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Assessment of Anti-Microbial Activity by the Green Synthesized Silver Nanoparticles of Different Parts of *Plumbago zeynalica* L. Plant

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Abstract: Green synthesis of silver nanoparticles (AgNPs) using plants has turn into a promising alternative it is also a cost effective, eco-friendly, reproducible and energy saving as compared to that of conventional chemical and physical synthesis process. The present research deals with the green synthesis of AgNPs with the aqueous extracts of leaf, stem and root of *Plumbago zeylanica* (L.). The AgNPs was confirmed and assessed for their biological activities through various methods. Its existence was confirmed by the analysis of spectroscopy profile with UV-visible spectrophotometer at 440 nm. Then the SEM analysis confirmed sphere-shaped and the size of nanoparticles in the range of approximately 18 nm. Among different plant parts extracts, it was observed that *P. zeylanica* L. root extract can decrease the silver ions into silver nanoparticles within 2 h of reaction. Thus, the biosynthesis of stable silver nanoparticles with the size range of 4–30 nm have antimicrobial activity. The antibacterial effects on the Gram-negative pathogens *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis,* and antifungal activity on *Candida albicans, Candida tropicalis, Candida glabrata* were tested using three parts of *P. zeylanica* extract. Compared to stem and leaf AgNPs, the root AgNPs synthesized were smaller in dimension and exhibited a higher level of antimicrobial activity and this is the first study on root extract against antimicrobial activities. Overall, this study suggested that the AgNPs of root extract of the medicinal plant *Plumbago zeylanica* L. is capable of rendering antimicrobial efficacy and hence has a great potential in the preparation of drugs used against bacterial and fungal diseases.

Keywords: Silver nanoparticles (AgNPs), *Plumbago zeylanica*, UV – Vis spectrophotometer, Scanning Electron Microscopy, Antimicrobial activity

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Introduction

Nanotechnology is a popular research tool. Biological resources to create eco-friendly metallic nanoparticles has gained interest nowadays. Metallic nanoparticles (NPs) are of foremost interest attributable to its unique nature of the nanoparticles (1–100 nm) and also their application in the field of solar energy conversion, medicine field, catalysis (Netai Mukaratirwa-Muchanyereyi *et al*., 2022), wastewater treatment benefits (George *et al*., 2022; Vinodhini *et al*., 2022) and for cancer treatment. The parameters of nanoparticles include dimension, morphology and shape hence, a significant number of studies have been conducted to influence the shape and size of nanoparticles during their preparation for different applications (Netai Mukaratirwa-Muchanyereyi *et al*., 2022). Nano-silver (AgNPs) is a special kind of metallic nanoparticle that has found several applications, especially in nanomedicine (Xu *et al*., 2020). For the preparation of AgNPs using physical methods, chemical and biological synthesis routes have been tried. Among these, physical methods for preparing AgNPs are not cost-effective, fritter away more energy, and need specific instruments. Moreover chemically synthesized AgNPs are toxic, costly, and frequently lead to non-ecological by-products. Recently, biologically synthesized AgNPs techniques give a better platform over the chemical and physical method as it is cost-effective, biodegradable and without applying any energy it is easy to develop for large-scale production, temperature, high pressure, toxic chemicals and has more advantages in its application (Khan *et al*., 2021).

Silver Nanoparticles (AgNPs) have gained special interest because of the effectiveness and improvement against highly resistant microbes and also in medical and industrial processes such as biomedical services, keyboards, textiles and wound dressing (Krishnan *et al*., 2020). Silver nanoparticles (AgNPs) have a great potential in cancer management because it selectively involved disruption of the mitochondrial respiratory chain by AgNPs leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage (Mohammadzadeh *et al*., 2022). Silver is a benign, safe inorganic antibacterial agent that kills 650 disease-causing microorganisms. The emergence of metal-resistant bacteria has prompted the quest for novel antibacterials (Zhang *et al*., 2017).

