

MODERN APPROACHES TO CHEMICAL SCIENCE: CONCEPTS AND TECHNIQUES

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Nanoparticles from Nature: Green Synthesis of Silver and Copper Nanoparticles Using *Pithecellobium dulce*

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Abstract

The green synthesis of nanoparticles has gained significant attention due to its environmentally friendly approach, utilizing plant extracts as reducing and stabilizing agents. This study explores the green synthesis of silver (Ag) and copper (Cu) nanoparticles from the leaf extract of the medicinal plant *Pithecellobium dulce*. The synthesis was carried out by mixing the plant leaf extract with metal salt solutions of silver nitrate (AgNO_3) and copper sulfate (CuSO_4) under ambient conditions. The formation of nanoparticles was confirmed through UV-Vis spectroscopy, which displayed characteristic absorption peaks at specific wavelengths corresponding to the plasmon resonance of silver and copper nanoparticles. The synthesized nanoparticles were further characterized using techniques such as FTIR, XRD, AFM, SEM, EDX and HRTEM. These results revealed the formation of spherical nanoparticles with an average size ranging from 10 to 50 nm. The antioxidant potential of the synthesized nanoparticles was evaluated against common bacterial strains, demonstrating notable activity. This study highlights the potential of *Pithecellobium dulce* leaf extract as a sustainable and eco-friendly source for the synthesis of metal nanoparticles with antimicrobial properties.

Keywords: Green Synthesis, Silver Nanoparticles, Copper Nanoparticles, *Pithecellobium dulce*, Antioxidant Activity, Nanoparticle Characterization

Introduction

The term “medicinal plant” includes various types of plants used in herbalism ("herbology" or "herbal medicine"). It is the use of plants for medicinal purposes, and the study of such uses. The word “herb” has been derived from the Latin word, “herba” and an old French word “herbe”. Now a days, herb refers to any part of the plant like fruit, seed, stem, bark, flower, leaf, stigma or a root, as well as a non-woody plant. Earlier, the term “herb” was only applied to non-woody plants, including those that come from trees and shrubs. These medicinal plants are also used as food, flavonoid, medicine or perfume and also in certain spiritual activities.

Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments.

Treatment with medicinal plants is considered very safe as there is no or minimal side effects. These remedies are in sync with nature, which is the biggest advantage. Now, after finding the role of herbs in medicine, lots of consumers started the plantation of tulsi and other medicinal plants in their home gardens. Medicinal plants are considered as a rich resource of ingredients which can be used in drug development either pharmacopoeial, non- pharmacopoeial or synthetic drugs. A part from that, these plants play a critical role in the development of human cultures around

the whole world. Moreover, some plants are considered as important source of nutrition and as a result of that they are recommended for their therapeutic values [1].

Why do plants have medicinal properties?

Plants produce many chemicals that are biologically active, not just in themselves, but also in other organisms. Some of these chemicals enhance their own survival. Below are several examples of active plant ingredients that provide medicinal plant uses for humans.

Alkaloids: This group is comprised of a wide variety of plants that contain nitrogen-bearing molecules that make them very active. Many of these plants have been used to create well-known drugs used for medicinal purposes. One such example, vincristine, which was derived from the Madagascar periwinkle (*Catharanthus roseus*), is used to treat some types of cancer.

Flavonoids: Flavonoids are found widely throughout the plant world and they have a wide range of medicinal uses and actions. They often act as pigments giving a yellow or white color to flowers and fruits. Some flavonoids have anti-viral and anti-inflammatory properties.

Minerals: Many plants have high levels of minerals because they can draw minerals from the soil and can convert them into a form that is more easily used by the human body. Mineral content is often the key factor in a plant's effectiveness as a medicine.

Phenols: Phenols are plant compounds that are thought to be produced to protect against infection and herbivory by

insects. They are often anti-inflammatory and antiseptic and can have anti-viral properties. Phenols vary in structure and range from salicylic acid (similar to aspirin) to complex sugar-containing phenolic acids.

Saponins: This group of active compounds obtains its name from the fact that like soap, they produce lather when placed in water. There are two main forms of saponins: steroidal and triterpenoid.

Tannins: Most plants produce tannins. Tannins serve as a deterrent to herbivory by insects and grazing animals given that they provide a harsh unpalatable flavor. Tannins are also useful in curing leather because of their tendency to contract and astringe tissues by binding with precipitating proteins.

Vitamins: Many plants contain high levels of useful vitamins. Many well-known fruits and vegetables have high levels of vitamin C and beta-carotene. Lesser-known vitamin containing plants like watercress, rose hips, and sea buckthorn have high levels of vitamins B, C, and E.

Volatile oils: Volatile oils are extracted from plants and are used to produce essential oils that play a very important role in medicinal botany. These oils are often very complex and can be comprised of 100 or more compounds. These oils have many uses. For example, tea tree oil is a strong antiseptic [2].

History and development of nanomaterials

Humans already exploited the reinforcement of ceramic matrixes by including natural asbestos nano fibers

more than 4,500 years ago [3]. The Ancient Egyptians were also using NMs more than 4000 years ago based on a synthetic chemical process to synthesize ≈ 5 nm diameter PbS NPs for hair dye [4]. Similarly, "Egyptian blue" was the first synthetic pigment which was prepared and used by Egyptians using a sintered mixture nanometer-sized glass and quartz around 3rd century BC [5]. Egyptian blue represents a multifaceted mixture of $\text{CaCuSi}_4\text{O}_{10}$ and SiO_2 (both glass and quartz). In ancient geographical regions of the Roman Empire, including countries such as Egypt, Mesopotamia, and Greece, the extensive use of Egyptian blue for decorative purposes has been observed during archaeological explorations. In 2003, Samsung introduced an antibacterial technology with the trade name Silver Nano™ in their washing machines, air conditioners, refrigerators, air purifiers and vacuum cleaners, which use ionic Ag NPs [6].

Nanotechnology

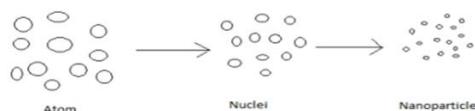
Nanotechnology an emerging field of nanoscience deals with nano size particles having a size of 1-100 nm. These nanomaterials are considered as "smart" materials used for constructing nanocarriers, which plays a vital role in drug delivery systems and possess high biocompatibility nature [7]. Nanoparticles have received special attention due to greater surface area to volume ratio and highly reactive than macromolecules [8]. Employing nanotechnology, green method for the synthesis of nanoparticles provides tremendous advantages as it is free of toxic chemicals and eco - friendly.

Classifications of nanoparticles

Nanoparticles can be broadly classified into two groups: Organic nanoparticles and Inorganic nanoparticles. Organic nanoparticle is carbon nanoparticle (fullerenes) and inorganic nanoparticles are magnetic nanoparticle, noble nanoparticle (gold and silver), semiconductor nanoparticle (titanium oxide and zinc oxide). Especially inorganic nanoparticles have created attention towards itself due to its superior material properties with versatile functions. Due to nano size feature, it easily used for chemical imaging drugs agents and drug. Its versatile function used for the cellular delivery as they are widely available, rich functionality, good biocompatibility. This is also a good carrier of targeted drug delivery and controlled drug release [9]. it is a completely advantageous material for medical science for example mesoporous silica combined with molecular medicines shows an excellent image on drug releasing. Gold nanoparticle is good carrier in thermo therapy of biological target [10]. Silver nanoparticle shows antimicrobial activity which heals the wounds and infectious disease.

Traditionally, researchers generally used two methods for the synthesis nanoparticles such as Bottom-up approach: The bottom-up approach is a nano-architectural phenomenon of self-assembly of materials from cluster-to-cluster, molecule-to-molecule or atom-to-atom on top of a base substrate. The main concern in the bottom-up approach is the adhesion of the surface layers to the base substrate. The most commonly used bottom-up methods are welding & riveting.

Fig1.1: Bottom High approach



Top-down approach: The Top-down method refers to a set of fabrication technologies starting with a block bulk material which share the same material with the base substrate. The most commonly used top-down methods are milling, drilling and grinding.

