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Green Synthesis, Characterization of AgNPs by Using Calotropis Gigantea Leaf Extract and Analysis of Antimicrobial Activity against Major Plant Pathogens

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Abstract

In recent study and well-developed research technology the nanoscience and nanotechnology play a vital role in clinical field such as it is the best way to detect treat and prevents various disease. Researcher identified various type of nanotechnologies with the help of different kind of nanoparticles .Silver nanoparticles is one of the unique nanoparticles which have the optical, electrical, and thermal properties and significant to biological and chemical science its applications include molecular diagnostic in clinical field, in antimicrobial coatings, and wound dressings now the biomedical device have silver nanoparticles provide protection against bacteria by releasing the low level of silver ions. The reactive oxygen species formed on the surface of silver nano particle that includes the cell death of mammalian and microbial cell. Silver nanoparticles have their chemical stability, catalytic activity which prove the silver nanoparticle as an antimicrobial and antifungal effect. Based on these effects, silver nanoparticles hold great potential in preventing wound inflammation and hence promoting wound healing properties. Silver nanoparticles (AgNPs) are one of the most vital nonmaterial among which are involved in biomedical applications which plays an important role in nanomedicine specially it has been focused on applications in cancer diagnosis and therapy. In this study we discuss the synthesis of AgNPs using the *calotropic Gigantea* leaf extract as well as we will see the antimicrobial activity against the plant pathogens which will give us the new aspect of treatment in clinical field.

Keywords
Silver Nanoparticles; nanotechnology; SEM; TEM; leaves extract- Calotropis Gigantea

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INTRODUCTION

Silver nanoparticles:

Nanoparticles are generally considered as particles with a size up to 100 nm, that have completely new or improved properties as compared to the bulk material that they are collected based on particular characteristics such as size, distributions and morphology. Currently developments in nanoscience and nanotechnology have brought potential building blocks for electronic, optoelectronics, medicines and solar cells. Nanoparticles of noble metals, such as gold, silver and platinum are broadly used in many fields and also directly come in contact with the human body, such as shampoos, soaps, detergents, shoes, cosmetic products, and tooth paste, besides medical and pharmaceutical applications.(9 , Anandalakshmi K,2017). Generally, Nanoparticles are synthesized and stabilized by using chemical methods such as chemical reduction, electro chemical technique, photo chemical reactions in reserves micelles and now a day's via green chemistry route. (8, Seeram. Hariprasada, (2015). Biological routes of nanoparticles synthesis using microorganism enzyme and plant, or plant extract have been suggested as possible eco-friendly alternatives to chemical and physical methods. Using plant for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell cultures. It can also be suitably scaled up for large-scale synthesis of nanoparticles. Specific surface area is relevant for catalytic reactivity and other related properties such as antimicrobial activity in silver nanoparticles. (7, S. Kaviya,2011)

Green Synthesis: -

Nanoparticle synthesis is generally carried out by a variety of physical and chemical methods, such as laser ablation, pyrolysis, chemical or physical vapour deposition, lithography electro-deposition, solar gel etc., which are not eco-friendly. Although the commercial methodologies have proven as efficient tools for synthesizing, but their continuous use may pose a great threat to human health and the environment because of the use of toxic and hazardous reagents and generation of toxic by-products in some instances. When compared to various physical and chemical methods, the synthesis is low cost, competent and fast method for producing nanoparticles. Now-a- day, green chemistry procedure are generally used in various biological systems such as yeast, fungi, bacteria and plant extract for synthesis of silver nanoparticles (Ag NPs) . The main reason for selection of the green synthesis method is, due its low cost, non-toxic, eco-friendly and also has great advantages. The green synthesised method is utilized to synthesis for Ag NPs using the various leaf extracts. (9, Anandalakshmi K12017)).

