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Antibiogram of silver nanoparticles synthesized from *Rhizobium* species

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ABSTRACT

In this study, we reported the antibacterial activity of silver nanoparticles. Here the nitrogen fixing bacterium Rhizobium species was isolated from rhizosphere soils of agricultural lands using spread plate technique. The pure isolates were inoculated in liquid broth and incubated in appropriate condition for the production of reducing agents. The extracellular components of those bacteria were separated from broth and used to reduce 1mM silver nitrate into silver nanoparticles. The reduction was initially confirmed by UV-Spectrophotometer size and agglomerations were determined by AFM analysis. The Antibiogram pattern of silver nanoparticles were expressed by disc diffusion method and it showed the silver nanoparticles exhibits increased antibacterial activity when combined with commercial antibiotics.

Key words: Silver nanoparticles, *Rhizobium*, UV-Spectrophotometry, AFM, Antibiogram

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INTRODUCTION

Nanotechnology is the branch of science, which controls the molecules at nano level, they are known as nanoparticles. It is the most emerging field for its vast and new applications in both applied and medical field [1]. The most important and distinct properties of nanoparticles are exhibiting the large surface area to volume ratio, so that they can interact with more molecules. Most of the nanoparticles are made from noble metals like Ag, Au, Pt and Pd. They are mostly used in Diagnostic probes, Display devices, Optoelectronic sensors and as catalysis in various reactions. Among those the silver was focused more for its chemical stability, good conductivity and potent antimicrobial activity [2]. Now days, the organisms causing various dreadful diseases are becoming multi drug resistant, because of the prolonged use of antibiotics and some unusual mutation in their genome. These types of resistant microbes are overcoming the conventional treatment methods. They develop new production mechanism for the synthesis of active compounds like enzymes, hormones and some secondary metabolites. Those compounds are acting against the commercial antibiotics and blocking their mode of action in particular ways. This type of inhibition has helps the organism to survive in the antibiotic presence medium and facilitates to cause severe infection. At this situation we need to develop new antimicrobial agents in order to overcome that problem. Silver is a well known drug used from ancient days. They were reduced by various methods using energy from light, sound, heat and used for treatment procedures. The antimicrobial capabilities of silver nanoparticles enable them as a potent alternate drug [2]. Due to their significant role in medicine, the researchers have shown more interest towards development of silver nanoparticles with defined properties. At present various Biological, Chemical and Physical methods are used for the production of silver nanoparticles. Among those methods the biological mediated synthesis has an increased interest because of their simple procedure and non-toxic to the environment [3]. Thus the present

study focused on the biosynthesis of silver nanoparticles from the soil bacterium, *Rhizobium* sp. and cross checking its antibacterial activities against the human pathogenic bacteria.

MATERIALS AND METHODS

Isolation of bacterial culture

The pathogenic bacteria were isolated from patients samples collected from the General Hospital, Sathyabama University, Chennai. The Blood, Pus, and Sputum samples were spread over nutrient agar plates and incubated at 37°c for 24 hrs. After incubation, single colony was taken for identification using various staining and Bio-chemical techniques [4].

The *Rhizobium* sp. used in this reduction reaction was isolated from Rhizosphere soil of an agricultural land used for the cultivation of *Arachis hypogea* (Ground nut). 1gm of soil sample was dissolved in 100ml of sterilized distilled water, from that 1ml of solution was drawn and serially diluted in 6 tubes containing 9ml of sterilized distilled water. 0.1ml of solution from those tubes were aseptically transferred to the plates containing Yeast Mannitol Agar and spread over the media using L-rod and incubated at 22°c for 36 hrs [4].

Identification of organisms

The pink, opaque, round colonies from YEMA media was selected individually and streaked again in the same Yeast Mannitol Agar in order to obtain pure culture. Those pure cultures were further examined by various analytic tests for the confirmation. The analysis includes gram staining, motility testing and various Bio-chemical analyses. The results for those tests were performed and tabulated for further studies [4].

Synthesis of silver nanoparticles

The identified *Rhizobium* colonies were inoculated in Yeast Mannitol Broth and incubated in orbital shaker at 180 rpm for 96 hrs. After the completion of required incubation period, the broth was centrifuged at 10,000rpm for 10 min in order to separate the cells. The cells were washed with milliQ water and suspended in 100ml of distilled water and mixed with silver nitrate at the concentration of 1mM and incubated in dark condition at 180 rpm for 96 hrs. After completion of incubation, the solution was centrifuged at 15,000 rpm for 15 min to concentrate the nanoparticles diluted in the water. The pellet was separated from supernatant and used as a crude source of silver nanoparticles for further analysis and characterization [5, 6].