Fig1.2: Top-down approach



Common Medicinal Plants used for Nanoparticle Synthesis

The green synthesis method is used to produce the nanoparticles as it is cost effective and main advantage is its eco-friendly approach compared to other methods. Some specific plant parts or whole plant especially angiospermic plants are used for the greater synthesis of nanoparticles [11]. Many of them reported that plant like *Musa sapientum* [12] *Clerodendrum inerme* [13] *Coriandrum sativum* [14] *Catharanthus roseus* [15] *Saururus chinensis* [16] are used for the synthesis of nanoparticles. Synthesis and Characterization of CuNPs using *Capparis zeylanica* leaf Extract. The leaf extract acts as both reducing and capping agent. The synthesized CuNPs were confirmed by the change of color after addition of leaf extract into the CuSO₄ solution. The biosynthesized CuNPs were characterized by using UV-Visible analysis, FTIR, XRD, SEM, EDX and

TEM analysis. The synthesized CuNPs were in cubical structure with the particle size in the range between 50-100 nm. The antimicrobial study of the CuNPs was established using both gram positive and gram-negative pathogens [17]. The synthesized CuNPs showed zone of inhibition against *E. coli*. The leaf extract shows higher antioxidant activity as found by DPPH and hydrogen peroxide assay. The size and the morphology were confirmed by TEM [18]. A study has reported the antibacterial activities of *Terminalia catappa* bark against pathogenic bacteria and exhibited the growth inhibitory activity in a dose dependant manner [19]. In some other work, the biologically synthesized copper nanoparticles were tested for antibacterial activity against two human pathogens viz., *E. coli* and *staphylococcus aureus* [20]. One of the literature studies reveal the shape of the nanopartiles which addresses various form of triangular, cubic and hexagonal polyshaped AgNPs which was distinct while compared with AuNP which was appeared like spherical [21].

Plant Extract as a Reducing Agent for Metal Nanoparticle Synthesis

Of several biological methods available for the preparation of metal nanoparticles, leaf extract method is simple and cost effective. Because of their reducing ability of metal salts to nano metal particles, different leaf extracts were used. Most of the bio nanocomposites were made using metal nanoparticles as fillers. Of these, AgNPs and copper nanoparticles (CuNPs) were found to possess good antibacterial activity [14]. However, AgNPs are expensive so slowly shifting towards using CuNPs for antibacterial

applications. Further, CuNPs can be used to form stable polymer nanocomposites due to their electrostatic interactions with the matrix [22]. There are several methods of generating metal nanoparticles such as physical, chemical and biological. Of these, biological method such as using leaf extract as a reducing agent is simple and economical [23]. The ability of leaf extract as a reducing agent was attributed to the presence of secondary metabolites in it. Most of the polymer composites and nanocomposites made using synthetic polymer matrices are non-biodegradable and pose many environmental problems. Hence, the trend is now shifted towards preparation of biodegradable nanocomposites using natural polymers. Most of the researchers concentrated on the synthesis of metal nanoparticles which can be subsequently used as nano fillers in different matrices to make nanocomposites. But often uneven dispersion of nanoparticles in a polymer matrix leads to their agglomeration. So whenever possible, it is advisable to in situ generate metal nanoparticles inside polymer matrices for their uniform distribution [24].

Silver Nanoparticles

The extraordinary optical properties of silver nanoparticles were used by glass founders as far back as in the time of the Roman Empire. This is evidenced by the so-called *Lycurgus cup* (4th century AD) now exposed in the British Museum. A detailed study of the composition of its bronze-mounted insets of stained glass, carried out in the late 20th century, revealed the presence of metal nanoparticles (with the average diameter of 40 nm) that consists of silver (70 %) and gold (30 %) alloy [25]. This

explained a remarkable feature of this bowl to change its color from red in transmitted light to grayish green in reflected light. In the preparation of this glass, nano silver was formed insitu.

Before the 1980s, the scientific and practical interest in silver nanoparticles was exclusively caused by the possibility of their use as highly dispersed supports for enhancing the signals from organic molecules in the Raman spectroscopy [26]. Fundamental studies carried out in the last three decade shows that silver nanoparticles exhibit a rare combination of valuable properties, namely, unique optical properties associated with the surface Plasmon resonance (SPR), well-developed surfaces, catalytic activity, high electrical double layer capacitance, etc. [27]. That's why they serve as a material in the development of new-generation electronic, optical and sensor devices. In the past 20 years, the trend miniaturization and the necessity of modernization of technological processes led to the substantial increase in the number of scientific publications devoted to the synthesis and ii of silver nanoparticles; at present, their synthesis is among the most actively developing trends of colloid chemistry.

Silver Nanoparticle Synthesis from and Leaf Extract

The *Pithecellobium dulce* extract and tissue culture derived callus have proved as reducing agents for synthesis of AgNPs. Presence of polyphenols and tannic acid plant derived compounds can effectively reduce the metal salts into nanoparticles. The variable size range of the nanoparticles is 5-20 nm. Approaching these plants based biological methods are single step and able to synthesize large quantity. The

callus extract and the leaf extract of *Pithecellobium dulce* plant, derived silver nanoparticles have higher antimicrobial activity against the clinical pathogens, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Micrococcus luteus* and *Klebsiella pneumonia* and fungi *Alternaria alternata*, *Candida albicans*, *Penicillium italicum*, *Fusarium equisetii* [28].

Application of Silver Nanoparticles

Silver nanoparticles have more applications in many areas, including biomedical, materials science, and catalysis. This is because of their unique properties when compared with their bulk solid. This part of the literature will give a brief description.

Human Health

Nanoparticles have many different effects on human health relative to bulk material from which they are produced [29]. Increase the biological activity of nanoparticles can be beneficial, detrimental or both. Many nanoparticles are small enough to have an access to skin, lungs, and brain [30,31]. Exposure of metal containing nanoparticles to human lung epithelial cells generated reactive oxygen species, which lead to oxidative stress and damage of the cells [32,33]. A study on toxic effects of silver nanoparticles was done on zebrafish as a model due to its fast development and transparent body structure. The results show a deposition of particles on organs and severe developmental effects. The biocompatibility and toxicity of silver nanoparticles were exhibited by observing single silver nanoparticles inside embryos at each development stage. The types of abnormalities in

zebra fish were strongly dependent on the dose of silver nanoparticles [34].

Environmental

Silver nanoparticles are of great concern to wastewater treatment utilities and to biological systems. The inhibitory effects of silver nanoparticles on microbial growth were evaluated at a treatment facility using an extant respirometry technique. The nitrifying bacteria were susceptible to inhibition by silver nanoparticles, which could have detrimental effects on the microorganisms in wastewater treatment. The environmental risk of silver nanoparticles was recently investigated by determining released silver from commercial clothing. The sock material and wash water contained silver nanoparticles of 10–500 nm diameter.

Catalytic Action

High surface area and high surface energy predetermine metal nanoparticles for being effective catalytic medium. Growing small particles of silver have been observed to be more effective catalysts than stable colloidal particles. These growing particles catalyzed the borohydride reduction of several organic dyes. The reduction rate catalyzed by growing particles is distinctly faster compared to that of stable and larger silver particles, which are the final products of growing particles. Catalysis is due to efficient particle-mediated electron transfer from the BH₄ ion to the dye. The catalytic activity of the particles depends on their size, E_{1/2} of the dye, and the dye-particle interaction. Catalytic activity of silver nanoparticles can be controlled by its size, as redox potential depends on the nanoparticle size [35].

Antimicrobial

Silver is a non-toxic, safe inorganic antibacterial agent being used for centuries and is capable of killing about 650 microorganisms that cause diseases. Silver has been described as being ‘oligodynamic’, that is, its ions are capable resistant a bacterostatic (growth inhibition) or even a bactericidal (antibacterial) impact. Therefore, it has the ability to exert a bactericidal effect at minute concentration [36]. It has a significant potential for a wide range of biological application such as antibacterial agents for antibiotic resistant bacteria, preventing infections, healing wounds and anti-inflammatory. Silver ions (Ag⁺) and its compounds are highly toxic to microorganism exhibiting strong biocidal effect on many species of bacteria but have a low toxicity towards animal cells.

