

Research Article



Biogenic Synthesis of AGNP's Using Pomelo Fruit – Characterization and Antimicrobial Activity against Gram +Ve and Gram –Ve Bacteria

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ABSTRACT

In the last decade synthesis of silver nanoparticles has been a subject of a lot of study and numerous methods are being developed for synthesis of nanoparticles of various shapes and sizes. Various biosynthetic strategies are being developed to employ biological sources such as fungi, bacteria, viruses, actinomycetes, algae and plant extracts to catalyze specific reactions. The use of citrus fruit extract to synthesize silver nanoparticles is an exciting possibility that relatively unexplored and under exploited. In the present study, the cost effective and ecofriendly technique of green synthesis of silver nanoparticles using *Pomelo* fruit (*Citrus maxima*) extract was demonstrated. The reduction process was simple, rapid and convenient to handle. Primary detection (color change of solution) indicated the formation of silver nanoparticles and was monitored by *UV-Vis Absorption Spectroscopy*. Characterization of silver nanoparticles was analyzed with *X-ray Diffraction (XRD)*, *Energy Dispersive Spectroscopy (EDS)*, *Scanning Electron Microscopy (SEM)*, *Fourier Transform Infrared Spectroscopy (FTIR)*, *Transmission Electron Microscope (TEM)* and *Particle Size Analysis (PSA)*. The antimicrobial activity of synthesized silver nanoparticles showed effective inhibitory activity against different multi-drug resistant human pathogens.

Keywords: Antimicrobial activity, *Citrus maxima*, Fourier Transform Infrared Spectroscopy, Transmission Electron Microscope.

INTRODUCTION

The pace of scientific research and technological advancement in the recent times is mostly due to the entry of Nanotechnology. With the birth of Nanotechnology in 1974, all fields of science have come up with a new dimension to the research in their respective fields of study. However, it is of recent times it made heavy strides and hastened the research activity in the areas of all sciences more so in the area of Life sciences. "Nanotechnology" mainly consists of the processing, separation, consolidation, and deformation of materials by one atom or one molecule. Nanoparticles of noble metals such as gold, silver and platinum are widely applied in products that directly come in contact with the human body such as shampoos, soaps, detergents, shoes, cosmetic products, and toothpaste, besides medical and pharmaceutical applications. Nanoparticle exhibit completely new or improved properties as compared to the larger particles of the bulk material that they are composed of based on specific characteristics such a size, distribution, and morphology.¹ Nanoparticles present a higher surface to volume ratio with their decreasing size. The specific surface area of nanoparticles is directly proportional to their biological effectiveness due to the increase in surface energy. The use of environmentally benign materials like plant leaf extract,² bacteria³ and fungi⁴ for the synthesis of silver nanoparticles offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and other biomedical applications as they do not use toxic chemicals for the synthesis protocol.

Biological synthesis provides advancement over chemical and physical method, as it is cost effective, and environment friendly, easily scaled up for large-scale synthesis and in this method there is no need to used high pressure, energy, temperature and toxic chemicals.

The surface Plasmon resonance plays a major role in the determination of optical absorption spectra of metal nanoparticles, which shifts to a longer wavelength with increase in particle size. The size of the nanoparticles implies that it has a larger surface area to be exposed to the bacterial cells and hence, it will have a higher percentage of interaction than bigger particles.^{5, 6} Nanoparticles with controlled size and composition are of fundamental and technological interest as they provide solutions to technological and environmental challenges in the areas in the area of solar energy conversion, catalysis, medicine, and water treatment. Thus, production and application of nanomaterial is from 1 to 100 nanometers (nm) is an emerging field of research.^{7, 8} Biological methods are considered as safe and ecologically sound for the nanomaterial fabrication as an alternative to conventional physical and chemical methods. Biological routes to the synthesis of these particles have been proposed by exploiting microorganisms⁹⁻¹³ and by vascular plants.¹⁴ The functions of these materials depend on their composition and structure. Plants have been reported to be used for synthesis of metal nanoparticles of gold and silver and of a gold- silver- copper alloy because of its distinctive



properties such as good conductivity, chemical stability, and catalytic and antibacterial activity.¹⁵

As most of the bacteria have developed resistance to antibiotics, there is a need for an alternative antibacterial substance.¹⁶ Silver is known for its antimicrobial properties, has been used for years in the medical field for antimicrobial applications, and even has shown to prevent HIV binding to host cells.¹⁷ The Agnps are also reported to be nontoxic to human and most effective against bacteria, viruses and other eukaryotic microorganisms at every low concentration and without any side effects.¹⁸ Silver nanoparticles may have an important advantage over conventional antibiotics in that they kill all pathogenic microorganisms, and no organism has ever been reported to readily develop resistance to it.¹⁹ Studies have indicated that biomolecules like proteins, phenols and flavonoids not only play a role in reducing the ions to the Nano size, but also play an important role in the capping of the nanoparticles.²⁰ The reduction of Ag⁺ ions by combinations of biomolecules found in these extracts such as vitamins enzyme/proteins, organic acids such as citrates, amino acids, and polysaccharides is environmentally benign, yet chemically complex.