Preliminary confirmation by UV-spectrophotometer

The initial characterization and confirmation of silver nanoparticles samples were done by UV-spectrophotometer. 10 µl of sample was mixed with 990 µl of distilled water and taken in quartz cuvette and scanned by UV-visible spectrophotometer (SHIMADZU) at the wavelength of 200-800ηm to find out its absorption maxima. The light emitted from photo spectrometer was allowed to pass through the nanoparticles solution. The solution will absorb maximum light at its particular wave length [7, 8].

AFM analysis

The concentrated pellet was slightly allowed to air dry in order to increase their density. From that sample 5 µl of silver nanoparticles solution was spread over the cover slip and allowed to air dried. After the completion of these preliminary procedures the slides were examined through Atomic Force Microscopy. This examination study will enable us to study the agglomerations, roughness, and particle size and homogeneity pattern of silver nanoparticles. Based on the results the production procedures have been optimized and standardized to get the fine nanoparticle samples with desired important qualities [9].

Antibiogram Studies

The standard Kirby-Bauer disc diffusion method was used to identify the anti-bacterial activities of silver nanoparticles. The bacterial Pathogens were inoculated in 1ml of nutrient broth and incubated in at 37°C after 3hrs of incubation they were swabbed over Muller Hinton Agar (composition: Beef extract-30%, casein hydrolysate-1.75%, starch-0.2%, agar-2.5%, P^H -7.0) plates using sterile cotton swabs. 3 discs of Ampicillin, 3 discs of Vancomycin and 1- Sterile Disc were placed at constant intervals. 20 µl of silver nanoparticles solution was added to the sterile disc and each one of antibiotic disc. 20 µl of 1mM silver nitrate was added to another two antibiotic discs. The remaining antibiotic discs serves as control [10, 11].

RESULTS AND DISCUSSION

Identification of organisms

The isolated *Rhizobium* sp. and the pathogens were identified by performing the various biochemical tests likes staining, motility, sugar fermentation, enzyme production and various standard tests [4]. The results of those performed tests were examined carefully for the identification of microorganisms and summarized in Table 1. Based on the result pattern of the individual test organisms, their genera and species were identified and marked as pure culture organisms. Out of these isolates, the medically important clinical pathogens were used for the detection of antibiogram pattern against biologically synthesized silver nanoparticles.

Table1: The biochemical test results of the bacterial pathogens

Sl No	Test name	Colony-4	Colony-7	Colony-8	Colony-12	Colony-15	Colony-17	Soil isolate
1	Gram Staining	-ve	- ve	- ve	- ve	+ve	+ ve	-ve
2	Motility	-ve	+ve	+ ve	+ ve	-ve	- ve	+ ve
3	Catalase	+ve	+ve	+ ve	+ ve	+ve	+ve	+ ve
4	Oxidase	-ve	- ve	+ ve	- ve	-ve	-ve	+ ve
5	Indole	-ve	+ve	- ve	-ve	-ve	-ve	- ve
6	Methyl red	-ve	+ve	- ve	+ve	-ve	+ve	- ve
7	Voges proskauer	+ve	-ve	- ve	-ve	+ve	+ve	+ ve
8	Citrate	+ve	-ve	+ve	-ve	+ve	-ve	- ve
9	TSI	A/A	A/A	K/K	A/A	K/A	K/A	A/A
10	Urease	- ve	-ve	- ve	-ve	-ve	-ve	- ve
11	Mannitol	-ve	-ve	- ve	-ve	-ve	+ve	+ve

From the above mentioned Table 1, the colony number 4 was identified as ***Klebsiella pneumoniae,*** the colony-7 was identified as ***Escherichia coli,*** the colony-8 was identified as ***Pseudomonas aeruginosa***, the colony-12 was identified as ***Shigella dysenteriae***, the colony-15 was identified as ***Bacillus subtillis*** and the colony- 17 was identified as ***Staphylococcus aureus***. Finally the pink coloured round colonies were confirmed as ***Rhizobium* sp**.

Synthesis of silver nanoparticles

The flasks containing cells with silver nitrate were continuously examined at constant intervals during incubation and observed for their colour change. The bacterial metabolites present in the solution will interact with silver nitrate and reduce in to silver nanoparticles. The reduction will increase the surface plasmon resonance and change the solution colour. The color change from white to pale yellow indicates the formation of silver nanoparticles **Fig-1**. The completion of reaction was confirmed by the formation of deep pale yellow color. The solution were taken and subjected for further analysis [5, 6].