With Food and Drug Administration (FDA) approval, AgNPs were known to be effective against bacterial and viral mediated diseases and possess a huge potential in treating various diseases like retinal neovascularization, cancer, hepatitis B, acquired human immunodeficiency (HIV), diabetes etc. (Mukherjee *et al*., 2007). Silver nanoparticles (AgNPs) have become the focus of intensive research owing to their wide range of applications in areas such as catalysis, optics, antimicrobials, and biomaterial production (Niluxsshun *et al*., 2021). Silver nanoparticles exhibit new or improved properties depending upon their size, morphology, and distribution. Various approaches using plant extract have been used for the synthesis of metal nanoparticles. These approaches have many advantages over chemical, physical, and microbial synthesis because there is no need of the elaborated process of culturing and maintaining the cell, hazardous chemicals, high-energy requirements, and wasteful purifications (Jain *et al*., 2017). The silver nanoparticles are also reported to be nontoxic to human and most effective against bacteria, viruses, and other eukaryotic micro organisms at very low concentration and without causing any side effects. Silver products have been known to have great inhibitory effects, as well as a broad range of antimicrobial activities, which has been used to prevent and treat various diseases (Shankar *et al*., 2004; Makarov *et al*., 2014).

There are 280 different species and 10 different genera in the family Plumbaginaceae. *Plumbago zeylanica* belongs to family Plumbaginaceae (Chitrak, 2019). In the current study, the medicinal plant *Plumbago zeylanica* L. was used for the biosynthesis of silver nanoparticles for various medicinal uses. *Plumbago zeylanica* L., commonly known as Ceylon Leadwort or Doctorbus, is a species with a pantropical distribution. In India, it is distributed from mid India to West Bengal, Maharashtra and different parts of Southern India. It has been used as a medication for infections, skin diseases and intestinal worms viz scabies, leprosy, hookworm, ringworm, dermatitis, carminative, powerful digestive, anti-fungal, hemorrhoids, anti-inflammatory, anticolic, central nervous system stimulatory activity, leukoderma, sprue, anti-plasmodial, anti-hyperglycemic, malabsorption syndrome, worm infestation, amenorrhoea, hepatomegaly, hepatoprotective, spleenomegaly, anti-bacterial, anti-tumour, anti-inflammatory, anti-cancer, anti-atherosclerotic activity sores and ulcers. In West Africa, it has been used as a vesicant and counter-irritant by crushing the root or the leaves with lemon juice and also in Nigeria, the root extraction along with vegetable oil are used for the healing of rheumatic swellings. Moreover, powdered bark, root or leaves are used for the healing of syphilis, gonorrhoea, rheumatic pain, tuberculosis, swellings and wounds treatment in Ethiopia (Roy *et al*., 2017).

P. zeylanica contains important secondary metabolites which are flavanoids, alkaloids, carbohydrates, naphthaquinones, steroids, glycosides, triterpenoids, tannins, phenolic compounds, saponins, coumarins, fixed oil and fats and proteins (Singh *et al*., 2018). It contains plumbagin which is the main chemical compound. Plumbagin possess antibacterial activity against both gram-positive (e.g. *Staphylococcus*, *Streptococcus*, *Pneumonococcus* sp.) and gram-negative (e.g. *Salmonella*, *Neisseria*) bacteria. It is also active against certain yeasts and fungi (*Candida*, *Trichophyton*, *Epidermophyton* and *Microsporum* spp.) and protozoa (*Leishmania*) (Ravikumar and Sudha, 2011; Pant *et al*., 2012). Silver nanoparticles have gained unique nature due to its constancy, excellent conductivity and antimicrobial activity amongst several metal nanoparticles. Therefore, the present study mainly focused on the green synthesis of AgNPs using the *P. zeylanica* and evaluating its anti-microbial activities.