The present study reports the synthesis of silver nanoparticles by cell free aqueous extract of *Citrus maxima* fruit which were biologically synthesized and the nanoparticles were characterized by UV-VIS, XRD, EDS, SEM, FTIR, TEM and PSA. The *Pomelo* (*Citrus maxima* or *Citrus grandis*) is a crisp citrus fruit native to South East Asia that belongs to family *Rutaceae*. *Pomelo* fruit is very rich in bioflavonoid, which is helpful in reducing pancreatic, intestinal and breast cancer. It has a high content of Vitamin C, which helps in retaining the elasticity of the arteries and improving the digestive system. Although the fruit has high ascorbic acid content, it produces an alkaline reaction once digested. It is extensively investigated for its pharmacological therapeutic effects; so far, there is no report on the synthesis of nanoparticles by using pomelo fruit extract and investigated against different multi drug resistant human pathogens.

MATERIALS AND METHODS

Experimental

Silver nitrate was obtained from Finar Production Company. All the glassware used in the present work sterilized in hot air oven before use.

Fruit material

Citrus maxima fruit belongs to family *Rutaceae* and commonly called as Pomelo. It had originated in the Southeast Asian countries as well as in China and the Indian sub-continent. The fruit is very rich in bioflavonoid and high content of Vitamin C. Fruits were collected from Srikakulam & Visakhapatnam areas, Andhra Pradesh, India.

Preparation of fruit extract

The fruit weighing 150gms was washed with ultra-pure water to remove dust, peel off the skin and the pulp was crushed into 300 ml sterile distilled water and filtered through Whatman No.1 filter paper, repeated the filtration process, and used for synthesis of silver nanoparticles.

Synthesis of nanoparticles

1mM aqueous solution of silver nitrate was prepared and used for the synthesis of silver nanoparticles. 10mL of fruit extract was added into 90mL of 1mM silver nitrate. The primary detection of synthesized silver nanoparticles was carried out in the reaction mixture by observing the color change from pinkish to black and optical density (O.D) at different time intervals were taken for 6 hours, using a UV – Visible Spectrophotometer. Then the solution is stored in dark at room temperature for 24 hours for the complete settlement of nanoparticles. After 24 hours the reaction mixture was centrifuged, at 10,000 rpm for 10 minutes, the supernatant was discarded. The suspension concentrated by repeated centrifugation. The supernatant was replaced by 10ml of distilled water each time and suspension stored for antibacterial assays and for the optical measurements.

Test organism

The multi-drug resistant human pathogens *Staphylococcus aureus*, *Staphylococcus albus* and *Pseudomonas aeruginosa* were obtained from Department of Microbiology, Rajiv Gandhi Institute of Medical Sciences (RIMS), Srikakulam, A.P, India and used for the invitro antibacterial sensitivity tests.

Analysis of silver nanoparticles

UV – Visible Spectra Analysis

The bioreduction of reaction mixture of pure silver ions was observed by observing the UV- Vis Spectrum at different time intervals taking 1mL of the sample, compared with 1mL of distilled water used as blank. UV – Vis spectral analysis has been done by using UV – VIS Spectrometer UV – 2450 (Shimadzu, Japan).

XRD Analysis

The silver nanoparticles were purified by repeated centrifugation of above synthesized brown suspension at 10,000 rpm for 10 minutes by freeze-drying. The freeze-dried nanoparticles were analyzed by XRD, to determine the characterization of the nanoparticles by using Xpert PRO, X – ray diffractometer (PANalytical BV) operation at a voltage of 40kv and the intensity of the diffracted x rays is measured as a function of the diffraction angle 2 θ . The crystalline domain size was calculated from the width of the XRD peaks, using the Scherer formula.

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

Where D is the average crystalline domain size perpendicular to the reflecting planes, λ is the x-ray

wavelength, β is the Full Width at Half Maximum (FWHM), and Θ is the diffraction angle.