Before incubation **After incubation**

Fig 1: Formation of silver nanoparticles

Confirmation by UV spectrophotometry

The reduced nanoparticles solutions were taken in quartz cuvette and scanned in UV spectrophotometer. The absorption maxima showing in the range of 400-450 nm indicates the reduction of silver nitrate into silver nanoparticles. The absorption maxima are directly proportional to the amount of surface plasmon resonance. The

nanoparticles solution increase active surface area when compare to the normal solution [7]. These increased active surface areas exhibit the maximum absorption at particular wave length respective to the compounds [8]. For this solution the peak was obtained at 420 nm and confirmed the formation of silver nanoparticles **(**Fig 2) [7, 8]**.**

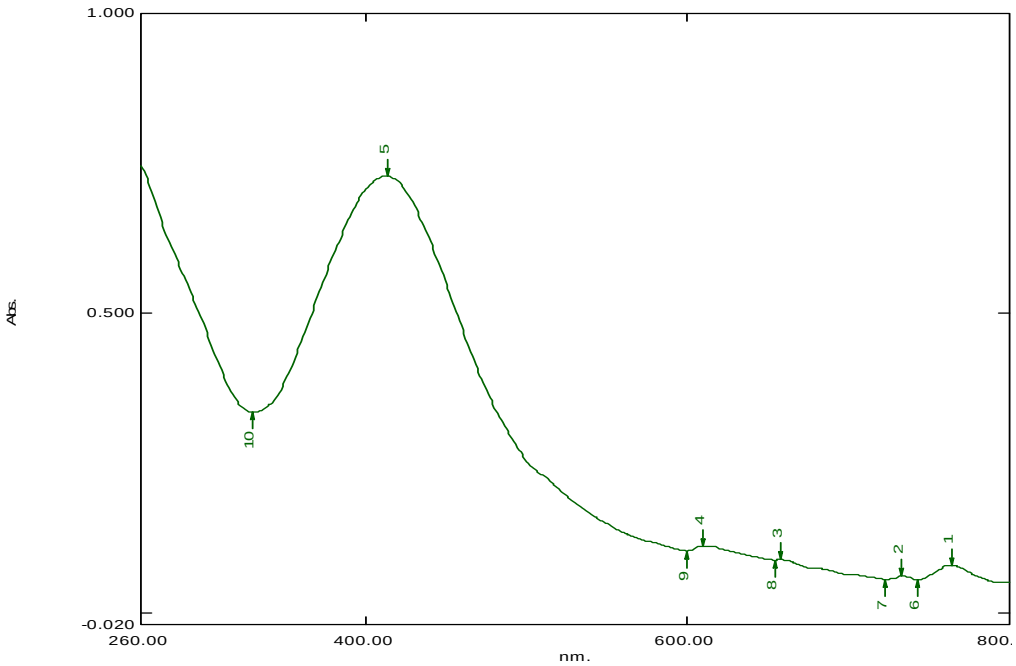

Fig 2: UV-spectrum for silver nanoparticles synthesized from *Rhizobium* sp

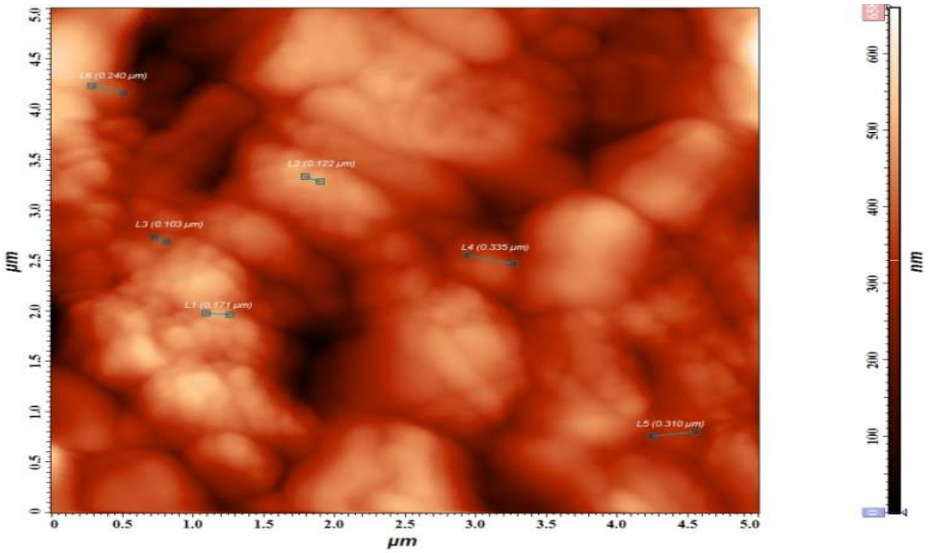

Fig 3: AFM image of silver nanoparticles synthesized from *Rhizobium* sp

AFM analysis

The size reduction of silver nitrate was monitored through Atomic Force Microscopy. The analysis showed the silver nanoparticles size was in between 100-350 nm range (Fig 3.) The image also showed that the nanoparticles are interacting together and form aggregates. These aggregates increased the size of nanoparticles when compared to the individual particles. The analysis also explained that the nanoparticles are conjugated with trace amounts of media components and other extra cellular secondary metabolites of bacteria. The study also explained the average roughness of sample was 340.5 nm due to the aggregations of nanoparticles with each other [9]. From the AFM

analysis it was concluded that the procedure has to be improved for the synthesis of reduced size nanoparticles compared to the existing one [9].