Metal nanoparticles have a high specific surface area and surface atoms, because of their outstanding physicochemical characteristics, including optical, catalytic, electronic, magnetic and antibacterial properties. Synthesis of metal nanoparticles is enormous due to their potential applicability in different areas such as electronics, chemistry, energy, and medicine development. Metal nanoparticles, particularly noble metals, have been studied mainly because of their strong optical absorption in the visible region caused by the group excitation of the free electron gas. The silver nanoparticles have a large area of interest as they have a large number of applications: nonlinear optics, spectrally selective coating for solar energy absorption, biolabeling, intercalation materials for electrical batteries as optical receptors, catalyst in chemical reactions, antibacterial materials, chemically stable materials and good electrical conductors.(11). Recent studies have indicated that bio molecules like phenols, proteins flavonoids and alkaloids not only play a role in reducing the ions to the nano size but also play an important role in the shaping the nanoparticles. There are many reports have been studied on the synthesis of Ag-NP's using extracts of many parts of medical plants such as leaf, root, bark, seed, fruits, flowers, stem as reducing agents. (8, Seeram. Hariprasad,2015).

WHY SILVER?

Silver is one of the basic elements that makes up our planet. It is a rare, but naturally occurring element, slightly harder than gold and very ductile and malleable. Pure silver has the highest electrical and thermal conductivity of all metals and has the lowest contact resistance. Silver can be present in four different oxidation states: Ag^0, Ag^{2+}, Ag^{3+}. The former two are the most abundant ones, the latter are unstable in the aquatic environment. Metallic silver itself is insoluble in water, but metallic salts such as $AgNO_3$ and Silver chloride are soluble in water. Metallic silver is used for the surgical prosthesis and splints, fungicides and coinage. Soluble silver compounds such as silver slats, have been used in treating mental illness, epilepsy, nicotine addition, gastroenteritis and infectious diseases including syphilis and gonorrhea. Although acute toxicity of silver in the environment is dependent on the availability of free silver ions, investigations have shown that these concentrations of Ag+ ions are too low to lead toxicity. Metallic silver appears to pose

minimal risk to health, whereas soluble silver compounds are more readily absorbed and have the potential to produce adverse effects. The wide variety of uses of silver allows exposure through various routes of entry into the body. Ingestion is the primary route for entry for silver compounds and colloidal silver proteins. Dietary intake of silver is estimated at 70-90µg/day. Since silver in any form is not thought to be toxic to the immune, cardiovascular, nervous or reproductive system and it is not considered to be carcinogenic, therefore silver is relatively non-toxic. Silver demand will likely to rise as silver find new uses, particularly in textiles, plastics and medical industries, changing the pattern of silver emission as these technologies and products diffuse through the global economy (5).

Properties of Silver Nanoparticles

1. Optical Properties

When silver nanoparticles are exposed to a specific wavelength of light, the oscillating electromagnetic field of the light induces a collective coherent oscillation of the free electrons, which causes a charge separation with respect to the ionic lattice, forming a dipole oscillation along the direction of the electric field of the light. The amplitude of the oscillation reaches maximum at a specific frequency, called surface plasmon resonance (SPR).

The absorption and scattering properties of silver nanoparticles can be changed by controlling the particle size, shape and refractive index near the particle surface. For example, smaller nanoparticles mostly absorb light and have peaks near 400 nm, while larger nanoparticles exhibit increased scattering and have peaks that broaden and shift towards longer wavelengths. Besides, the optical properties of silver nanoparticles can also change when particles aggregate and the conduction electrons near each particle surface become delocalized.

2. Antibacterial Effects

The antibacterial effects of silver nanoparticles have been used to control bacterial growth in a variety of applications, including dental work, surgery applications, wounds and burns treatment, and biomedical devices. It is well known that silver ions and silverbased compounds are highly toxic to microorganisms. Introduction of silver nanoparticles into bacterial cells can induce a high degree of structural and morphological changes, which can lead to cell death. Scientists have demonstrated that the antibacterial effect of silver nanoparticles is mostly due to the sustained release of free silver ions from the nanoparticles, which serve as a vehicle for silver ions.

