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ANTIBACTERIAL AND ANTIVIRAL PROPERTIES OF SILVER NANOPARTICLES SYNTHESIZED BY MARINE ACTINOMYCETES

Janardhan Avilala and Narasimha Golla *

Department of Virology, Applied Microbiology Laboratory, Sri Venkateswara University, Tirupati - 517503, Andhra Pradesh, India.

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Correspondence to Author:
Narasimha Golla

Department of Virology, Applied Microbiology Laboratory, Sri Venkateswara University, Tirupati - 517503, Andhra Pradesh, India.

E-mail: gnsimha123@rediffmail.com

ABSTRACT: Present work has been conducted to evaluate antibacterial and antiviral activity of silver nanoparticles using marine Actinomycete, *Nocardiopsis alba*, isolated from mangrove soil. Formation, size, and shapes of silver nanoparticles (AgNPs) were characterized by UV-visible spectroscopy, X-Ray Diffraction (XRD), FTIR and Transmission electron microscope (TEM). From the UV-visible spectroscopy, the absorption peak was found at 420 nm. The SEM images confirmed that the sample contains spherical silver nanoparticles. The XRD analysis confirmed that the silver nanoparticles are crystalline, which was confirmed by the FT-IR peak at 564 cm^{-1} corresponding to the silver nanoparticles vibration present in the crystalline structure. The silver nanoparticles have significant antibacterial activity against *Pseudomonas aeruginosa, Klebsiella pneumonia, Streptococcus aureus, and E. coli.* and antiviral activity against new castle viral disease (NDV) in cattle The findings of the present study suggest that the silver nanoparticles possess a good antibacterial a d antiviral activity and could have great importance as a therapeutic agent in current nanomedicine.

INTRODUCTION: Nanobiotechnology combines biological principles with physical and chemical approaches to produce nano-sized particles with specific functions. Silver, Aluminum, Gold, Zinc, Carbon, Titanium, Palladium, Iron, Fullerenes, Copper have been routinely used for the synthesis of nanoparticles. However, former three metals are the most popular metals in bio nonmaterial synthesis. Although an array of physical, chemical and biological methods have been used for synthesis noble metal nanoparticles of particular shape and size for various applications, they remain expensive and involve the use of hazardous chemicals [1].

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A large body of evidence stated that the synthesis of nanoparticles using biological process has generated great interest, due to their unusual optical, chemical, photoelectrochemical and electronic properties [2]. Biological synthesis of nanoparticles by microorganisms, such as bacteria, fungi, actinomycetes and plant extract has been suggested as possible eco-friendly alternatives to chemical and physical methods [3].

The silver nanoparticles have wide applications in medical and agricultural fields [4]. The inorganic nanoparticles are found to be effective in scavenging oxygen-based free radicals, antibacterial property [5]. Actinomycetes are widely distributed in natural and manmade environments, and they are a rich source of antibiotics and bioactive molecules. Although, actinomycetes are well exploited for antibiotics and other high-value metabolites, they are less exploited in nanoparticles synthesis.

Limited reports are available on extracellular biosynthesis of nanoparticles from actinomycetes [6]. The present work focused on biosynthesis, characterization of silver nanoparticles and their therapeutic importance as an antibacterial and antiviral agent.

MATERIAL AND METHODS:

Sample Collection: Mangrove soil was collected from the mangroves forest at Pennar region of Bay of Bengal, Nellore District, Andhra Pradesh, and India and the collected soil sample were used for the isolation of actinomycetes.

Isolation of Actinomycetes: Starch casein nitrate agar (SCA), was used for the selective medium for isolation of actinomycetes. The actinomycetes culture was identified based on microscopic and macroscopic observations and finally identified as *Nocardiaopisis alba* by molecular identification (16S rDNA sequencing) method.