SEM Analysis

The morphological characterizations of the samples were done using JEOL (JSM – 6610LV) SEM machine. Thin films of the sample were prepared on carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 minutes. In the analysis an electron beam is focused into affine probe and subsequently raster scanned over a small rectangular area. As the beam interacts with the sample it creates various signals all of which can be appropriately detected.

EDS Analysis

Energy Dispersive Spectroscopy Analysis determined the presence of elemental silver. In order to carry out EDS Analysis, thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid and performed on JEOL JSM – 6610LV SEM instrument equipped with a thermo EDS attachment.

TEM Analysis

Transmission Electron Microscopic (TEM) Analysis was performed with JEOL 1200 EX instrument operating at 120kv voltage. Thin film of the sample were prepared on a carbon coated grid by dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper. Later on, film on the TEM grid was allowed to dry by placing it under a mercury lamp for 5 minutes for the characterization of size and shape of synthesized silver nanoparticles.

FTIR Analysis

To remove any free biomass residue or compound that is not the capping ligand of the nanoparticles, the residual solution of 100ml after reaction was centrifugal at 10,000 rpm for 10 minutes and the resulting suspension dispersed in 10ml sterile distilled water. The centrifuging and dispersing process was repeated three times. The purified suspension was freeze dried to obtain powder. Finally, FTIR IR Analyzer 1 PRESTIGE 21 (SHIMADZU) analyzed the dried nanoparticles.

PS Analysis

The particle size ranges of the nanoparticles were determined by using particle size analyzer (Nano partica SZ – 100, horiba, Japan). Particle sizes were arrived based on measuring the time dependent fluctuation of scattering of laser light by the nanoparticles undergoing Brownian motion.

Antibacterial Assays

The antibacterial assays by agar diffusion method was done against human pathogenic bacteria (gram + ve & gram – ve) like *Staphylococcus aureus*, *staphylococcus*

albus and *Pseudomonas aeruginosa* lawn cultures on Mueller Hinton (MH) agar plates using turbidity standards and fresh overnight cultures were used for inoculating silver nanoparticles. 5 μ L and 10 μ L of sample was inoculated on MH agar plates. The zone of inhibition around area of silver nanoparticle was measured after 18 – 24h with Antimicrobial Sensitivity Measuring scale and the absence of growth on the plates around the silver nanoparticle area confirmed antibacterial activity.

RESULTS AND DISCUSSION

The *Pomelo (Citrus maxima)* fruit extract was used for the synthesis of silver nanoparticles. The reaction started with in first hour of the incubation with silver nitrate (1mM). This was confirmed by the appearance of black color in the reaction mixture.

Visual Inspection

It is well known that silver nanoparticles exhibit black color in aqueous solution due to excitation of Surface Plasmon vibrations. It was found that aqueous silver ions when exposed to *Citrus maxima* fruit extract were reduced in solution, there by leading to the formation of silver hydrosol. The fruit extract was pink in color before the addition of silver ions and this changed to black color, suggested the formation of silver nanoparticle. The flasks were observed periodically was change in color from pink to different shades of black (Table – 1).

Table 1: Visual Inspection (Color Change)

Time	Fruit Extract + AgNO ₃
0 minutes	-
10 minutes	-
30 minutes	+
1 hour	+
2 hours	+
4 hours	++
8 hours	++
16 hours	+++
24 hours	+++

- : No color change; + : Color change; ++ : Brown; +++ : Black

UV – Visible Spectrum Analysis

This is an important technique to preview the morphology and stability of nanoparticles. The fruit mediated silver nanoparticles sample showing an optical absorption band peaked at about 420nm typical absorption for metallic silver nano clusters due to the Surface Plasmon Resonance. Plasmon bands are broad with an absorption tail in the longer wavelength.

XRD Spectrum Analysis

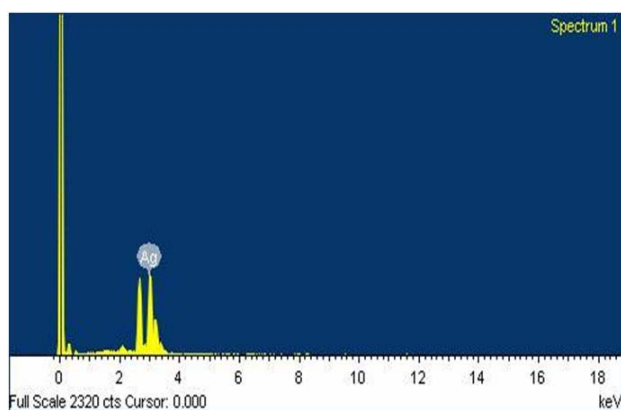
The XRD Spectrum pattern of the dried silver nanoparticles is as shown in figure. The diffraction peaks are observed it can be indexed to the (111), (200), (311)

and (222) reflections of face centered cubic structure of metallic silver ions respectively revealing that the synthesized silver nanoparticles are composed of pure crystalline silver.

