruginosa* ATCC 27853 and $AgNO_3$ solution, turned pale yellow to dark brown (Figure 1).

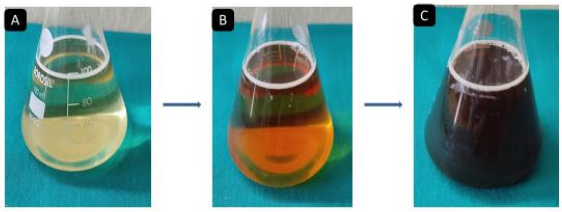

Figure 1: shows the formation of silver nanoparticles in mixture broth

A- Culture supernatant of *P. aeruginosa* + AgNO3 solution

B- Culture supernatant of *P. aeruginosa* + AgNO3 solution after 48 hours of incubation

C- Culture supernatant of *P. aeruginosa* + AgNO3 solution after 96 hours of incubation.

The confirmation of the nanoparticle synthesis and stability of the AgNPs in colloidal solution was monitored by UV visible spectroscopy.[15] In the present study, the instrument used for spectroscopy was UV visible spectrophotometer (Agilent technologies, Cary 60, Serial No: G6860A, Model No: MY17030038). The maximum absorption of UV rays was seen at 422 nm and it was 0.539 (Figure 2).

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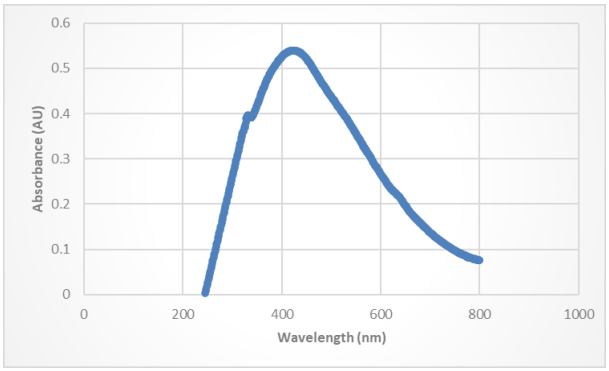

Figure 2: shows the maximum absorption of UV rays at 422 nm in UV spectroscopy

FTIR is a non-invasive, cost effective and simple technique to identify the role of biological molecules in the reduction of silver nitrate to silver nanoparticles.[19] The instrument used to get the spectra was Fourier Transform Infrared Spectrometer, BRUKER, Model No: ALPHA 100508 (Figure 3).

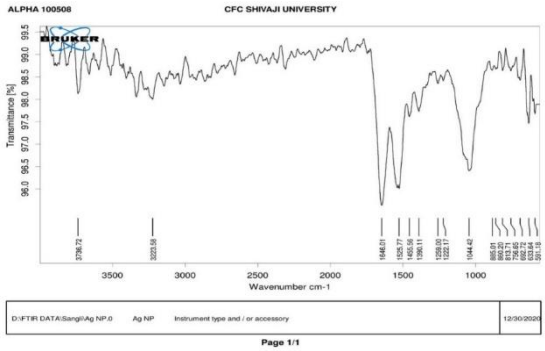

Figure 3: shows FTIR spectrum of silver nanoparticles

There were 2 absorption bands in a single bond region i.e., at 3736.72 cm -1 and at 3223.58 cm -1, indicating hydrogen bond. This confirms the existence of hydrate (H2O), hydroxyl (-OH), ammonium or amino group. Absorption bands below 1700 cm -1 indicates amides or carboxylates functional group. Range between 1670 - 1620 cm -1 indicates unsaturated bond, specifically the peak at 1650 cm -1 is for double bond carbon or olefinic compounds (C=C). Absorption bands at 1525.77 cm -1 and 1455.56 cm -1 indicates amides or carboxylate aromatic rings and methyl (- CH3) or methylene C- H) bend respectively whereas band at 1390.11 cm -1 indicates phenol or tertiary alcohol (OH) bend.

Scanning Electron Microscope (SEM), model: JEOL JSM – 6360, JAPAN was used to reveal the size and shape of biosynthesized AgNPs. The shape of biosynthesized AgNPs was spherical and the maximum and minimum size of AgNPs was 57.774 nm and 8.137 nm respectively. The mean size of nanoparticles was 26.187 ± 9.109 nm (Figure 4)

In agar well diffusion method, all the ESBL producers (100%) showed antibacterial activity at a concentration of 20 µg/ml and above but some of them (21.21%) showed bactericidal effect at 10 µg/ml and above (Figure 5, Table 2).