3. Sensors

Peptide capped silver nanoparticle for colorimetric sensing has been mostly studied in past years, which focus on the nature of the peptide and silver interaction and the effect of the peptide on the formation of the silver nanoparticles. Besides, the efficiency of silver nanoparticles based fluorescent sensors can be very high and overcome the detection limits.

4. Optical probes

Silver nanoparticles are widely used as probes for surface-enhanced Raman scattering (SERS) and metal-enhanced fluorescence (MEF). Compared to other noble metal nanoparticles, silver nanoparticles exhibit more advantages for probe, such as higher extinction coefficients, sharper extinction bands, and high field enhancements.

5. Catalyst

Silver nanoparticles have been demonstrated to present catalytic redox properties for biological agents such as dyes, as well as chemical agents such as benzene. The chemical environment of the nanoparticle plays an important role in their catalytic properties. In addition, it is important to know that complicated catalysis takes place by adsorption of the reactant species to the catalytic substrate. When polymers, complex ligands, or surfactants are used as the stabilizer or to prevent coalescence of the nanoparticles, the catalytic ability is usually decreased due to reduced adsorption ability. In general, silver nanoparticles are mostly used with titanium dioxide as the catalyst for chemical reactions (27).

Silver nanoparticles (AgNPs) are increasingly used in various fields, including medical, food, health care, consumer, and industrial purposes, due to their unique physical and chemical properties. These include optical, electrical, and thermal, high electrical conductivity, and biological properties. Due to their peculiar properties, they have been used for several applications, including as antibacterial agents, in industrial, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopedics, drug delivery, as anticancer agents, and have ultimately enhanced the tumor-killing effects of anticancer drugs . Recently, AgNPs have been frequently used in many textiles, keyboards, wound dressings, and biomedical devicesAgNPs have been shown much interest because of their therapeutic applications in cancer as anticancer agents, in diagnostics, and in probing. (26)

Anticancer Activity of Silver Nanoparticles

The antimicrobial efficacy of silver nanoparticles (SNPs) has been demonstrated through several studies, although only a few anticancer studies have been conducted in this regard. Since the food and drug administration (FDA) approved its usage in human body, SNPs could be used as potential antimicrobial and anticancer agents, especially in emergent situations such as treating burns and healing of wounds.

Antimicrobial Effects of Silver nanoparticles

Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death.

Toxicity of silver nanoparticles:

The unique physical and chemical properties of silver nanoparticles make them excellent candidates for a number of day-to-day activities, and also the antimicrobial and anti-inflammatory properties make them excellent candidates for many purposes in the medical field. However, there are studies and reports that suggest that nanosilver can allegedly cause adverse effects on humans as well as the environment.

It is estimated that tonnes of silver are released into the environment from industrial wastes, and it is believed that the toxicity of silver in the environment is majorly due to free silver ions in the aqueous phase. The adverse effects of these free silver ions on humans and all living beings include permanent bluish-gray discoloration of the skin (argyria) or the eyes (argyrosis), and exposure to soluble silver compounds may produce toxic effects like liver and kidney damage; eye, skin, respiratory, and intestinal tract irritations; and untoward changes in blood cells.

Economic and social important of calotropis Gigantean

The medicinal plant is providing a main role cultural and biological significant human; mostly they provide foods sources, remediation and energy sources. Now a day the main use of medicinal plant in drug manufacturing has become a global trend and have mostly estimated the plants used in medicinally cases, the plants c. gigantea is perennial xerophytes woody shrub which is able to in harsh condition of heat, drought and poor soils. This member of plant like calotropis gigantea are secrete milk like latex is known mainly heal wounds and stop bleeding of fresh cut.