Energy Dispersive Spectroscopy Analysis

The additional support of reduction of Ag^+ ions to elemental silver, as confirmed by EDS Analysis, showed a peak at 3 keV in the silver region, which confirms the presence of elemental silver in figure. The optical absorption is typical for the absorption of metallic silver nano crystals due to Surface Plasmon Resonance (Figure 1).

Figure 1: EDS analysis



EDS Analysis shows a peak in the silver region, which confirms the presence of elemental silver.

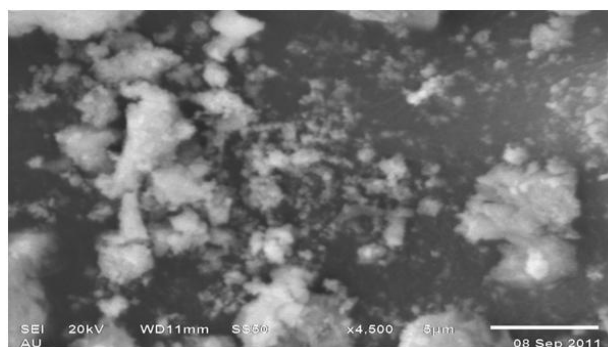
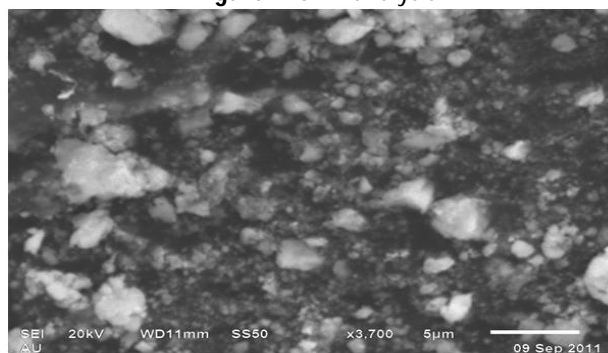
Scanning Electron Microscopy Analysis

The Scanning Electron Microscopy has been employed to characterize the size, shape and morphologies of formed silver nanoparticles. The SEM images of samples are shown in figure respectively. From the images, it is evident that the morphology of silver nanoparticles is nearly spherical it is in agreement with the shape of Surface Plasmon Resonance band in the UV – Vis Spectra. The average particle size range analyzed from the SEM images is observed to be 2.5nm to 5.7nm. Usually biosynthesized silver nanoparticles are covered by biomolecules. In figure, small silver nanoparticles are seen attached to the surface of very large biomolecules (Figure – 2).

Transmission Electron Microscopy Analysis

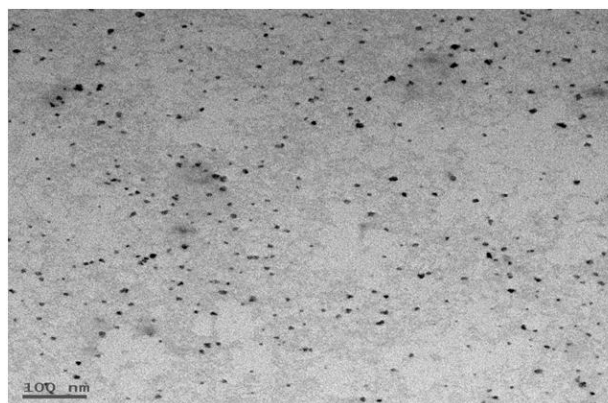
The representative TEM picture recorded from the silver nanoparticle film deposited on a carbon coated copper TEM grid is shown in figure. Figure showed individual silver nanoparticles as well as number of aggregates. The morphology of the nanoparticles is highly variable; most of the Agnps are spherical and are in the range of 2.5nm to 5.7nm in size (Figure – 3). The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent. The separation between the silver nanoparticles seen in the TEM image could be due to capping by proteins present in plant material.

Figure 2: SEM analysis



The SEM images of sample show the morphology of silver nanoparticles is nearly spherical.

Figure 3: TEM analysis



TEM image shows individual round silver nanoparticles as well as number of aggregates in the range of 2.5nm to 5.7nm in size.