Biosynthesis of Silver Nanoparticles: The actinomycetes were used for silver nanoparticles biosynthesis. The culture flasks were incubated on an orbital shaker (Scigenics-India) at 27 °C and agitated at 220 rpm. The actinomycetes biomass was harvested after 5 days of growth and centrifuged (Thermo -Scientific) at 12000 rpm for 10 min. The supernatant was collected for further reaction to synthesis nanoparticles. The sample was added separately to the reaction vessel containing silver nitrate ($AgNO_3$) at a concentration 0.1M and control (without the silver nitrate) supernatants also run along with the experimental condition. The reaction between this supernatant and Ag^+ ions was carried out in bright conditions for 24 h [7].

Characterization of Silver Nanoparticles: The nanoparticles were separated by centrifugation at 12000 rpm for 10 min. The supernatant was used for analysis and characterization of silver nanoparticles.

UV-VIS Spectral Analysis: The reduction of pure Ag+ ions was monitored by measuring the UV-Vis spectrum of the reaction medium diluting with a small aliquot of sample into distilled water. UV-Vis spectral analysis done with UV-VIS spectro-photometer between the range of 300-600 nm (Shimadzu, UV-2450).

FTIR Analysis: To identify the bio molecules functional groups attached to silver nanoparticles were identified by Fourier transform infrared (FTIR) spectra of supernatant mixed with KBr at a ratio of 1:100 and the recorded on FTIR spectroscopy using a diffuse reflectance accessory the scanning data were obtained with a range between 500-4000 cm^{-1}.

XRD Analysis: The elemental morphology and crystal structures were determined by drop coated films of $AgNO_3$ on a glass plate and employed with X-ray diffractometer (INEL X-ray diffractometer)

TEM Analysis: The silver nanoparticles were fixed in 2.5% (w/v) aqueous glutaraldehyde, centrifuged, suspended in 1.5 ml of 0.1 M phosphate buffer (pH-7.2) at 4 ºC and post-fixed in 1% Osmium tetraoxide at 4 ºC in 0.1 M phosphate buffer (60 min) for TEM. Samples were dehydrated using a graded series of acetone. Micrographs were taken with a HITACHI 7500 transmission electron microscope operated at 100 kV.

Antibacterial Activity: The antibacterial efficacy of silver nanoparticles was tested against pathogenic bacterial strains, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Bacillus subtilis*, by agar well diffusion method [8]. For this, 24 h old Luria broth (LB) cultures of tested bacterial strains were spreader on sterile LB agar plates using sterile spreader followed by placing of silver nanoparticle solution (100 µl/well). Simultaneously streptomycin standard antibiotic disks were placed as control and the plates were incubated at 37 ºC for 24 h. After that, the zone of inhibition surroundings well was measured. Experiments were carried in duplicates and average values were recorded.

Antiviral Activity: Anti-viral activity of silver nanoparticles was tested in an embryonated chicken egg. The eggs were obtained from Poultry Division, Sri Venkateswara Veterinary University, Tirupati and were incubated at 37 ºC in an egg incubator. Lasota strain of NDV was obtained from the Department of Virology, Sri Venkateswara University, Tirupati. Titers of the NDV were determined by inoculating chick embryonating eggs and calculated median egg infectious (EID_{50})

of the virus as per the method of Young *et al*., (2002) [9]. From this, 100 EID_{50}/0.1mL of the viral stock were prepared to proceed for the experiments. This viral stock was stored at -40 ºC.

Preparation of Virus Inoculum (Virus/ Compound Mixture: A 1:2 v/v dilution of the 100 EID_{50}/0.1 mL of the virus with predetermined silver nanoparticles concentrations were made to put extract final concentration in the virus/compound mixture at 10 mg/mL. The virus/compound mixtures were kept at 4 ºC for 1 h to react.