The main significant of c. gigantea have the ability of both blood coagulation and thinning that the latex contain thrombin is an enzyme responsible for clot formation and plasmin is dissolving it easily. Gigantea are also known as treating heart failure since they rich in cardiac glycosides are major cardiocative compound belonging to triterpenoids class of compound.

Pesticides

Some kind of plants compound such as alkaloids, nicotine and lupitin in the extract of calotropis gigantea latex produced high mortality against mosquito larvae which make it very efficient in the control of several mosquito species some kind of gigantea plant have highly toxic to the white garden terrestrial snails thepapisana.

Renewable energy sources

The extraction of calotropis gigantea could use as a substitute of petrochemical feed stocks, some studies is now concluded that c. gigantea have high density fluid that rich in hydrocarbon. The main ratio of carbon and hydrogen in the extraction of c. gigantea were similar to the crude oil and the heat value content was comparable to the crude oil and fuel oil or may be gasoline. The oil contains of gigantea plant have more saturated fatty acids have been increasing with the increased temperature and drought.

Molecular cases

The calotropis gigantea plant have high polymorphism among the tested population in which the most diverse gene pool , there was a significant difference in primary metabolites carbohydrate and protein and secondary metabolites cardiac glycosides and flavonoids among the different population of given sample which were taken for molecular studies.

MATERIAL AND METHODS

Plant material: -Fresh **Calotropis Gigantea** leaves

Chemicals: - Silver nitrate ($AgNO_3$), Distilled water, Ethanol

Glassware: - Chopping Knife, Conical flask, Petri plates, Beaker, Spreader, Puncture, Culture tubes

Instruments: - Autoclave, Centrifuge, Spectrophotometer, Hot plate, Transmission

electron microscopy, Laminar air flow, Weighing machine

Cultures: - Pure cultures of gram positive –*Bacillus subtilis*, *Staphylococcus sps*. And gram-negative bacteria of - *Klebsiella sps.*

1. Collection of leaves

A major source of plant materials is forest and also occurs from rural areas, agriculture land. First collect the plant and separate the good and healthy leaves. They are washed with several times with tap water and washed several times with distilled water, after they dried at room temperature 37°C for removal of moisture.

Figure: 1 sample of C. gigantea

2. Preparation of leaf Extract:

10-15gm of leaves were weighed and sliced into small pieces, and then 100-200ml of double distilled water was added and then boiled. After boiling the solution it is cooled. The extract was filtered with what man no.1 filter paper. The extract was stored at 4^0C for further usage.

Image:2 Boiling of leaf

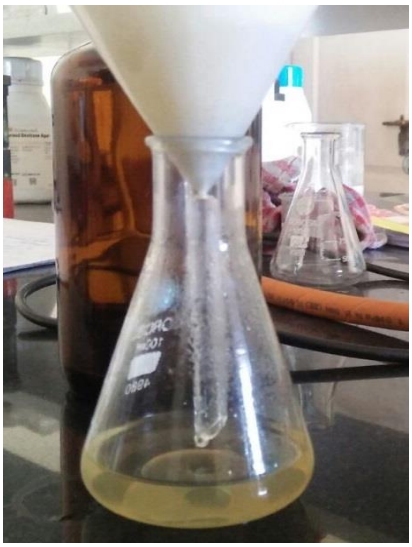

Image:3 Plant extract

3. Preparation of AgNO3 Solution

Required molar $AgNO_3$ solution was prepared by accurate amount of silver nitrate was dissolved in required volume of water. Generally, for the preparation of silver nanoparticles we use 1mM silver nitrate solution. The solution was stored at dark color bottle for present to auto oxidation. [0.0168g $AgNo_3$ /100ml D.W]

4. Green Synthesis of leaves Silver Nanoparticles

Generally, 80 or 90 ml of AgNO3 was added to 20 or 10 ml of leaf extract and follow some physical techniques like heat, stirring then the solution was incubated some time. The color change was observed, it is indicated by formation of silver nanoparticles, which was confirmed by UV-Visible spectrophotometer. The formed silver nanoparticles were centrifuge separated and dried.