FTIR Analysis

FTIR analysis was used for the characterization of the resulting nanoparticles. The wave number (or) frequency (cm^{-1}) of absorption band of peak assigned to the type of vibration, intensity and functional groups of the silver nanoparticles synthesized using fruit extract are shown in figure. Different functional groups were involved. The peaks in the region 4000 to 3600 cm^{-1} and 3400 to 2850 cm^{-1} were assigned to O-H stretching of alcohol and phenol compounds, and aldehyde – C – H – Stretching of alkanes, respectively. The peaks in the region of 1860 to 1550 cm^{-1} and 1450 to 1375 cm^{-1} correspond to N – H bend of primary and secondary amides and C – H (– CH_3 – bend) of alkanes, respectively. The peaks at the region of 1350 to 1000 cm^{-1} correspond to C – N – stretching vibration of the amine or – C – O – Stretching of alcohols, ethers, carboxylic acids, esters and anhydrides. FTIR

analysis reveals that the carbonyl group from amino acid residues and proteins has the stronger ability to bind metal indicating that the proteins could possibly form a layer covering the metal nanoparticles (i.e., capping of silver nanoparticles) to prevent agglomeration and there by stabilize the medium. This suggests that the biological molecules could possibly perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium.

Particle size analysis

The particle size range of the nanoparticles along with its polydispersity was determined by Particle size analyzer. The range of particle size is in between 2.5nm to 5.7 nm.

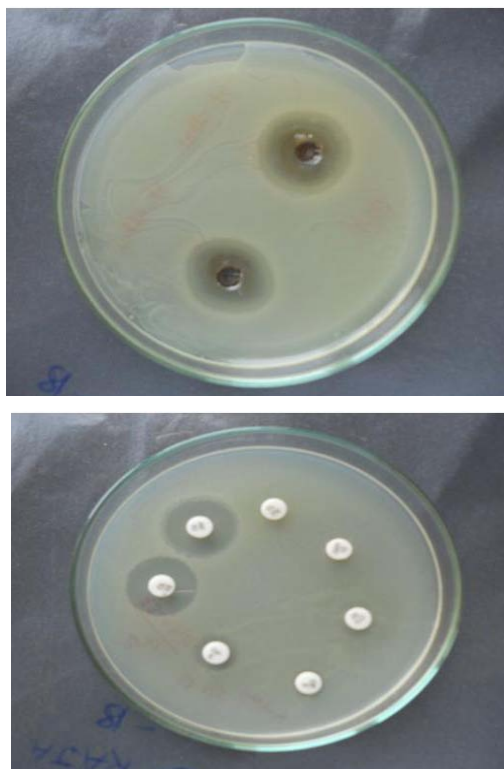
Antibacterial assays

Silver nanoparticles exhibited antibacterial properties against bacterial pathogens such as *Staphylococcus aureus* with 18mm zone of inhibition, *Staphylococcus albus* with 19mm zone of inhibition and *Pseudomonas aeruginosa* with 25mm zone of inhibition which compared to other antibiotics (Table 2), by close attachment of the nanoparticles with the microbial cells (Figure 4).

Table 2: Zone of Inhibition

Micro organisms	Antibiotics zone (mm)	AgNP'S ZONE (mm)
<i>S.aureus</i>	3mm	18mm
<i>S.albus</i>	12mm	19mm
<i>P.aereginosa</i>	13mm	25mm

Figure 4: Antibacterial Assays



Microbial nanoparticles showed very strong inhibitory action against gram + ve & gram – ve bacteria like *S.aureus*, *S.albus* & *P.aereginosa* (1st slide) compared to other antibiotics (2nd slide)

CONCLUSION

The present work indicates that the citrus fruits are useful in the extracellular synthesis of silver nanoparticles.

Extra cellular synthesis of highly stabilized silver nanoparticles by using *Citrus maxima* fruit and the antibacterial effect of biosynthesized AgNP'S against gram +ve & gram –ve bacteria are reported. UV – Visible absorption spectroscopy, which shows an intense peak at 420nm, made the characterization of AgNP'S. XRD pattern confirms the FCC structure of AgNP'S. EDS Analysis gives the optical absorption peak approximately at 3 keV. The SEM & TEM micrographs shown spherical shaped structures with size ranging between 2.5 nm to 5.7 nm. The process that is discussed in the present work for production of nanoparticles is environmental friendly because it is free from toxic chemicals; it is also low cost protocol. Use of citrus fruit in synthesis of other cost effective metal nanoparticles is an exciting, neoteric & tremendous future possibility.

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