Antiviral Assay: Antiviral activity of silver nanoparticles was carried out by developing chick embryos. Nine-day-old embryonated chicken eggs were used for this study. The eggs were swabbed with 70% alcohol and transferred into sterile trays. The swabbed eggs were placed in the micro safety cabinet where they were punched and inoculated with the nanoparticles/virus mixture via the allantoic route [10]. The nanoparticles/virus mixture is prepared by suspending 0.1 mL of (New Castile virus) NDV in 0.1 mL of nanoparticle solution. Virus dissolved in saline solution without nanoparticles is used as controls. This mixture (inoculum) is incubated for 1 h at 4 ºC before using for inoculation. The treated viruses and controls were inoculated *via* CAM and the yolk sac of 9-11 day old chick embryos. After inoculation, the eggs were sealed with molten wax and incubated at 37 °C. Allantoic fluid from treated eggs was collected for a spot test and haemagglutination test to detect NDV in the eggs.

Haemagglutination (HA) Test: The haemagglutination test was carried out by the method of Thayer and Beard [11]. For this, a known volume of red blood cells (RBC) and NDV in PBS buffer in microtiter plate was incubated in 30 min in room temperature after incubation haemagglutination was observed.

RESULTS:

Biosynthesis of Silver Nanoparticles: Silver nanoparticles were synthesized by actinomycetes isolated from mangrove soil. The actinomycetes culture filtrate upon addition of silver nitrate solution (1 mM) changes from colorless to brown **Fig. 1** is an indication of the formation of silver nanoparticles.

FIG. 1: COLOR CHANGE OF AgNPs (BIO REDUCTION OF SILVER NITRATE)

Characterization of Silver Nanoparticles: The silver nanoparticles are primarily characterized by UV-Vis spectrum. The culture filtrate was subjected to UV spectrometry, and absorption peak was obtained at 420 nm it is an indication of the presence of silver nanoparticles **Fig. 2**.

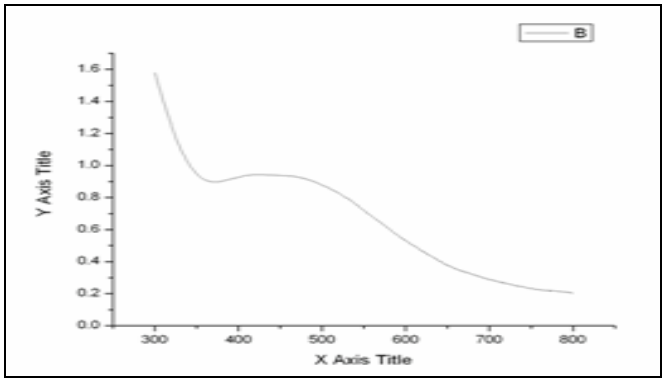

FIG. 2: UV-VIS SPECTRUM OF AgNPs

TEM Analysis of Silver Nanoparticles: The Agnps were subjected to transmission electron microscopy (TEM) to understand their topology and size and the results revealed the formation of polydispersed spherical silver nanoparticles in the range of 20 to 60 nm with an average size of 32.5 nm **Fig. 3**.

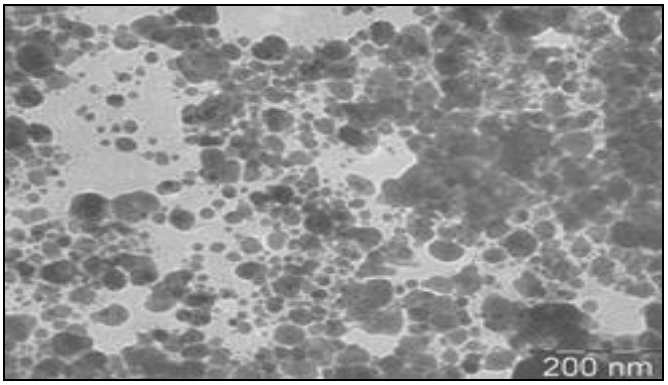

FIG. 3: TEM IMAGE OF SILVER NANOPARTICLES

FTIR Analysis: The FTIR measurements reveal seven bands at 3421, 2923, 1629, 1379, 1162, 1058 and 564 cm–1 that correspond to the N-H, C-H, C=C CH_3, C-C, S=O, and C-Cl respectively **Fig. 4**.