Our study showed the MIC value of AgNPs against all the ESBL producers was in a range of 2.30 µg/ml to 3.28 µg/ml (Figure 6, Table 3)

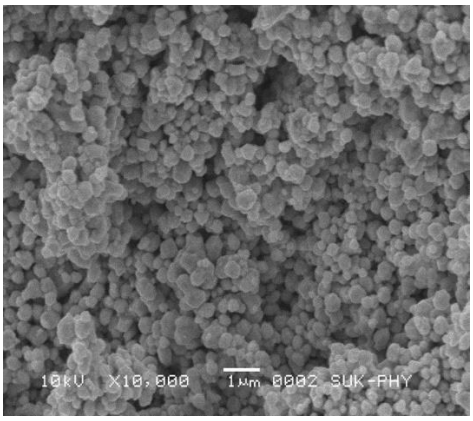

Figure 4: shows Scanning electron microscopic image of AgNPs

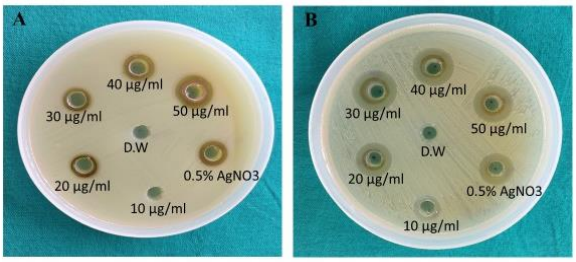

Figure 5: shows the antibacterial activity of AgNPs against ESBL producer by Agar well diffusion assay:

a) Zone of inhibition at 20 µg/ml and above

b) Zone of inhibition at 10 µg/ml and above

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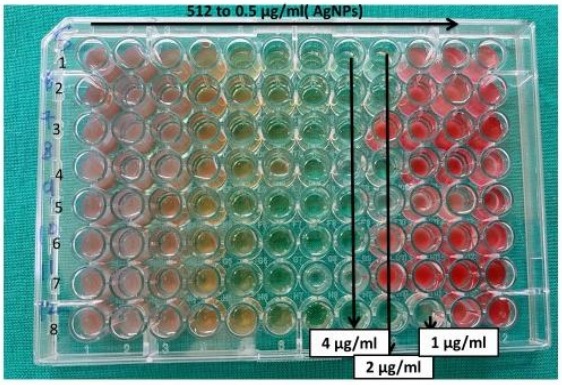

Figure 6: shows the MIC of AgNPs for ESBL producers

Table 2

Antibacterial activity of silver nanoparticles at different concentrations against ESBL producers

(Agar well diffusion assay)

ESBL Producer	Concentrations of silver nanoparticles	
	10 µg/ml (No) (%)	**≥20 µg/ml (No) (%)**
***E.coli* (N=295)**	52 (17.62)	295 (100)
***Klebsiella pneumoniae* (N=233)**	35 (15.02)	233 (100)
***Klebsiella oxytoca* (N=18)**	8 (44.4)	18 (100)
***Proteus mirabilis* (N=29)**	27 (93.10)	29 (100)
Total (N=575)	**122 (21.21)**	**575 (100)**

Our study showed the MIC value of AgNPs against all the ESBL producers was in a range of 2.30 µg/ml to 3.28 µg/ml (Figure 6, Table 3)

4. Discussion

In the present study (Table 1), highest percentage of ESBL producers were isolated from pus sample (57.69%) followed by urine (52.92%). Most of the studies showed ESBL production was seen higher in urine followed by pus, sputum and then other body fluids [4,20,21] while according to a study [5], ESBL cases were higher in Sputum followed by blood, pus and urine.

Among isolated ESBL producers, *Klebsiella pneumoniae* was most predominant (53.4%), followed by *E.coli* (50 %). Apart from these, *Klebsiella oxytoca* and *Proteus mirabilis* (41.86 % and 51.78 % respectively) were also isolated from different clinical specimens. Our results are in accordance with the results

Table 3

MIC of silver nanoparticles against ESBL producers

ESBL Producer	MIC OF Silver Nanoparticle (µg/ml)
***E.coli* (N=295)**	2.30 ± 1.16
***Klebsiella pneumoniae* (N=233)**	2.34 ± 1.68
***Klebsiella oxytoca* (N=18)**	3.28 ± 2.40
***Proteus mirabilis* (N=29)**	2.59 ± 1.82

of the studies conducted in Nepal [22] and Puducherry [5], which stated *Klebsiella pneumoniae* was predominant among ESBL producer (16.55%, and 50.9% respectively) followed by *E.coli* (13.51% and 32.9% respectively). Many studies showed *E.coli* was most prevalent among ESBL producer followed by *Klebsiella spp.* and *Proteus mirabilis.* [4,20,21]