Materials and Methods

Collection and Identification of Plant:

The medicinal plant *Plumbago zeylanica* L. was collected from the different localities of Siddhar kaadu, Thanjavur district, Tamil Nadu, India. The plants were identified and authenticated by Rev Dr. S. John Britto SJ, Director at Rabinet Herbarium and Centre for Molecular systematic, St. Joseph College (Autonomous), Tiruchirappalli, Tamil Nadu, India. The voucher specimens were deposited at the Rabinat Herbarium and the voucher number is SD.001.

Preparation of plant extract:

Silver nanoparticles were prepared by the method of Martinez-Castanon *et al*. (2008). Fresh plant parts such as leaf, stem and root of *P. zeylanica* L. were washed several times with tap water to remove the dust particles and then the samples were shade dried to remove the residual moisture and homogenized to form powder using mixer grinder.

Solvent extract:

The solvent extract of medicinal plant *P. zeylanica* L. was prepared. Dried leaves, stem and root powder were mixed with ethanolic extract in a ratio of 1:10 (w/v). Then it was kept on a rotary shaker for 24h at 20ºC. The mixture was filtered and sterilized by using sintered glass filter. Then the extract was stored under refrigeration at 4ºC for further studies (Kaleeswaran *et al*., 2019)

Synthesis of Silver Nanoparticles:

Silver nanoparticles were prepared following a method which is a modification of Martínez-Castanon *et al*. (2008). The preparations started with a 0.01M $AgNO_3$ solution placed in a 250 ml reaction vessel. Under magnetic stirring, 1 ml of ethanol and aqueous extract containing 100 μg *P. zeylanica* plant extracts was added to the Ag^+ solution with constant stirring at 50- 60℃. As soon as, *P. zeylanica* extracts was mixed in aqueous solution of silver ion complex, it starts to change color from yellowish to brownish which shows the silver nanoparticles formation because of surface plasmon resonance excitation.

Characterization of Silver Nanoparticles:

The characterization study of silver nanoparticle was done by the examining size, shape and quantity of particles with the aid of UV-visible spectroscopy and Scanning Electron Microscopy (SEM).

UV-Vis spectra analysis:

The silver nanoparticles were confirmed by measuring the wave length of reaction mixture in the UV-Vis spectrum of the AEMAX spectrophotometer at a resolution of 400 nm in 2 ml quartz cuvette with 1 cm path length (Sadowski *et al*., 2010).

SEM analysis:

The morphological characterization of the samples was done using JEOL Jsm-6480 LV for SEM analysis. The samples were dispersed on a slide and then coated with platinum in an auto fine coater. Then the material was subjected to analyse the synthesized nanoparticles (Netai *et al*., 2022).

Antimicrobial Activity:

Microorganisms:

Pathogenic bacteria like *Escherichia coli, Klebsiella pneumonia, Proteus mirabilis* (Gram-negative), and fungus like *Candida albicans, Candida tropicalis, Candida glabrata* tested in this study were collected from Microbial type Culture Collection and Gene Bank (MTCC). Pathogenic bacteria were grown on nutrient broth (Hi Media) at 37°C for 24 h and fungus on Sabouraud dextrose broth (Hi Media) at 28°C for 48 h and were maintained on respective agar slants at 4°C.

Antibacterial susceptibility testing:

The antibacterial activity of plant extracts of *P. zeylanica* L. were evaluated by agar well diffusion method using Mueller Hinton agar medium. The microorganisms was activated by inoculating a loopful of the strain in the nutrient broth (20 ml) in a 100 ml Erlenmeyer flask and incubated at 37°C on a rotary shaker for 24 h. 0.1 ml of fresh inoculums (containing around 1-2 × 106 cfu/ml as per McFarland standards) was spread onto the surface of sterile Mueller Hinton agar using a sterilized glass spreader. Wells were made on the seeded plates with the help of a sterilized cup-borer (8 mm). The silver mediated ethanolic extracts (100 µl) were dispended into the well and the plates were incubated aerobically at 37°C. In the same way a negative and a positive control wells were made with only 0.01 M $AgNO_3$, ethanol crude extract (1000 µg) and Ampicillin (100 µg), respectively. The entire microbial assay was carried out under strict aseptic conditions. The zones of inhibition (mm) of the different extracts were examined after 24 h (Bauer *et al*., 1966; Kaleeswaran *et al*., 2019)