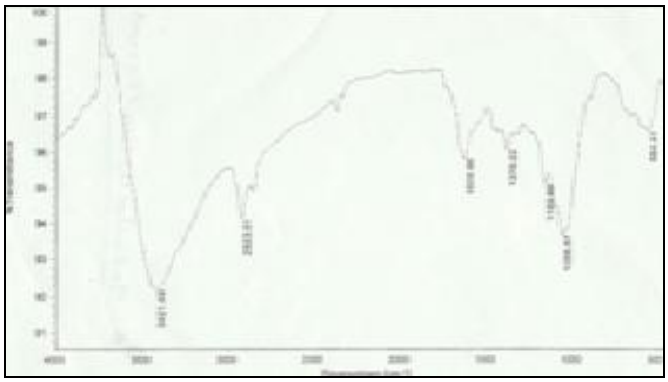

FIG. 4: FTIR SPECTRUM OF SILVER NANOPARTICLES

XRD Analysis: The XRD analysis of silver nanoparticles shows some Bragg reflections, that corresponds to (111), (200), (220), (311) and (222) reflections of face-centered cubic structure of silver **Fig. 5**.

The size of the silver nanoparticles formed in the process was estimated from the Debye-Scherrer equation, and the estimated mean size of the particle was 32 nm.

Antibacterial Activity: The silver nanoparticles exhibited good antibacterial activity against bacterial pathogens like *Bacillus subtitles*, *Pseudomonas aerugionsa*, *Klebsiella pneumonia*, *Escherichia coli* and *Streptococcus aureus* **Table 1** activity were compared with standard antibiotic streptomycin.

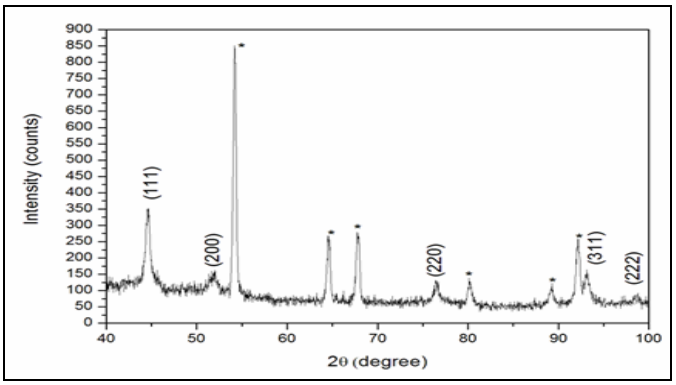

FIG: 5: XRD SPECTRUM OF SILVER NANOPARTICLES

TABLE 1: ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES

Microorganism	Zone of inhibition (cm)
Pseudomonas aeruginosa	0.7
Klebsiella pneumonia	1.0
Bacillus subtilis	1.2
Escherichia coli	1.5
Streptococcus aureus	1.3
Standard antibiotic (Streptomycin)	1.2

Antiviral Activity: Antiviral activity of silver nanoparticles was studied in new castle viral disease (NDV). For this embryonated eggs were treated with various concentration of nanoparticle suspension and egg mortality was tabulated **Table 2.** Simultaneously the HA and HI test results were also recorded. The nanoparticle suspension at 0.3 and 0.5 ml shows better antiviral activity.