The results of different studies clearly indicate that the prevalence of ESBL producers vary greatly geographically and it is rapidly changing over time. Mainly it is based on different risk factors, local reasons, and rationale use of Beta lactam antibiotics.[23]

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Similar to our study, there are some studies which have used *Pseudomonas aeruginosa* ATCC 27853 for the biosynthesis of AgNPs.[8,11] Previous research studies, [9,24] have shown that NADH and NADH- dependent nitrate reductase enzyme secreted by bacteria is a key factor in the biosynthesis of nanoparticles. *Pseudomonas aeruginosa* is known to secrete nitrate reductase, which might be responsible for the bio reduction of silver ions and the subsequent formation of AgNPs.[24]

Physicochemical parameters such as pH, incubation time, temperature, and Ag ion concentration play an important role in AgNPs synthesis.[11]

In the present study, alkaline pH was used for the biosynthesis of AgNPs. Similarly, a study stated that stable and small nanoparticles formed at alkaline pH. The nucleation of the nanoparticles is caused due to lower pH while at higher pH, electrostatic repulsion can be seen among nanoparticles and that will lead to formation of smaller nanoparticles.[25] Some studies,[8,11] have also reported at alkaline pH, small, stable and spherical AgNPs were produced due to the fast reaction rate.

Silver nitrate ($AgNO_3$) concentration can also affect the size and morphology of the AgNPs. In our study, 0.4 g/l concentration of $AgNO_3$ was used and the same concentration was also used by other studies and they have observed, at this concentration of $AgNO_3$, there was an increase in the silver ions reduction rate to AgNPs. At the concentration below 0.4 g/l, AgNPs synthesis was reduced and at higher concentrations of $AgNO_3$ aggregation of nanoparticles can be seen.[8,11]

Generally, the conversion rate of $AgNO_3$ to AgNPs can be increased at higher temperatures. Higher concentration of AgNPs was clearly observed with increasing temperature, with maximum UV visible absorption observed at 70 ^{0}C similar to our study. Temperature also has an effect on particle size. When the temperature increases, smaller particles would be produced.[11]

Similar to our observation, some studies [8,9,11,15], have also observed, colour of the solution containing $AgNO_3$ and culture supernatant of *Pseudomonas aeruginosa* ATCC 27853 turned pale yellow to dark brown, after incubation of 96 hours, due to reduction of silver ions suggested the formation of AgNPs. As the incubation time increases, AgNPs synthesis also increases and subsequently the colour intensity of the solution also increases, following which the absorbance spectrum declines. After 96 hours of incubation, precipitation of AgNPs was observed and no further increase in the absorbance was observed, indicating the complete conversion of silver ions into AgNPs.

In the present study, the maximum absorption of UV rays was seen at 422 nm and it was 0.539. This result is in accordance with the other studies [9,14], and there in the absorption spectrum of AgNPs, a wide peak at 420-430 nm was seen and it is allocated to surface plasmon resonance indicating the presence of AgNPs with a good AgNPs dispersion without aggregation and might be responsible for sphere-shaped nanoparticles.[19]

Like our results of FTIR spectrum, other studies [8,11,14] showed the peaks at 3226.39 cm $^{-1}$, 1653 cm $^{-1}$ to 1639.16 cm $^{-1}$, 1452 cm $^{-1}$ to 1403 cm $^{-1}$, 1397 cm $^{-1}$, 1387.41 cm $^{-1}$, 1331 cm $^{-1}$, 1042 cm $^{-1}$ and they were assigned to stretching vibrations of primary amines, carbonyl group (C=O), C-O stretching vibrations of aromatic and aliphatic amines, nitro compounds, phenol or tertiary alcohol, alcohol (C-O) groups and -C-N- stretching respectively.

It was observed that, biosynthesized AgNPs are more stable due to the coating by microbial components and the presence of proteins in the media. These Protein components bind to the AgNPs through free amino, carbonyl groups or cysteine and cause coating of AgNPs which helps to stabilize the nanoparticles and prevent agglomeration.[8,11,14,15]

In the present study, SEM showed the mean size of AgNPs was 26.187 ± 9.109 nm and they were spherical in shape. These results are in agreement with the other previous studies [8,11,15] They have found the shape of biosynthesized silver nanoparticles from *Pseudomonas aeruginosa* was spherical, having monodispersed morphology and the size of same nanoparticles was ranging from 20-50 nm.