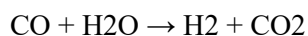
Copper nanoparticles

In recent years, the preparation of copper nanoparticles has received increasing attention from many researchers since copper nanoparticles are viewed as possible replacements for Ag and Au nanoparticles because of their useful anti-microbial, anti-biotic and anti-fungal (fungicide) agent and for their potential electrical, dielectric, magnetic, optical, imaging, catalytic, biomedical and bioscience properties [37]. Although copper is one of the most widely used materials in various applications, its synthesis in nano sizes is challenging due to its high tendency for oxidation. Unlike gold and silver, copper is extremely sensitive to air, and the oxide phases are thermodynamically more stable. Therefore, the formation of a surface oxide layer on copper nanoparticles is inevitable. The presence

of copper oxides on the surface of nanoparticles is not desirable for many industries, such as electronics that count on copper as a good alternative for current expensive metals. The electrical conductivity of copper nanoparticles decreases dramatically if they become impure with oxide phases. One can rarely find a method in literature that produces pure copper nanoparticles, unless the whole procedure was done under an inert atmosphere [38]. The achievement in synthesis of pure copper nanoparticles by reducing copper salt in the presence of surfactant [39].

Application of Copper nanoparticles

Metallic nanoparticles can be used in heat transfer systems to improve efficiency. Fluids containing metallic nanoparticles with a thermal conductivity of about three times that of a pure fluid could double the fluid's heat transfer rate. It is reported that adding only 0.3 volume percent of copper nanoparticles, with average diameter of less than 10 nm, to ethylene glycol increased its thermal conductivity up to 40% [40]. A major problem facing fuel-cell technologies is formation of high levels of carbon monoxide (CO) which is produced during hydrogen production. One way to eliminate the CO byproduct is to combine it with water to produce hydrogen gas and carbon dioxide (CO₂) in a process known as the "water- gas shift" reaction (See reaction below).



With the assistance of proper catalysts, the water-gas shift reaction can convert a large portion of carbon monoxide into carbon dioxide. For this purpose, to achieve greatest catalytic activity, nanoparticles (2 - 4 nm) of either gold or

copper supported on a metal oxide (zinc oxide, ZnO and cerium oxide, CeO₂) have been used. Although, gold nanoparticles show the greatest catalytic activity in water-gas shift reaction, copper is almost as reactive, and its cost is much lower [41].

Antibacterial activity

AgNPs are able to physically interact with the cell surface of various bacteria. This is particularly important in the case of Gram-negative bacteria where numerous studies have observed the adhesion and accumulation of AgNPs to the bacterial surface. Many studies have reported that AgNPs can damage cell membranes leading to structural changes, which render bacteria more permeable [42].

The correlation between the bactericidal effect and AgNP concentrations is bacterial class dependent [43]. Indeed, *Pseudomonas aeruginosa* and *Vibrio cholera* were more resistant than *E. coli* and *Salmonella typhi*, but at concentrations above 75 µg/mL, the bacterial growth was completely abolished [44].

AgNPs have been shown to be definitely an effective antibiotic against *E. coli*, *S. typhi*, *Staphylococcus epidermidis* and *S. aureus* [45]. Increasing scientific evidence has demonstrated that AgNP activity would depend not only on their concentration and size [46], but also on their shape [47]. The effect of nanoparticles with spherical, rod-like and triangular shapes against *E. coli*. They showed that all of them had antimicrobial activity, with the triangular nanoparticles being qualitatively more effective. Probably the triangular shape gives a greater positive charge to the nanoparticles, which together with the

active facets on a triangular-shaped particle is able to ensure a greater activity. It has been suggested that AgNPs also interfere with bacterial replication processes by adhering to their nucleic acids [46]. All factors which influence the activity of AgNPs (concentration, size, shape, UV radiation and the combination with various antibiotics) should be taken into consideration when preparing AgNPs for clinical use [48]. Notwithstanding the many conflicts in the literature regarding the effects of antibacterial AgNPs, it is likely that it is the result of a combined effect of each contributing feature, which provide a broad spectrum of antibacterial activity and decrease the probability of developing resistance [49].

Phytochemical screening

Among the 120 active compounds currently isolated from the higher plants are widely used in modern medicine, today 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived [50]. The phytochemical interaction and trace components may alter the drug response in ways that cannot currently be replicated with a combination of few purative active ingredients. Pharmaceutical researchers recognize the concept of drug synergism but note that clinical trials may be used to investigate the efficacy of a particular herbal preparation, provided the formulation of that herb is consistent [51]. There is

evidence that using some alternative medicines especially those evolving herbs, metals, minerals or other materials involves potentially serious risks including toxicity [52]. With the development of natural product chemistry, the potential of chemotaxonomy is now being increasingly obvious. The application of chemical data to systematics has received serious attention of a large number of biochemists & botanists [53]. The screening of plant extracts of plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic proto types [54]. Hence during the present investigations phytochemical screening of certain native plants of Agra city is carried on with a view to analyse the presence of chemical constituents that included primary & secondary metabolites, with a view to recommend their application in pharmaceutical industry.

Enzyme

All enzymes are protein [55]. Enzymes are the proteins that are produced by living organisms to regulate their biochemical processes. Enzymes can be also known as catalysts that speed up biochemical processes without itself being consumed [56]. Enzyme can be classified into six major types according to the reaction types catalyzed. The six major types of enzymes are tabulated in Table 1.1 [57].

Class	Type of chemical reaction catalyzed	Example
Hydrolase	Hydrolysis reaction	Digestive enzymes
Isomerase	Rearrangement of atoms within a molecule	Fumarases
Ligase	Joining two molecules by forming a new bond.	Citric acid synthetases
Lyase	Nonhydrolytic removal of a groups	Decarboxylases, Aldolases
Oxidoreductase	Oxidation or reduction reactions which hydrogen or oxygen atoms are gained or lost.	Dehydrogenase, Cytochrome oxidase
Transferase	Transfer of function group	Transaminase

Table 1.1: Classification of enzymes.

Enzymes with Special Characteristics

Special characteristics of microbial enzymes include their capability and appreciable activity under abnormal conditions, mainly of temperature and pH. Hence, certain microbial enzymes are categorized as thermophilic, acidophilic or alkalophilic. Microorganisms with systems of thermostable enzymes that can function at higher-than-normal reaction temperatures would decrease the possibility of microbial contamination in large scale industrial reactions of prolonged durations [58-60]. The quality of thermo stability in enzymes promotes the breakdown and digestion of raw materials; also, the higher reaction temperature enhances the penetration of enzymes [61]. The complete saccharification and hydrolysis of polysaccharides containing agricultural residues requires a longer reaction time, which is often associated with the contamination risks over a period of time. Therefore, the hydrolytic enzymes are well sought after, being active at higher temperatures as well as retaining stability over a prolonged period of

processing at a range of temperatures. The high temperature enzymes also help in enhancing the mass-transfer and reduction of the substrate viscosity [62,63] during the progress of hydrolysis of substrates or raw materials in industrial processes. Thermophilic xylanase are considered to be of commercial interest in many industries particularly in the mashing process of brewing. The thermo stable plant xerophytic isoform of laccase enzyme is considered to be useful for their applications in textile, dyeing, pulping and bioremediation [64,65].

Antioxidant

Antioxidant research is an important topic in the medical field as well as in the food industry. Many plants, particularly medicinal ones, have been extensively studied for their antioxidant activity in recent decades. Antioxidants from aromatic, spicy, medicinal, and other plants were studied to develop natural antioxidant formulations for food, cosmetic, and other applications [66]. It is believed that an increased intake of food rich in natural

antioxidants is associated with lower risks of degenerative diseases, particularly cardiovascular diseases and cancer [67]. There are three major classes of plant chemicals: terpenoids, phenolic, and alkaloids [68]. Among these three groups, phenolic compounds are the most important for dietary applications and the most extensively researched ([69]. Phenolic compounds include phenolic acids (hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins), and flavonoids. These compounds can scavenge excess free radicals and effectively reduce oxidative stress, which protect plants, fruits, and vegetables and protect DNA, protein and lipids in the human body from oxidative damage. They have been used as antioxidants by humans, thus preventing diseases from being induced. Finding new and safe antioxidants from natural sources is of great interest for applications as natural antioxidants, functional foods, and nutraceuticals.