Image 4. (a) Plant extracts (b) Plant extract+$AgNO_3$ and boil, colour changes

After adding plant extract and AgNO3 in 1:8 ratio containing 2 molar concentration of prepared AgNO3 solution. Then keep for boiling the mixture for 30-40 minutes. And then colour changes can be observed. Cool the solution and store at in fridge for further use. But freshly prepared only good for observe characteristics including TEM, SEM, UV spectroscopy, and antimicrobial activity.

5. Antimicrobial activity by Agar-well diffusion method:

The assay was conducted by agar well diffusion method. About 15 ml of Nutrient agar medium was poured in the sterilized petri dishes and allowed to solidify. 50µl 24hrs active bacterial strains were taken and test strain was spread over the medium using a sterilized glass spreader. Using flamed sterile borer, wells of 4 mm diameter were punctured in the culture medium and required concentrations (100µl) of AgNPs solution were added to the wells. The plates thus prepared were left for diffusion of extracts into media for 24-48 hours incubated at 37oC. After incubation for 48h, the plates were observed for zones of inhibition. The diameter of zone of inhibition was measured and expressed in millimeters. Plant aqueous extract was used as negative control.

***Xanthomonas* plant pathogens: -**

Xanthomonas species can cause bacterial spots and blights of leaves, stems, and fruits on a wide variety of plant species. Pathogenic species show high degrees of specificity and some are split into multiple pathovars, a species designation based on host specificity.

Citrus canker

Caused by Xanthomonas citri subsp. citri is an economically important disease of many citrus species (lime, orange, lemon, pamelo, etc]

Bacterial leaf spot has caused significant crop losses over the years. Causes of this disease include *Xanthomonas euvesicatoria* and *Xanthomonas perforans* = [*Xanthomonas axonopodis* (syn. *campestris*) pv. *vesicatoria*], *Xanthomonas vesicatoria*, and *Xanthomonas gardneri*. In some areas where infection begins soon after transplanting, the total crop can be lost as a result of this disease.

Figure: 5 leaf cracker

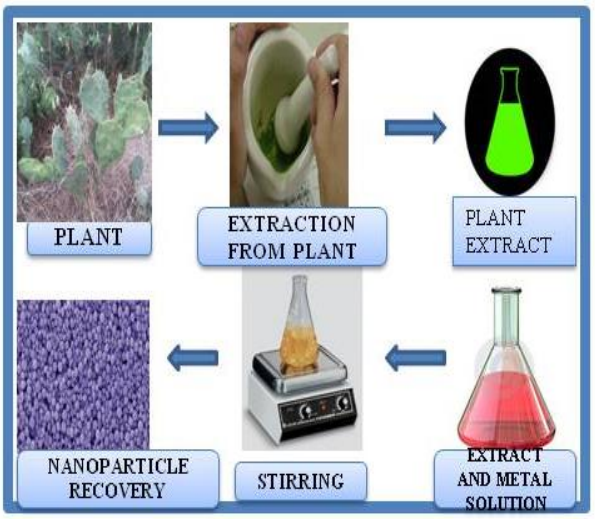

Figure: 6 synthesis of silver nanoparticles

RESULTS AND DISCUSSION

Characterization of AgNps:

U.V-Visible Spectral Study

Formation and stability of AgNPs sterile distilled water is confirmed using UV-Visible spectrophotometer in a range of wavelength from 200 to 800 nm. The production of silver nanoparticles by reduction of silver ions due to the addition of leaves extract. The band observed in spectrum, confirmed by silver nanoparticles. Various plants leave extracts were giving peaks at different wavelength. Most of the plants give band at 400-490nm region. (3-22). Different plant leaves show following absorption values.