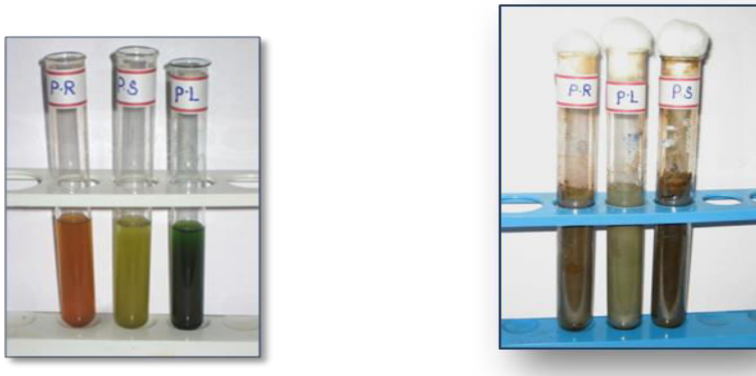
Antifungal activity:

The antifungal activity of herbal-mediated silver nanoparticles were tested by means of well diffusion method using Sabouraud dextrose agar. The pre-sterilized medium of each Petri plate containing cut wells were inoculated with respective ethanolic extract of silver nanoparticles (1000 µg) and with respective pathogen disc at the edges of the plate. These plates were incubated at 28°C for 7 days. This test was performed against fungal phytopathogens such as *Candida albicans, Candida tropicalis* and *Candida glabrata* using miconazole (50 µg) as control (Vinodhini *et al*., 2022).

Results

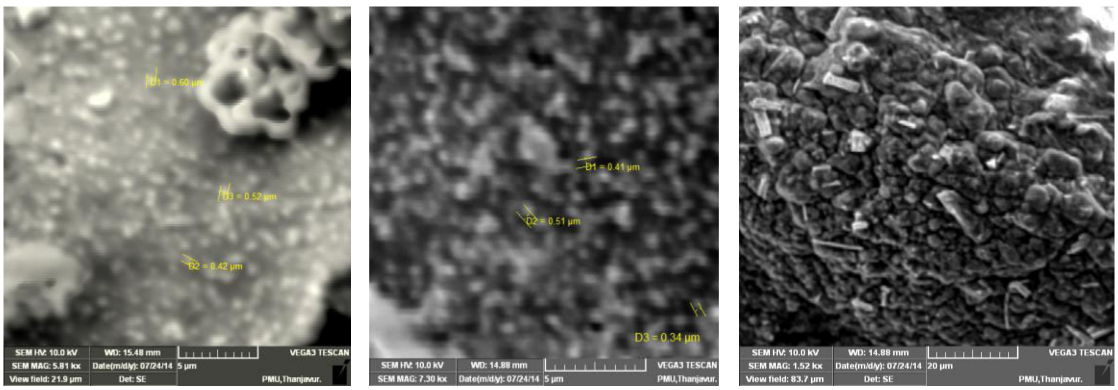
Synthesis of silver nanoparticles:

In the present study, silver nanoparticles were synthesized using the leaves, stem and root of *P. zeylanica* L. After the addition of $AgNO_3$ the colour changes from colourless to different color, depends on the medicinal plant extract. Silver nanoparticles reveal the brown color in aqueous solution as a result of excitation of surface plasmon vibrations in silver nanoparticles. Plant extract was mixed in the aqueous solution of the silver ion complex; it started to change the color from yellowish brown color to dark brown due to reduction of silver ion, which may be the indication of formation of silver nanoparticles (Fig. 1a). Almost all the herbal mediated silver nano solutions after incubation time showed the colour change from light to dark (Fig. 1b).



(a) (b)

Fig. 1: (a) Adding of silver nitrate to the extracts of *P. zeylanica* L. plant, (b) Synthesis of silver nanoparticles of *P. zeylanica* L. extracts.