In the present study, three commonly used antioxidant evaluation methods such as DPPH radical scavenging activity, reducing power assay and phosphomolybdenum method were chosen to determine the antioxidant potential of seeds.

Aromatic plants are well known for their antioxidant and antimicrobial properties that prevent food degradation and alteration [70], as they are rich in phenolic substances, usually referred to as polyphenols, which are ubiquitous components of plants and herbs. Halliwell and Gutteridge [71] defined antioxidants as compounds that—when present in low concentration in relation to the oxidant—prevent or delay the

oxidation of the substrate. Their importance in the safeguarding of health, and the protection from coronary heart disease and cancer, has recently been established, thus constituting them as functional food preservatives. Polyphenols are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Some show metal chelation properties [72,73]. In addition, some have antimicrobial activity [74].

Objectives

- To study the synthesis of silver copper nanoparticles from the leaf extract of the medicinal plant *Pithecellobium dulce*.
- To study the isolation of enzyme from the leaves of *Pithecellobium dulce* by standard methodology and synthesis silver nanoparticles from the enzyme.
- To study the formation and absorption properties of biosynthesized nanoparticles by UV-visible (UV-vis) spectroscopy techniques.
- To identify the functional groups of phytochemicals in the leaf extract by Fourier Transform Infrared (FT-IR).
- To study the size, structure and phase synthesized nanoparticles by using X-ray diffraction (XRD) techniques.
- To study the surface morphology (shape and size) of the nanoparticles by Atomic force microscopy (AFM), Scanning Electron Microscopy (SEM) with Energy dispersive X-Ray Analysis (EDAX) and Transmission electron microscopy (TEM) techniques.

- To study the antioxidant activity of green synthesis silver nanoparticles from enzyme by phosphomolybdenum method.

Literature Review

- Hutchison et al. (2008) [75] Nanoscience involves the study of materials on the nanoscale level between approximately 1 and 100 nm in length in at least one dimension.
- Rosi and Mirkin et al (2005) [76] The study of how to control the formation of two- and three-dimensional assemblies of molecular scale building blocks into well-defined nanostructures or nanomaterials.
- Uskokovic et al (2008) [77] Nanotechnology is the application of science and technology to control matter at the molecular level, which is also referred to as the ability for designing, production, characterization and application to structures, devices and systems by controlling shape and size at the nanometer scale.
- Burda et al., (2005) [78] Nanotechnology emerges from the physical, chemical, biological and engineering sciences where novel techniques are being developed to probe and manipulate single atoms and molecules. Nanomaterials have broad applications in a variety of fields because of their unusual and size dependent optical, magnetic, electronic and chemical properties.
- Hodes et al, (2007) [79] Nanoparticles are characterized by an extremely large surface area to volume ratio, and their properties are

determined mainly by the behaviour of their surface.

- Hsiao et al., (2006) [80] Nanobiotechnology is an emerging area of opportunity that seeks to fuse nano/micro fabrication and biosystem to the benefit of both. Increasing awareness towards green chemistry and other biological processes has led to the development of simple and eco-friendly approaches towards the synthesis of nanomaterial.
- Goyer et al, (1997) [81] Copper is one of a relatively small group of metallic elements that are essential to human health as it is a constituent of many enzymes involved in numerous body functions and is a constituent of hair and of elastic tissue contained in skin, bone and other body organs.
- Airey and Verran et al, (2007) [82] A number of recent studies have explored the potential benefit of using copper in place of stainless steel on surfaces to reduce bacterial loads in a number of settings including hospitals and the food industry.
- Moya et al., (2006) [83] Copper nanoparticles can easily oxidize to form copperoxide. If the application requires the copper nanoparticles to be protected from oxidation, the copper nanoparticles are usually encapsulated in organic or inorganic materials such as carbon and silica.
- Esteban-Cubillo et al., (2006) [84] Colloidal copper has been used as an antimicrobial agent for decades. Copper monodispersed nanoparticles (2-5nm) embedded into a polysilicate called sepiolite

- (Mg₈Si₁₂O₃₀ (OH)₄(H₂O)₄.8H₂O) have revealed a strong antibacterial activity and were able to decrease the microorganism concentration by 99.9%.
- Cioffi et al., (2005) [85] Copper nanoparticles (about 6 nm) embedded in polyvinylmethylketone films exhibit a noticeable inhibitory effect on the growth of microorganisms (*E. coli* and *S. cerevisiae*).
 - Theivasanthi and Alagar et al, (2011) [86] Almost all properties of nanoparticles are due to their small sizes and they are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications.
 - A.Mills et al., (1997) [87] described Photocatalysis generally involves the process of photosensitization, where a photochemical reaction occurs in one chemical species due to the absorption of photonic energy by another species called photosensitize.
 - C.Haefeli et al. (1984) [88] The first evidence of bacteria synthesizing silver nanoparticles was established using the *Pseudomonas stutzeri* AG259 strain that was isolated from silver mine.
 - M. Hussein et al., (2006) [89] There are some microorganisms that can survive metal ion concentrations and can also grow under those conditions, and this phenomenon is due to their resistance to that metal.
 - R.vaidyanathan et al. (2010) [90] The enzyme converts nitrate into nitrite. In in vitro synthesis of silver using bacteria, the presence of alpha-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) - dependent nitrate reductase would remove the downstream processing step that is required in other cases.
 - P. Mohanpuria et al. (2008) [91] When in comparison with bacteria, fungi can produce larger amounts of nanoparticles because they can secrete larger amounts of proteins which directly translate to higher productivity of nanoparticles.
 - P. Mukherjee et al. (2001) [92] Trapping of Ag⁺ ions at the surface of the quinine cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system.
 - A.Ahmad et al. (2003) [93] The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. Considering the example of *F. oxysporum*, it is believed that the NADPH-dependent nitrate reductase and a shuttle quinine extracellular process are responsible for nanoparticle formation.
 - A. Jha et al. (2009) [94] In the case of mesophytes, it was found that they contain three types of benzoquinones:cyperoquinone, dietchequinone, and remirin. It was suggested that the phytochemicals are involved directly in the reduction of the ions and formation of silver nanoparticles
 - Look et al., (2001) [95] It is found in paints, cosmetics, plastic and rubber manufacturing, electronics and pharmaceuticals. More recently however, it has again gained large

- interest for its semiconducting properties.
- Lovely et al., (1987) [96] It can also be suitably scaled up for large-scale synthesis of nanoparticles. It is well known that biological systems can provide a number of metal or metal containing particles in the nanometer size range. The synthesis of magnetite nanoparticles by magnetotactic bacteria.
 - Shang et al., (2007) [97] Although the protein may retain most of its native structure after adsorption on the NP surface, in some cases the thermodynamic stability of the protein is decreased, making the protein more sensitive to chemical denaturants such as urea.
 - Zhang et al., (2009) [98] Recent study reported that some nanomaterials catalyze the formation of protein fibrils as evidenced from the fact that interactions between proteins and nanophase materials could induce modifications in protein structure, leading to the growth of extended assemblies.
 - Jin et al. (2001) [99] It was reported that prismatic structure of AgNPs could be obtained by irradiation of a conventional 40-W fluorescent light on previously prepared spherical particles (8.0 ± 1.7 nm).
 - Metraux and Mirkin et al., (2005) [100] Compared with photo-induced synthesis, thermal synthesis (or chemical reduction methods) of silver nanoprisms were reported to be able to achieve AgNPs of similar shape and size.
 - Pal et al. (2007) [101] Synthesis of this specific structure was also appealing to consider due to a proposed mechanism in which different crystal facets may induce different extent of antimicrobial effects.
 - Typical procedures for synthesis and characterization of truncated triangular silver nanoplates were reported by Chen and Carroll (2002) [102]. Three phases were involved in the synthesis route: seeding, growth and aging. First, sodium borohydride (NaBH_4) was used to reduce Ag^+ into $\text{Ag}(0)$ with sodium citrate as stabilizing agent.
 - Ahmad et al., (2011) [103] During glycolysis NAD (Nicotinamide adenine dinucleotide) formation occurs, which is a co-enzyme found in all living cells. NAD is an oxidizing agent which accepts electrons from other molecules and gets reduced.
 - The green synthesis of metal NPs, reduction, capping and stabilization steps occur and the biomolecules such as enzymes, proteins, sugars etc. due to presence in the plant extract reported by Singh et al. (2015) [104].
 - [105] Though the exact mechanism involved in AgNPs production by plants is not fully deciphered. It is believed that the biomolecules are involved directly in the reduction of the ions and formation of AgNPs (Jha et al., 2009).
 - [106] The biochemical and molecular mechanisms of AgNPs green synthesis remain undiscovered, for instance biochemical mechanisms underscored the significance of phytoconstituents which may

mediate green synthesis (Chung et al., 2016).