The properties of AgNPs depend on size and shape of NPs, stabilizers, surrounding media and also the preparation method. The shape and size of AgNPs depends on the controlled thermodynamic conditions such as: temperature, concentration of silver precursor and the pH.[26]

In our study (Table 2), all the ESBL producers (100%) showed antibacterial activity at a concentration of 20 µg/ml and above but some of them (21.21%) showed bactericidal effect at 10 µg/ml and above. Similar to our results, other studies also [27,28], reported the zone of

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inhibition of AgNPs against *E.coli* was seen at a concentration of 20 µg/ml, 10 µg/ml and above respectively. According to a study [16], zone of inhibition of AgNPs against ESBL producing GNB was observed at a concentration of 20 µg/ml and maximum activity was recorded at 40 µg/ml.

There are many theories developed for the antibacterial activity of AgNPs. AgNPs bind to the functional groups of the bacterial cell such as hydroxyl, thiol and carboxyl group and disturbs the respiratory enzyme. Moreover, nanoparticles entered the bacterial cells and release silver ions and leads to inhibition of DNA replication and cell division and finally results in bacterial cell death.[17,29,30] Also, bactericidal activity of AgNPs is due to the cellular leakage that inhibits cell growth and ability of replication.[17]

Similar to our study (Table 3), there are some studies carried out to calculate the MIC of AgNPs against some gram-negative bacteria. According to a study [16], the MIC values of AgNPs for all ESBL isolates varied from 4-8 µg/ml, similar to our study where we found MIC values for the same group of bacteria was in a range of 2.30 µg/ml to 3.28 µg/ml. A study reported MIC of AgNPs for *Klebsiella pneumoniae* and *E.coli* was 11 µg/ml and 3 µg/ml respectively [28], which is somewhat higher than the values we have found against the same bacteria (2.34±1.68 µg/ml and 2.30±1.16 µg/ml respectively). Another study [17], reported the MIC of AgNPs against Gram negative bacteria at concentrations ranging between 4 - 32 µg/ml. According to other studies [27,30], MIC of AgNPs against *E.coli* was 50 µg/ml and 3.4 µg/ml respectively.

The bactericidal effect of AgNPs is due to the attachment of AgNPs to the cell membrane surface. It is reported that the positive charge of silver ions can attract the negatively charged bacterial cell membrane by the electrostatic interaction [7,27] and change the physicochemical properties of the cell membranes which leads to disturbance in permeability, osmoregulation, electron transport and respiration.[30]

So, in this study, it was investigated that AgNPs have an antibacterial activity against ESBL producing GNB. These findings suggest, biosynthesized AgNPs may play a major role in the treatment of the infections caused by these bacteria in the near future. The main limitation of this study was we could not study the biological compatibility of AgNPs as it requires in vivo studies. Recently, several studies focused on toxicity and adverse effects caused by AgNPs using experimental animals. But, the appropriate dose of AgNPs required for system or local treatment of an infection was not studied and verified. Similarly, pharmacological and pharmacokinetic studies of AgNPs are required for their potential use in the medical field.

Thus, Nanotechnology is still an open field which requires further exploration in order to determine if AgNPs in single or in combination with the other conventional antibiotics can be effective for the local and systematic therapy of infectious diseases without showing any side or adverse effects

5. Conclusion:

Nanotechnology is an advanced alternative to develop new antimicrobial compounds. Synthesis of nanoparticles can be carried out by physical, chemical and biological methods. Biological synthesis of nanoparticles is simple, environmentally friendly and cost effective. *Pseudomonas aeruginosa* potentially reduces $AgNO_3$ to AgNPs. The stability of *Pseudomonas* nanoparticles is most among all the biosynthesized nanoparticless. SEM showed biosynthesized AgNPs were spherical and mean size of these nanoparticles was 26.187 ± 9.109 nm. Nanoparticles produced from *Pseudomonas aeruginosa* ATCC 27853 have antibacterial activity against ESBL producing gram negative bacilli at the concentration of 10 and 20 µg/ml. MIC of these nanoparticles against the same group of bacteria was in a range of 2.30 µg/ml to 3.28 µg/ml. So, we can conclude, in the future, biosynthesized AgNPs can be used as an alternative to conventional antibiotics against ESBL producing GNB. To overcome the limitations of this study, more studies on its toxicity and appropriate dose for clearance of an infection are required.

Hence, Nanotechnology can provide a good platform to modify and develop the important properties of silver in the form of silver nanoparticles having promising applications in various fields.

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Ethical approval:

This study is approved by Institutional Ethical Committee with the reference number BV(DU)/MCH/2146/20-21 dated 28/01/2021.

Author contribution:

Conceptualization, Data Curation, and original draft writing: Pradnya Jadhav

Investigation and supervision: Shubhangi Gadgil

Review, Data validation and editing: Shilpa Shah

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