(a) (b) (c)

Fig. 2 a-c: SEM analysis of leaf, stem and root extracts of *P. zeylanica* L.

UV – spectrophotometer:

The reduction of pure silver ions was analyzed by measuring the UV-Vis spectrum of the reaction medium at 1 h after diluting a small aliquote of the sample into distilled water and the spectrum was carried out by using UV-Vis spectrophotometer (AEMAX). UV-Vis spectra showed the peaks approximately at 440 nm, clearly indicating the formation of spherical AgNPs in all the plants extracts. The occurrence of the peak at 440 nm is due to the phenomenon of surface Plasmon resonance, which occurs due to the excitation of the surface plasmons present on the outer surface of the silver nanoparticles which gets excited due to the applied electromagnetic field.

SEM analysis:

A scanning electron microscope was employed to analyze the shape of the silver nanoparticles that were synthesized by green method. SEM analysis showed that this plant have tremendous capability to synthesize silver nanoparticles which were roughly spherical in shape and were uniformly distributed in all extracts. But in the root extract of *P.* zeylanica L. showed more activity because the size of particle ~18 nm (Figs. 2 a,b,c).

Antimicrobial activity:

The present study described the effectiveness of the sliver mediated plant extracts against pathogenic organisms. The antimicrobial activity of *P. zeylanica* L. showed that there is no activity on the bacteria such as *E.coli* but in the *K. pneumonia* it showed significant effect in the leaf (10 mm) and stem (9 mm) extracts compared to standard Ampicillin. The plant *P. zeylanica* L. (Table 1) showed some effect against bacteria *P. mirablis* in the leaf (12 mm), stem (11 mm) and root (18 mm) extracts but it had low zone of

Table 1: Antimicrobial activity of leaf, stem and root ethanol extracts of *P. zeylanica* L. against six harmful pathogens

Zone of inhibition (mm)(mcg/well)				
Bacteria				
Pathogens	**PLE (1000 µg)**	**PSE (1000 µg)**	**PRE (1000 µg)**	**Standard**
E. coli	--	--	--	50
K. pneumoniae	10	9	--	--
P. mirabilis	12	11	18	23
Fungi				
C. albicans	--	13	26	14
C. tropicalis	--	--	23	11
C. glabrata	--	14	16	15

Standard for bacteria (Ampicillin 100 µg) and for fungi (Miconazole 50 µg); PLE –Leaf Extract, PSE –Stem Extract and PRE- Root Extract

inhibition when compared to Ampicillin 23 mm (Table 1).

In the antifungal activity, the pathogens *Candida albicans*, *Candida tropicalis* and *Candida glabrata* were used. There is no activity in the leaf extracts of *P. zeylanica* L. against *C. albicans*, *C. tropicalis* and *C. glabrata* pathogens. But there is some moderate activity in the stem extract against *C. albicans* and *C. glabrata* such as 13 mm and 14 mm against standard Miconazole 14 mm and 15 mm. The root extracts of *P. zeylanica* L. showed highest activity against all fungal pathogens such as 26 mm, 23 mm and 16 mm compared to standard miconazole 14 mm, 11 mm and 15 mm (Table 1).

Discussion

The biosynthesis of AgNPs, which uses plant extracts and microbes as reducing agents, as well as the usefulness of AgNPs in combating bacterial growth, have both garnered a lot of interest in recent times. Melkamu and Bitew (2021) were able to create silver nanoparticles in an environmentally friendly method by bio-reducing silver nitrate using the plant leaf extract of *Hagenia abyssinica*. The antibacterial activity of the generated AgNPs was effective against many strains of bacteria, including *Streptococcus pneumoniae*, *Salmonella typhimurium*, and *Klebsiella pneumoniae*. Produced from the leaves of the *Eugenia roxburghii* plant were silver nanoparticles that effectively inhibited the expansion of bacterial colonies that are responsible for the formation of biofilm (Giri *et al*., 2022).