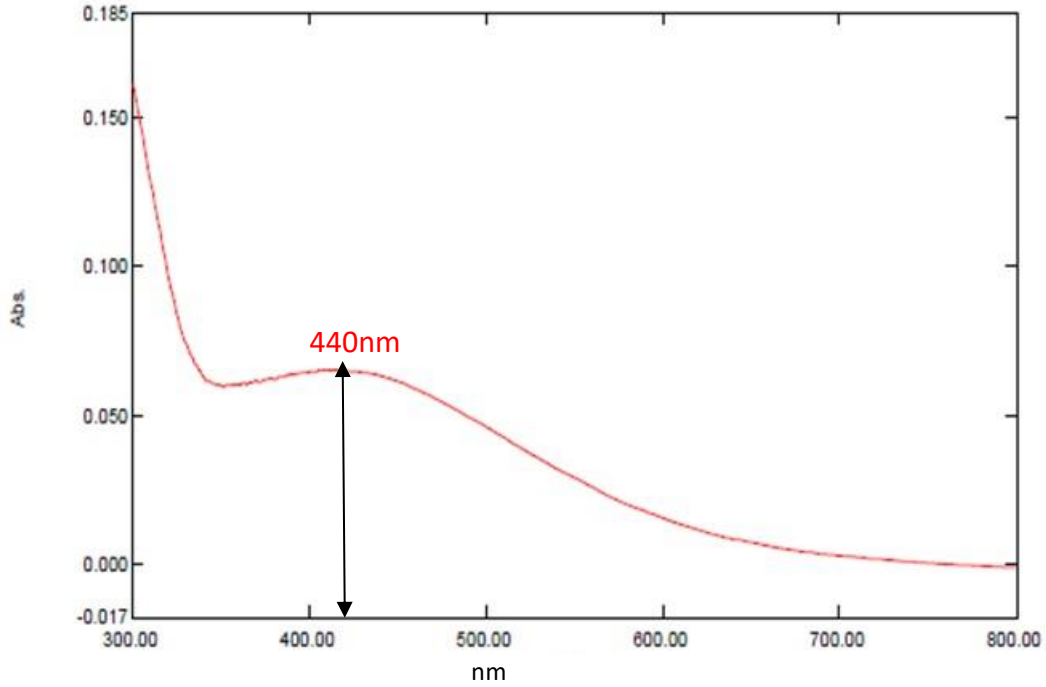

Image: 7 UV spectrophotometer range of AgNPs

As per the review of literature shows Normal range of UV for silver nanoparticles will be between 200-800nm. Synthesized AgNPs with ***C.Gigantea*** leaf plant extract shows UV ranges between 400-450 nm and preliminary confirmed by UV results that it successfully synthesized AgNPs with ***C.Gigantea*** leaf extract can form silver nanoparticles.

SEM [Scanning Electron Microscopy]:

Microscopy Methods:

Scanning Electron Microscopy also uses a high energy electron beam, but the beam is scanned over the surface and the back scattering of the electrons is looked at. The sample must again be under a vacuum and foe SEM it must be electrically conductive at the surface. This can be achieved by sputter coating a non-conductive sample. This requirement can be restrictive and again this technique can be time consuming and expensive. Environmental SEM is available where samples can be looked at again in a low-pressure gas environment as opposed to a vacuum. Scanning Transmission Electron Microscopy combines the ideas of looking at the surface of the sample and into the sample with an electron beam.

Analysis

SEM analysis shows uniformly distributed silver nano particles on the surfaces of the cells. The suspended silver nano particles in sterile distilled water were used for scan electron microscope analysis by fabricating a drop of suspension onto a clean electric stub and allowing water to completely evaporate. SEM analysis gives size of silver nanoparticles. Majority cases a large size silver nanoparticle was observed due to agglomerisation of smaller ones.