TABLE 2: ANTIVIRAL ACTIVITY OF SILVER NANOPARTICLES

Concentration (mL) of silver nanoparticles	No. of Eggs	Mortality				HA Test	
		24 h	48 h	72 h	96 h	+ve	-ve
0.1	5	0/5	1/5	2/4	2/2	5	0
0.2	5	0/5	0/5	1/5	2/4	3	2
0.3	5	0/5	1/5	¼	2/3	4	1
0.4	5	0/5	0/5	2/5	1/3	3	2
0.5	5	0/5	1/5	¼	2/3	4	1
Control	5	0/5	0/5	0/5	0/5	0	0

DISCUSSION: The silver nanoparticles were synthesized using the cell-free filtrate of marine actinobacteria *Nocardiapsis Alba*. Initially, the formation of silver nanoparticles was confirmed by observing the color change of the reaction mixture. The appearance of a brown color from the colorless filtrate in the reaction vessels after 24 h of incubation at room temperature suggested the formation of silver nanoparticles.

Similar findings were made Jaidev and Narasimha [7]. Silver nanoparticles are yellowish brown due to excitation of surface plasmon vibrations within themselves [12]. The reduction of silver ions in the silver nitrate solution upon addition of filtrate manifests in the color change leading to the formation of silver nanoparticles. The exact mechanism responsible for the synthesis of silver nanoparticles is yet to be known in detail, however,

it was hypothesized that the silver ions require NADH-dependent nitrate reductase enzyme for their reduction, [13, 14] which was secreted by the actinomycetes in its extracellular environment. The presence of NADH-dependent nitrate reductase enzyme in extracellular cell filtrate of the microbes used for the synthesis of nanoparticles has been confirmed, and the mechanism has been studied [15, 16].

The reduction of Ag^+ ions was observed in the UV spectrum. The size and shape of the silver nanoparticles reflect the absorbance peak. The size of the nanoparticles has a linear correlation with the peak intensity. Our findings reinstate the reports of Gole *et al.*, [17] which stated that proteins could bind to nanoparticles either through free amine groups or cysteine residues or electrostatic binding of enzymatic negatively charged carboxylate groups present in the cell wall of actinomycetes. The XRD analysis of the silver nanoparticles shows the number of Bragg reflections, that corresponds to (111), (200), (220), (311) and (222) reflections of face-centered cubic structure of silver. The size of the silver nanoparticles formed in the process was estimated from the Debye-Scherer equation and the size was confirmed with 32 nm by transmission electron microscope (TEM). The reflections observed in this analysis indicates that the presence of silver metal with very small size [18]. It was surprisingly found that these silver nanoparticles were more efficient as anti-microbial than the most conventional antibiotics today.

Several types of research have reported the possible inhibitory action of silver nanoparticles on various gram positive and gram negative bacterial strains [19]. The antibacterial activity of silver nanoparticles against methicillin-resistant *Staphylococcus aureus* [20], *Escherichia coli* [21] and *Bacillus subtitles* [22] has been reported. The silver nanoparticles can act as effective antiviral agents. Antiviral activity was carried out on New Castile viral disease (NDV) virus using embryonated eggs. The eggs were treated with different concentrations of nanoparticle suspension, and the egg mortality was tabulated **Table 2**. Simultaneously the HA and HI test results were also recorded. The nanoparticle suspension at 0.3 and 0.5 ml shows better antiviral activity. The size of silver nanoparticles range from 1 to 10 nm can inhibit HIV and HSV by interacting with gp120 and competition for the binding of the virus to the cells [23-27]. The nanoparticle size range from 10-80 nm can inhibit the Respiratory syncytial virus and monkeypox virus by blocking the viral attachment to host cell [28, 29].

CONCLUSION: In the present investigation the silver nanoparticles were synthesized by an actinomycete, *Nocardiapsis alba* isolated from mangrove soil. Formation of AgNPs confirmed by UV-Vis spectrophotometer and functioned groups by FTIR, and the size and shape of silver nanoparticles confirmed by XRD and transmission electron microscope. The silver nanoparticles showed very good antibacterial and antiviral activities against bacterial and viral strains which case the disease in humans and cattle. The present study proved that the silver nanoparticles act as antibacterial and antiviral agents for current nanomedicine.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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