- Li et al., (2007) [107] Silver ions are then reduced by proteins, lead to changes in their secondary structure and the formation of silver nuclei, consequently silver nuclei grow by the reduction of silver ions and their aggregation at nuclei.
- Safeopour et al., (2009) [108] AgNPs are used in the development of new technologies in the areas of electronics, material sciences and medicine and because of their extensive applications in various areas more research is being conducted on the AgNPs by the scientists throughout the world.
- Harikumar et al, (2016) [109] The results obtained from the effect of bacterial load indicated that there was a decrease in antibacterial activity as the bacterial concentration increased. Flow test was conducted for the antibacterial filter by passing contaminated water through the filter and no bacteria were detected.
- Sunitaojha et a, (2017) [110] reported the capping of phytochemicals and thermal stability of RcAgNPs were assessed by FTIR spectra and TGA analysis, respectively. It also showed antibacterial activity against both gram positive and gram negative strains. RcAgNPs were non-toxic against normal cell line (mouse fibroblast cell line L929) at lower concentrations (80 µg ml⁻¹).
- Shivan and payamalle and hosakatteni Ranjan amurthy et al, (2016) [111] The aim of present study was the evaluation of

antibacterial activity of *G. xanthochymus* seed extracts against some pathogenic bacteria like *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli* and to analyze the mechanism of its antibacterial action by atomic force microscopy (AFM).

- [112] Khalid et al., (2018) The medicinal importance of these plants depends upon the chemically vital and active substances that produce specific physiological action on the human body. Flavonoids, tannin, phenolic compounds and alkaloids are the most important bioactive components of plants.
- [113] Sathyaprabhu et al., (2018) The enzyme extracted from different parts of the sample show an optimum activity at pH 6 along with a molecular weight ranging between 60 to 100 kDa. In conclusion, the study suggests faba bean can serve as best source of protease enzyme.
- [114] Charalamposproestos et al., (2013). The antioxidant capacity of the plant extracts was measured by their ability to scavenge free radicals such as (a) DPPH(2,2-diphenyl-1-picrylhydrazyl) and, (b) ABTS (2,2'-azinobis-(3-ethylbenzothiaziline-6-sulfonate). The Folin-Ciocalteu method proved the existence of antioxidants in the aromatic plant extracts.

Method and Materials

Pithecellobium dulce

It originated from Mexico, then went to America, Central Asia and then to cooking, contains high wholesome esteem and various medical advantages for body. Besides being a viable normal

cure, it is more moderate contrasted with high-cost medicines in clinics and restorative centres. Studies have concluded that hydro alcoholic fruit extract of *Pithecellobium dulce* can be used safely for experimental and clinical trials. This study was carried out to evaluate acute and sub-acute toxicity profile of HAEPD in 2010. According to the studies performed in 2012, scientists have validated the antimicrobial potential of traditionally important plant, *Pithecellobium dulce*. The bark and pulp of Manila Tamarind is used as a traditional remedy against gum ailments, toothache, and haemorrhage. Bark extract is also used against dysentery, diarrhea, and constipation. An extract of leaves is used for gall bladder ailments and to prevent miscarriage. Seeds when grounds are used to cleanse ulcers. Numerous studies have been performed on anti-oxidant, anti-inflammatory, anti-diabetic, anti-cancer properties of Manila tamarind. It provides relief from pain, eczema, fever, cold, sore throat, pigmentation, acne and pimples.



Pithecellobium dulce

Collection of Plant material and preparation of extract

Pithecellobium dulce leaves collected from natural geographical landscapes of Thoothukudi, in India. The *Pithecellobium dulce* leaves were washed thoroughly then sliced into small pieces and 20g leaf was boiled with 100 mL of sterile distilled water for 15 min

in a 500 mL beaker. The extract obtained was filtered through Watmann filter paper No.1. The filtrate was collected and stored at 4°C. Then the solution was used for the reduction of silver ions (Ag⁺) to silver nanoparticles (AgNO₃).

Preparation of silver nitrate solution

Seventeen milligrams (17 mg) of silver nitrate (Analytical grade) 99.9% AgNO₃, MW = 169.87 g/mol) were weighed using electronic balance and transferred into 500 ml Erlenmeyer flask. The silver nitrate was slowly dissolved by gently swirling the flask containing distilled de-ionized water. After all the solid has dissolved, more water was slowly added to bring the level of solution exactly to a volume mark of 100 ml. The prepared 1 mM silver nitrate solution was stored at 4°C in amber colored bottle.

Synthesis of silver nanoparticles

A volume of 20 ml of aqueous extract of *Pithecellobium dulce* leaves was added to 80 ml of 1 mM of silver nitrate solution in 100 ml Erlenmeyer colored for reduction of Ag⁺ions and stabilization of AgNPs. The reactions were carried out in darkness (to avoid photo activation of AgNO₃) at room temperature. Complete reduction of AgNO₃ to Ag⁺ ions was confirmed by visual change of colour from pale green to reddish brown indicated the formation of silver nanoparticles (AgNPs). The synthesis silver nanoparticles stored at 4°C for future use.

Preparation of copper sulfate solution

24.9mg of copper sulfate (Analytical grade) 99.9% CuSO₄, MW = 249.68 g/mol) were weighed using electronic balance and transferred into 500 ml Erlenmeyer flask. The copper sulfate

was slowly dissolved by gently swirling the flask containing distilled de-ionized water. After all the solid has dissolved, more water was slowly added to bring the level of solution exactly to a volume mark of 100 ml. The prepared 1 mM copper sulfate solution was stored at 4°C in amber colored bottle.

Synthesis of copper nanoparticles

20mL of *Pithecellobium dulce* leaf extract was added to 80ml of 1 mM aqueous CuSO₄.5H₂O solution and kept under magnetic stirrer for 2hours (60°C). Complete reduction of CuSO₄ to Cu²⁺-ions was confirmed by visual change of colour from pale blue indicated the formation of copper nanoparticles (CuNPs). The synthesis copper nanoparticles stored at 4°C for future use.

Phytochemical screening

The active Phytochemicals present in the floral extract were determined by various tests.

Test for alkaloids

A fraction of extract was treated with Wagners test reagent (1.27g of iodine and 2g of potassium iodide in 100mL of water) and observed the formation of reddish-brown color. It indicates presence of alkaloids.

Test for Flavonoids

A small amount extract was treated with aqueous sodium hydroxide and hydrochloric acid and observed. No yellow orange color. It indicates absence of flavonoids.

Test for Tannins

Few mL of extract was treated with 10% alcoholic ferric chloride solution and

observed the formation of blue colour solution. It indicates presence of tannins.

Test for Phenols

The fraction of extract treated with 5% ferric chloride and observed of deep blue color. It indicates presence of Phenols.

Test for Saponins

To the small amount of the extract, 10 mL of distilled water was added and shaken for about 30 seconds and observed the formation of foam, it indicates the presence of saponins.

Test for Steroids

2 ml of chloroform and concentrated H₂SO₄ were added with the 5 ml aqueous plant crude extract. In the lower chloroform layer red color appeared that indicated the presence of steroids.

Test for oils and Fats

a. Spot test: A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

b. Saponification test: A few drops of 0.5 N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on a water bath for 2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats

Test for Phytate

Four grams of ground sample was soaked in 100 cm³ of 2% HCl for 3 h and then filtered through two layers of filter paper 25 cm³ of the filtrate was placed in a 250 cm³ conical flask and 5 cm³ of 0.3% NH₄SCN solution was added as an indicator, 53.5 cm³ of distilled water was then added to reach

the proper acidity. This mixture was titrated against FeCl_3 solution, which contains about 0.00195 g of Fe iron per cm^3 of FeCl_3 solution. The result was multiplied by factor 1.95 to obtain phytate P. phytate P result was multiplied by factor 3.55 to convert to phytate.