Antibiosis study

The current study proved that the antibacterial properties of silver nanoparticles against the common clinical pathogens. Additionally we concluded that the combination of silver nanoparticles along with the commercial antibiotics like Ampicillin and Vancomycin gave the enhanced bactericidal properties when compared to their individual activities (Fig 4 & Table 2), which was agreed with the previous authors [10, 11, 12].

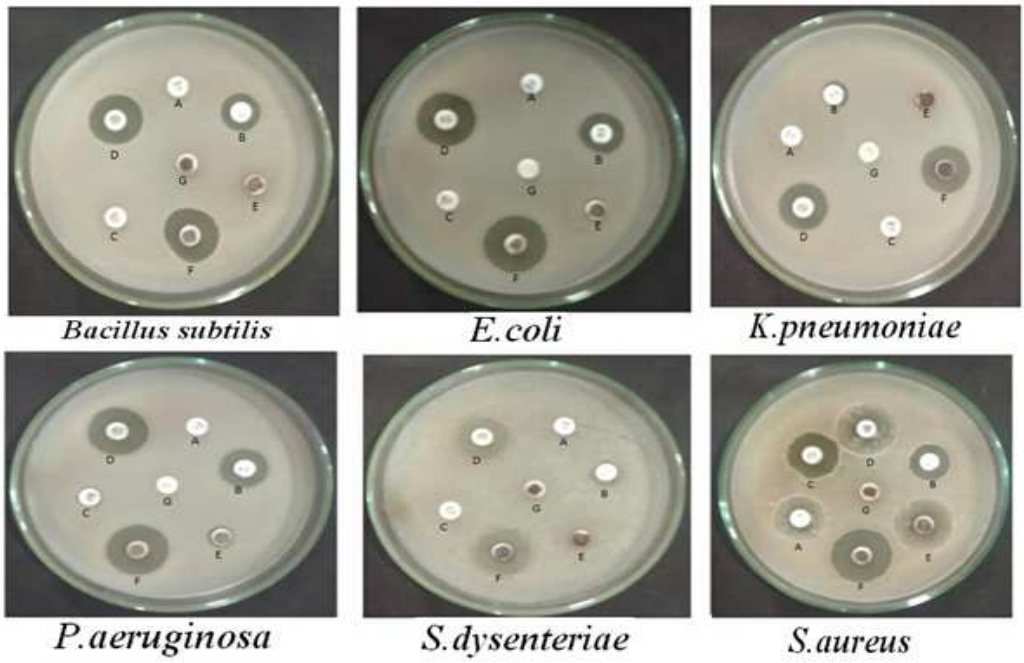

Fig 4: Bactericidal properties of silver nanoparticles
A-Amp; B-Van; C-Amp+1mM $AgNO_3$; D-Van+1mM $AgNO_3$; E-Amp+AgNPs; F-Van+AgNPs; G-AgNPs

Table 2: Zone of Inhibition of Silver Nanoparticles against Various Pathogens

Clinical Pathogens	Zone of Inhibition in mm						
	Amp 10 mcg **(A)**	Van 30 mcg **(B)**	Amp + AgNPs **(E)**	Van + AgNPs **(F)**	Amp + 1mM $AgNO_3$ **(C)**	Van + 1mM $AgNO_3$ **(D)**	AgNPs **(G)**
K. pneumoniae	-	8	7	16	1	10	5
E. coli	-	14	6	21	8	15	5
P. aeruginosa	-	16	8	21	8	19	6
S. dysenteriae	-	12	8	19	-	12	5
B. subtilis	-	13	-	19	-	15	5
S. aureus	20	14	20	23	17	15	5

Amp- Ampicillin; Van- Vancomycin; AgNPs- Silver Nanoparticles; $AgNO_3$- Silver Nitrate.

CONCLUSION

From this study it was confirmed that the silver nanoparticles synthesized from *Rhizobium* sp. have the promising and potent antibacterial activity against the clinical pathogens. It was put an platform to extend the research towards the development of nanoparticles as a novel drug to cure the diseases caused by the drug resistant organisms, and also the study proved that the nanoparticles will exhibit synergistic activity with the commercial antibiotics. In specific way, the nanoparticles exhibited good interactions with Vancomycin when compared with Ampicillin. Finally it confirmed that the synthesized silver nanoparticle has potential antibacterial activity against tested pathogens but its potency was increased when combined with commercial antibiotics.

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