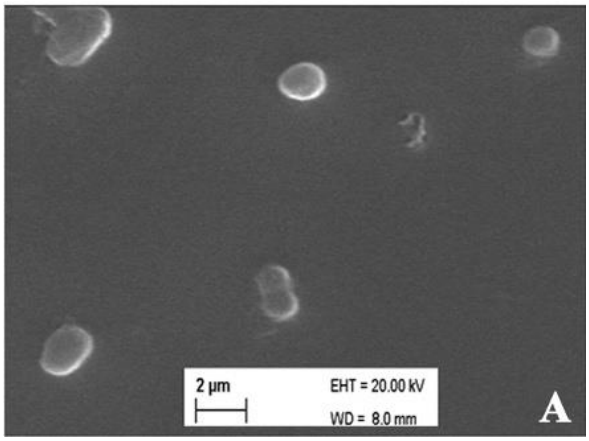

Image 7. SEM analysis result of *C.Gigantea*
Size - 2um
EMT – 20 KV
WO – 8.0mm

TEM [Transmission Electron Microscopy]

Microscopy Methods

Transmission Electron Microscopy (TEM) uses an electron beam to interact with a sample to form an image on a photographic plate or specialist camera. The sample must therefore be able to withstand the electron beam and also the high vacuum chamber that the sample is put into. The sample preparation can be difficult as a thin sample on a support grid must be prepared. The process can also be time consuming and this, along with the cost, is the main criticisms of TEM. High-Resolution TEM (HRTEM) looks at the interference of the electron beam by the sample rather than the absorbance of the beam as with ordinary TEM. This gives a higher resolution which is beneficial when studying nanoscale samples. However, it does require understanding of the sample to allow interpretation of the results, as the phase-contrast resulting information can be difficult to interpret. This can therefore restrict the use of HRTEM. Environmental TEM allows TEM to be carried out in-situ by using the relevant gaseous atmosphere as opposed to the vacuum used for TEM.

Analysis: -

TEM analysis give the information about the morphology of the silver nano particles. generally silver nano particles are spherical or crystal structures. Tem also give average mean size of silver nano particles. TEM measurements were performed on JEOL model JEM 2100 instrument operated at an accelerating voltage at 120 kV.