Test for Terpenoids

Take 1 ml of extract of each solvent and add 0.5 ml of chloroform followed by a few drops of concentrated sulphuric acid, formation of reddish-brown precipitate indicates the presence of terpenoids in the extract.

Test for Carbohydrates

Take 1 ml of extract, add few drops of Molisch's reagent and then add 1 ml of concentrated sulphuric acid at the side of the tubes. The mixture was then allowed to stand for 2 to 3 minutes. Formation of red or dull violet colour indicates the presence of carbohydrates in the sample extract.

Enzyme extraction

- It is very important to use fresh, desiccated ammonium sulfate. This ensures uniform and rapid dissolution.
- The day before use, place ammonium sulfate overnight in ca. 120°C drying oven in a large beaker or drying dish (ammonium sulfate decomposes at 220°C).
- Clean grinder carefully and grind dry ammonium sulphate to a fine powder. Wear a dust mask (if you don't need a mask, the powder is not dry enough).
- Use ground powder immediately. For 70% saturation use 43.6 grams/100 mLs.

- Add the powder slowly but steadily with thorough mixing. Do not allow clumps to form.
- Allow precipitate to form for 30 minutes at 4°C with stirring.
- Recover precipitate by centrifugation. Solutions highly saturated in ammonium sulfate are quite dense and it can be difficult to pellet the precipitate. For CBF3 prep, spin at 40K in type 45 rotor for 1 hour.
- Remove supernatant, respin briefly to clear remaining ammonium sulfate.
- Resuspend pellets in a volume of buffer equal to the volume of the extract.

Antioxidant assay method

Phosphomolybdenum assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of Prieto (1999). This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue colour.

Reagent preparation

Reagent was prepared by adding 0.588ml of sulphuric acid, 0.049g ammonium molybdate and 0.036g sodium phosphate. The final volume was made up to 10ml with Dis. H_2O .

Working procedure

10mg of plant extract was dissolved in 1ml of DMSO. 100 μl from the prepared sample was taken and 1ml of reagent

solution was added to it and incubated in a boiling water bath at 95°C for 90 min. After 90 min, the absorbance of the solution was read at 695 nm. Ascorbic acid (10mg/ml DMSO) was used as standard. The Phosphomolybdenum reduction potential (PRP) of the studied extracts were reported in percentage.

Instrumentation

UV-Vis spectrophotometer

The synthesized silver Nanoparticles were characterized by JASCO variant 630 spectrometer (fig 3.1) within a range of wavelength 200-900nm.

Principle of UV spectroscopy

UV spectroscopy obeys the Beer-Lambert law, which states that: when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution. The expression of Beer-Lambert law is $A = \log(I_0/I) = Ecl$.

Where,

A = absorbance

I_0 = intensity of light incident upon sample cell

I = intensity of light leaving sample cell

C = molar concentration of solute

L = length of sample cell (cm.)

E = molar absorptivity

From the Beer-Lambert law it is clear that greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of

light absorption. This is the basic principle of UV spectroscopy.

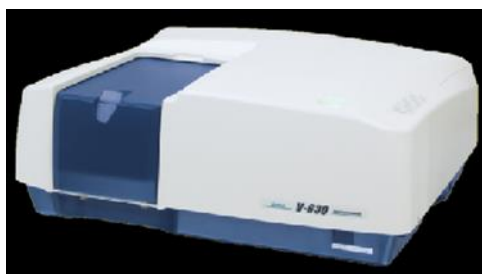


Fig. UV spectroscopy

Fourier transform infrared (FTIR) spectroscopy

The silver nanoparticles were analysed by for the detection of different functional groups using Thermo scientific Nicolet iS5ATR-iD1 Spectrometer (fig 3.2). The FTIR was recorded in the range of 400–4000 cm^{-1} .



Fig. FTIR Spectrometer

- In FTIR analyses, Infrared light from the light source passes through a Michelson interferometer along the optical path. The Michelson interferometer comprises a beam splitter, moving mirror, and fixed mirror. The light beam split into two by the beam splitter is reflected from the moving mirror and fixed mirror, before being recombined by the beam splitter.
- As the moving mirror makes reciprocating movements, the optical path difference to the fixed mirror

changes, such that the phase difference changes with time. The light beams are recombined in the Michelson interferometer to produce interference light.

- The intensity of the interference light is recorded in an interferogram, with the optical path difference recorded along the horizontal axis.

$f = 2V_m \nu / C$ interferogram frequency

$v_m = \delta / 2t$. mirror velocity

XRD Analysis

The phase variety and grain size of synthesized Silver nanoparticles was determined by X-ray diffraction spectroscopy (fig3.3) (Philips PAN analytical). The synthesized silver nanoparticles were studied with $\text{CuK}\alpha$ radiation at voltage of 30 kV and current of 20 mA with scan rate of 0.030/s. Different phases present in the synthesized samples were determined by X'pert high score software with search and match facility. The particle size of the prepared samples were determined by using Scherrer's equation as follows

$$D \approx \frac{0.9\lambda}{\beta \cos\theta}$$

Where D is the crystal size, λ is the wavelength of X-ray, θ is the Bragg's angle in radians and β is the full width at half maximum of the peak in radians. The crystallite size of synthesized product was determined using X-ray diffractometer Bruker AXS D8 Advance operating at a voltage of 40 kV and a current of 30 mA with $\text{CuK}\alpha$ radiation operating between 10 and 80° of 2 θ angles at scanning rate of 2° per min. Using Scherrer equation one can

calculate crystallite size from XRD data.
Crystallite size = $0.94 \times \text{X ray wavelength} / \beta \cos(\theta)$



Fig. XRD Analysis

Scanning Electron Microscopy (SEM)

This electron microscopy-based technique determines the size, shape and surface morphology with direct visualization of the nanoparticles. Therefore, scanning electron microscopy offers several advantages in morphological and sizing analysis. However, they provide limited information about the size distribution and true population average. During the process of SEM characterization, solution of nanoparticles should be initially converted into a dry powder. This dry powder is then further mounted on a sample holder followed by coating with a conductive metal (e.g. gold) using a sputter coater. Whole sample is then analyzed by scanning with a focused fine beam of electrons. Secondary electrons emitted from the sample surface determine the surface characteristics of the sample. This electron beam can often damage the polymer of the nanoparticles which must be able to withstand vacuum. Average

mean size evaluated by SEM is comparable with results obtained by dynamic light scattering. In addition, these techniques are time consuming, costly and frequently need complementary information about sizing distribution. In this research study, Joel JSM-6480 LV SEM (fig3.4) machine was employed to characterize the average particle size and sound structure of nanoparticles. Compositional analysis on the sample was carried away by the energy dispersive X-ray spectrometry (EDS) attached with the SEM. The EDS analysis of Ag sample was served by the SEM (JEOLJSM 5800) machine. The EDS normally reveals the presence of phases.



Fig. Scanning Electron Microscopy

Transmission Electron Microscope

Transmission electron microscopy techniques can provide imaging, diffraction and spectroscopic information, either simultaneously or in a serial manner, of the specimen with an atomic or a sub-nanometer spatial resolution. TEM operates on different principle than SEM, yet it often brings same type of data. The sample preparation for TEM is complex and time consuming because of its

requirement to be ultra-thin for the electron transmittance. High-resolution TEM imaging, when combined with nano diffraction, atomic resolution electron energy-loss spectroscopy and nanometer resolution X-ray energy dispersive spectroscopy techniques, is critical to the fundamental studies of importance to nanoscience and nanotechnology. During the TEM characterization nanoparticles dispersion is deposited onto support grids or films. After dispersion they are fixed using either a negative staining material (phosphotungstic acid or derivatives, uranyl acetate, etc., or by plastic embedding). This is done to make nanoparticles withstand against the instrument vacuum and facilitate handling. Alternatively, nanoparticles sample can also be exposing to liquid nitrogen temperatures after embedding in vitreous ice. When a beam of electrons is transmitted through an ultra-thin sample it interacts with the sample as it passes through the surface characteristics of the sample are obtained.