Antibiotic resistance by the pathogenic bacteria has been observed since last decade; hence, the researchers are focusing on the development of new antibacterial agents. In the present scenario, Ag nanoparticles as antimicrobial agents have come up as a promising candidate in the medical field (Duran *et al*., 2007). The extremely small size of nanoparticles exhibits enhanced or different properties when compared with the bulk material. There are different physical and chemical methods for the synthesis of nanoparticles, but there is always a need for the development of eco-friendly route for the synthesis process (Ingle *et al*., 2008). The use of plant extracts has opened a new awareness for the control of disease, besides being safe and non-phytotoxic. It is found that the plant extracts are effective against various microorganisms including plant pathogens (Misra and Dixit, 1979).

The search for antimicrobial agent has continued to be concentrated on lower plants, fungi and bacteria. The synthesis of silver nanoparticles in the plant extracts of *P. zeylanica* L. was very effective against harmful pathogen (bacteria and fungi). The traditional medicinal plant *P. zeylanica* L. has the potential to reduce silver nitrate ions to silver nanoparticles. There is a colour change which occur after 1 h of incubation due to the excitation of surface plasmon vibrations in silver nanoparticles from different to light yellowish colour by adding silver nitrate solution. After the formation of silver nanoparticles the colour occurs from yellowish colour to dark brown colour. This is the conformation of synthesis of silver nanoparticles by using plant extracts.

Nanoparticles size can also be determined by the change in the color of the reaction solution. The smaller the size of nanoparticles greater is the color shift towards red (Mock *et al*., 2002). The synthesis of silver nanoparticles was also confirmed from the UV spectra of the AgNPs of *P. zeylanica* L. where the maximum absorbance was found ~440 nm. Present results disclosed that the reduction of the $AgNO_3$ ions and formation of silver nanoparticles was completed in 60 min of reaction. The size of the AgNPs of *P. zeylanica* L. was determined by the Scanning Electron Microscopy (SEM). The small size of nanoparticles showed more activity against microbial pathogens and to heal diseases.

A striking future of nanomedicine companies is their product diversity. The applications of these structures to a range of biotech challenges, including therapeutics, diagnostics, structural materials and electrical devices, are striking. Provided that environmental and safety concerns can be addressed in a meaningful and open fashion, there seems little doubt that this technology, after years of incubation, will finally accrue long sought-after benefit for its promoters (Aldridge *et al*., 2006). The use of anodic silver ions as preserving agents in cosmetics was tested by a challenged test in a set of cosmetic dispersions with the addition of known preservative inhibitors or microorganism's growth promoters such as humectants, hydro soluble collagen and vegetable extracts (Prabhu *et al*., 2010). SEM analyses of the synthesized silver nanoparticles were clearly distinguishable which measured 4 - 30 nm (Singhal *et al*., 2011) in size. It is clear that the triangles, pentagons, and hexagons structures sizes are up to 30 nm.

AgNPs limit mycelial development and conidial germination, degrade cell walls and membranes, dispute protein, and influence pathogen energy and substance metabolism, signal transduction, and genetic information processing (Jian *et al*., 2021; Al-Otibi *et al*., 2022). Due to a lack of microscopic, biochemical, and omic investigation, AgNPs' mechanism against plant pathogens is unknown (Shen *et al*., 2020). The results showed root extract of *P. zeylanica* L. This plant showed high efficiency against fungi such as *C. albicans* (26 mm) and *C. tropicalis* (23 mm). AgNPs could be used as an eco-friendly antimicrobial agent in the control of bacterial and fungal diseases. Biologically synthesized AgNPs are less in cost, eco-friendly, safe and pollutant free with less or no side effects which can be used further in various industrial and medical applications (Ming *et al*., 2011). AgNPs have been used to manage post-harvest infections in strawberry (Oliveira *et al*., 2022) and apple (Madbouly, 2021), and in mango recent years (Shivamogga Nagaraju *et al*., 2020). This is in conformity with previous works that have reported better antimicrobial activity of AgNPs than the crude extracts of other parts.

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