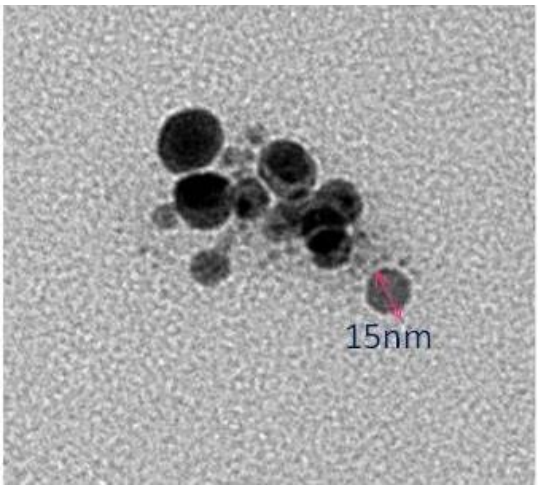

Image.8 Particle size 15nm

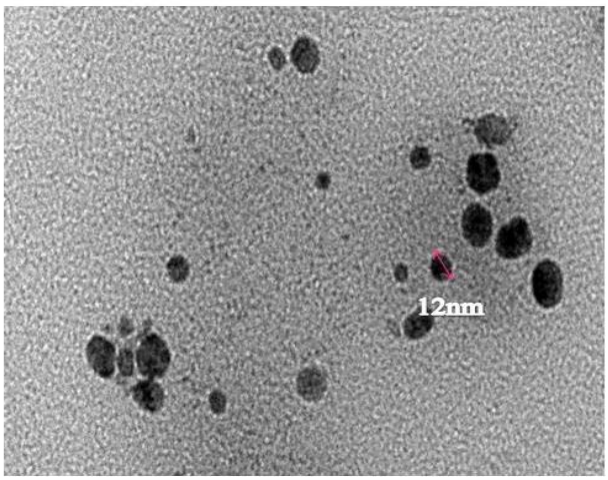

Image.9 Particle size 12nm

Anti-Microbial Activity: -

Many researchers have tried to measure the activity of metal ions against microorganisms. Even though copper and zinc show antimicrobial properties, these nanoparticles have limited usefulness as an antimicrobial agent for several reasons, including the interfering effects of salts and the antimicrobial mechanism. These limitations can be overcome with silver nanoparticles, which exhibit efficient antimicrobial property compared to other salts due to their large surface area providing better contact with microorganisms. The nanosize space allowed expansion of the contact surface of silver with the microorganisms and this nanoscale has applicability for medical devices as surface-coating agents (Kim et al., 2004). The nano-silver can inhibit the growth of a wide variety of microorganisms. Recently, health care providers and researchers took a renewed interest in silver because the pathogens, when exposed, showed increased resistance capability to antibiotics. In addition, the nanoscale technique development for producing silver nanoparticles may assist medical use, especially in applications where fighting germs is a major concern

Analysis: -

The anti-microbial activity of biosynthesized silver nano particles was tested by Disc diffusion method. The silver nano particles formed from different medical plants leaves extracts show different biological activity. Some plants and its biological activities are given below. Antibacterial activity of the synthesized Ag NPs was studied by the standard well method. The overnight grown bacterial suspensions of –

1. *Klebsiella pneumoniae,*
2. *Bacillus subtilis (plant endophytic bacteria)*
3. *Staphylococcus chromogenes (plant major pathogens)*

Were standardized using gel puncher. 5 mm diameter well made by gel puncher. The dilutions of biosynthesised Ag NPs varying from 5 mg, 10 mg and 15 mg/mL were prepared with two-fold symmetry. 20 mL of molten sterilized nutrient agar solution was poured into each petri plates and seven organisms were grown in them. The tested organisms were inoculated in four well (5 mm diameter), which is contains in different dilutions of Ag NPs (5 mg, 10 mg and 15 mg/mL) solutions. Each petri plate was loaded with 1 or 2 well. The plates containing the bacteria and Ag NPs were incubated at 37°C, and then examined for confirmation appears as a clear area around the well. The diameter of such zones of inhibition was measured using a metre ruler, and the mean value for each organism was recorded and expressed in millimeters.

Image.10 Bacillus subtitles

Image.11 Klebsiella sp.

Bacteria	Concentration (µl)	Zone of Inhibition (AgNPs/ Plant extract)
Bacillus subtilis	200µl	14mm 6mm
Klebsiella sps.	200µl	20mm 15mm
Xanthomonas sp.	200µl	15mm 8mm
Staphylococcus chromogenes	200µl	10mm No inhibition

Table 1 Zone of inhibition

Gram positive bacteria - *Bacillus subtilis* showing zone of inhibition foe both AgNPs and plant extract also but another bacteria *Staphylococcus chromo gene* is showing zone of inhibition for only AgNPs not for plant extract.

Gram negative bacteria - *Klebsiella sps.* And *Xanthomonas sp*. showing higher zone of inhibition for both AgNPs and plant extract

CONCLUSION

Silver nanoparticles (AgNPs) were successfully obtained from bioreduction of silver nitrate solutions using *C.Gigantea*. AgNPs have been appropriately characterized using UV-visible spectroscopy, SEM, TEM analysis. Silver nanoparticles also aided in plant germination and growth by sequestering nutrients for them and could hence be implemented for agricultural purposes. Hence, due to their benign and stable nature and antimicrobial property, these AgNPs may be well utilized in industrial and remedial purposes. However, plant uptake and utilization of AgNPs require more detailed research on many issues like uptake potential of various species, process of uptake and translocation and the activities of the AgNPs at the cellular and molecular levels.

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