The size and morphology of the synthesized AgNPs were determined by high resolution transmission electron microscopy (HR-TEM, JEOL JEM 2100). (fig 3.6)



Fig. Transmission electron microscopy

Atomic Force Microscopy

This technique is also known as scanning force microscopy (technique that forms images of surfaces using a probe that scans the specimen), very high-resolution type of scanning probe microscopy, with reported resolution on the order of fractions of a nanometer, more than 100 times better than the optical diffraction limit. The atomic force microscopy is based on a physical scanning of samples at sub-micron level using a probe tip of atomic scale and offers ultra-high resolution in particle size measurement. Depending upon properties, samples are usually scanned in contact or noncontact mode. During contact mode, the topographical map is generated by tapping the probe on to the surface across the sample and probe hovers over the conducting surface in non-contact mode. One of the prime advantages of AFM is its ability to image non-conducting samples without any specific treatment. The morphology was investigated by Atomic Force Microscope (AFM) with Nano surf Easy scan 2 AFM (fig 3.7).



Fig. Atomic Force Microscopy

Results and Discussion

UV-VIS Spectroscopic Analysis.

The UV-Vis absorption spectra of AgNPs prepared from leaves extract of *Pithecellobium dulce* are shown in Figure 4.1. The absorption band of

AgNPs occurs at 426 nm. The AgNPs exhibit a yellowish-brown color in aqueous solution due to the excitation in UV-visible spectrum depending upon the particle size.

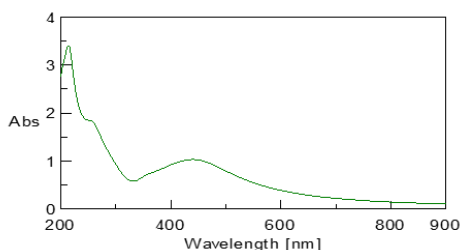


Fig. UV-Vis absorption spectra of AgNPs

UV VIS Spectroscopic analysis for CuNps

UV-VIS spectroscopy It is usually recognized that the UV-Vis spectroscopy could be used to examine the size and shape-controlled nanoparticles in aqueous suspensions. The reduction of copper sulphate to copper nanoparticles was monitored by measuring the UV-Visible spectrum of the reaction medium immediately after diluting a small aliquot of the sample into distilled water. Absorption spectra of copper nanoparticles formed in the reaction media has absorbance peak at 325nm (Fig 4.2).

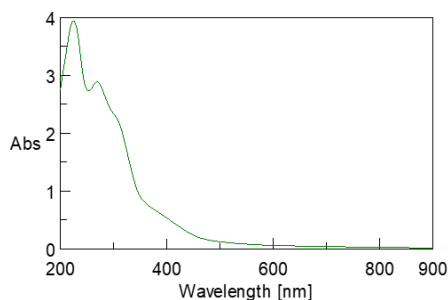


Fig. UV VIS Spectroscopic analysis for CuNps

UV-Visible spectral study in AgNps synthesized from enzyme

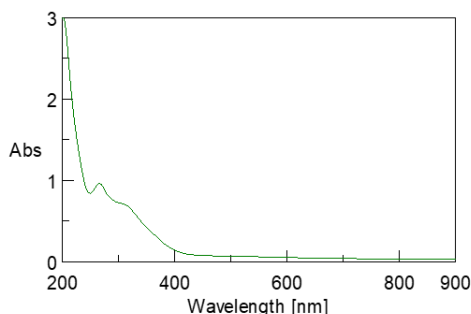


Fig UV-Visible spectral study in AgNps synthesized from enzyme

UV-visible spectroscopy is also of use to detect the presence of large particles (with a hydrodynamic radius higher than 200 nm) in a protein preparation. This can be done by monitoring the absorbance signal above 320 nm, where aggregate-free protein samples are not supposed to absorb light, and the signal can be attributed exclusively to the scattering of light by large aggregates present in the sample.

The UV/Vis spectrum of AgNPs showed a surface plasmon absorption band with maximum absorbance at 267, 314 nm (fig. 4.3).

FTIR Analysis.

The FTIR spectra were carried out to identify the possible biomolecules responsible for capping and reducing agent for the formation of metal nanoparticles. In Figure 4.4, FTIR spectra of silver and copper nanoparticles show strong absorption band at 1603 cm^{-1} and 1616 cm^{-1} , respectively, and it is attributed to binding of $\text{NHC}=\text{O}$ to metal ions. Other peaks include 2922 cm^{-1} (secondary amine), 1383 cm^{-1} (C-N stretching vibration of aromatic amine), 1138 cm^{-1} ,

821 cm^{-1} , 764 cm^{-1} , and 595 cm^{-1} for silver nanoparticles and 1383 cm^{-1} , 1074 cm^{-1} , and 601 cm^{-1} for copper nanoparticles. The presence of peak at 3186 cm^{-1} and 3341 cm^{-1} could be due to O-H group in poly phenols or proteins or polysaccharide. It has been reported that proteins can bind to metal nanoparticles through the free amine groups or carboxylate ion of amino acid residues.

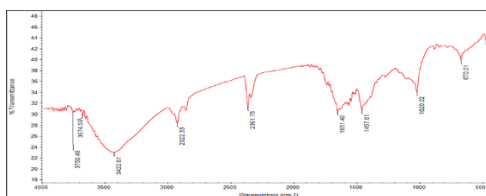
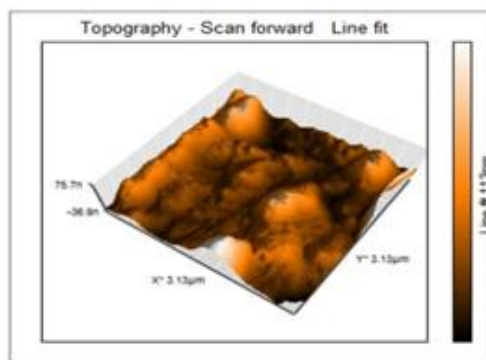


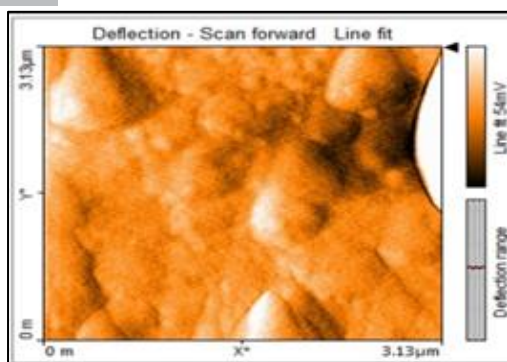
Fig. FTIR Spectrum of Pithecellobium dulce.

AFM Studies of AgNPs and CuNps

Topography of the silver nano particles synthesized from pithecellobium dulce extract were given in the Fig.4.5. Triangular shapes of different sizes were seen in the topography. Spherical shape was reported for the silver nano particles synthesized using aqueous solution of propolis.



a) 3D view

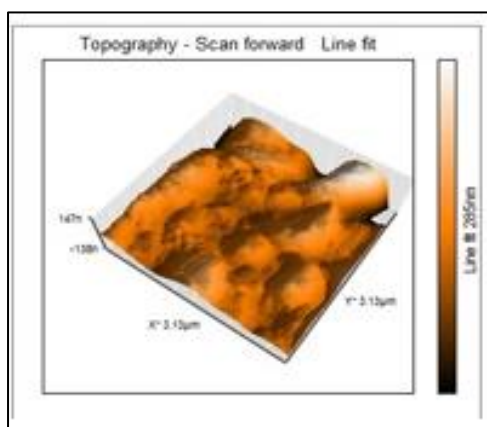


(b) normal view

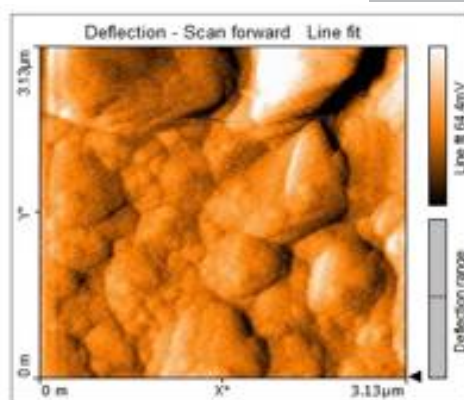
The silver nanoparticle which was synthesized by *Pithecellobium dulce* extract showed large agglomerated particles. Surface roughness of the silver nanoparticles synthesized from *Pithecellobium dulce*. This increase in roughness may be due to the formation of less agglomerated particles.

AFM images were taken using Nano Surf easy scan 2 AFM (BT02218).

Topography of the copper nanoparticles synthesized from *Pithecellobium dulce* extract were given in the Fig 4.6. Triangular shapes of different sizes were seen in the topography.



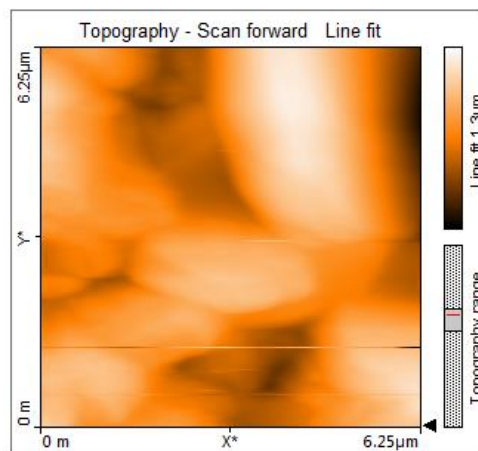
(a)

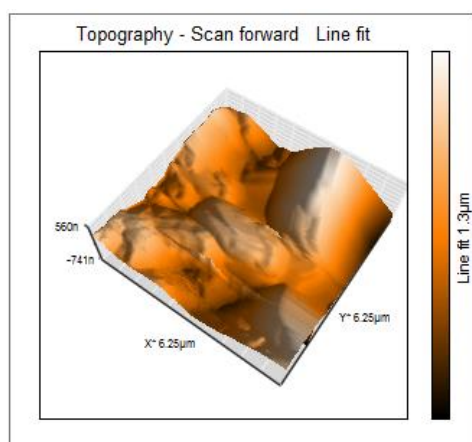


(b)

AFM studies of AgNPs from Enzyme

Oval shape particles of different diameters are clearly seen on the topography. This resulted due AFM topographies of silver nano particles prepared from *Pithecellobium dulce* leaf is given in the figure 4.7. to the aggregation of the particles. Surface is uneven with small peaks and small dents. Roughness values are calculated for the surface of area 39.37 pm².



**Fig 4.7**

X-Ray Diffraction Analysis

The powder XRD of the metal nanoparticles is recorded between 2 θ values 20° and 90° and exhibits crystalline nature and is consistent with

earlier reports showing possible peaks of silver and copper metal in Figure 4 [15, 25]. Bragg's diffraction peaks for silver nanoparticles are observed at 38.38°, 44.48°, 64.66°, 77.56°, and 81.66° corresponding to 111, 200, 220, 311, and 222, respectively, representing face centered cubic structure of silver. Bragg's reflection for copper nanoparticles are observed in XRD pattern with value of 43.6°, 50.7°, and 74.45° representing 111, 200, and 220 planes of FCC structure of copper. The average crystallite size of AgNPs and CuNPs was calculated to be about 31 nm and 26 nm respectively, using Scherrer formula. Crystallite Size Calculation. The average crystallite size has been calculated by using Debye-Scherrer formula.

Table. X-Ray diffraction studies of AgNPs

pos[2 θ]	Height (cts)	FWHM left [2 θ]	d-spacing[Å]	Rel.Int[%]	size(nm)
51.2141	288.42	0.3921	6.621	98.73	23.54
58.3251	202.31	0.3824	6.5281	96.32	17.26
68.2492	201.14	0.3862	6.0814	82.48	15.14
72.8997	114.47	0.3984	5.2048	68.42	21.19
79.0433	61.84	0.2941	4.2804	51.58	31.17
82.8249	41.81	0.4102	4.182	28.01	41.07
99.3021	52.54	0.4201	4.084	21.92	17.81

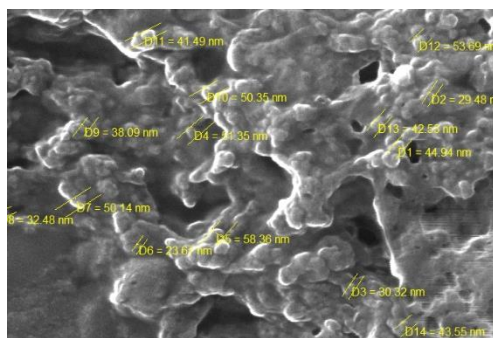
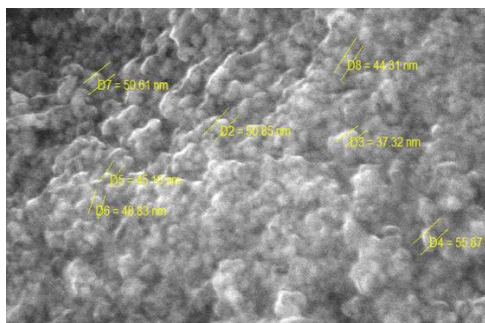
162.3211	41.51	0.4442	4.014	15.02	23.81
142.4284	28.54	0.4012	3.9942	11.92	32.81

Table X-Ray diffraction studies of CuNPs

pos[2 θ]	Height (cts)	FWHM left [2 θ]	d-spacing[Å]	Rel.Int[%]	size(nm)
38.1759	200.85	0.3553	2.3551	31.98	24.72
44.3197	56.29	0.6764	2.0421	8.96	13.25
46.2811	338.76	0.1716	1.9601	53.95	52.61
54.8576	83.39	0.1698	1.6722	13.28	53.08
57.5224	89.84	0.1913	1.6009	14.31	49.5
64.5007	45.22	0.4181	1.4435	7.2	23.48
77.0516	55.22	0.9851	1.2367	8.79	10.77

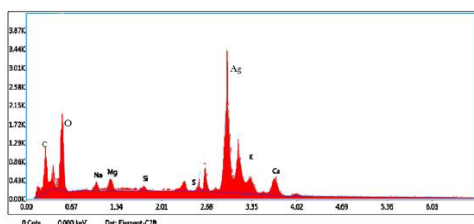
SEM Analysis.

The surface morphological and nano structural studies using SEM are shown in Figure 4.8. The SEM micrographs clearly show well aggregates of silver nano-particles of *Pithecellobium dulce* with average particle sizes ranging from 20 to 65 nm and copper nanoparticles with 30–65 nm in size.

**EDAX Analysis.**

Below figure shows the elemental profile of synthesized silver and copper nanoparticles using leaves extract of *Pithecellobium dulce*. The EDX analysis of silver nanoparticles shows an intense signal at 3 keV indicating the presence elemental silver in examined samples. The elemental analysis of the silver nanoparticles shown in the figure revealed a strong silver signal (66.43%)

along with weak signals of O (22.22%) and Cl (8.12%). Furthermore, two small peaks of Si (1.67%) and Al (1.56%) were also observed in the examined sample. The EDX analysis of copper nanoparticles possesses metallic copper (19.09%) with some other impurities, that is, O (74.32%) and Si (6.59%). This includes elemental peaks at 1.00, 8.00, and 9.00 keV for copper.



EDAX of AgNPs

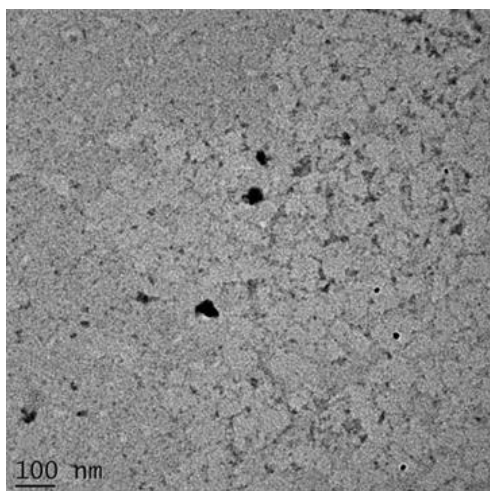
Element	weight %	Atomic %	Net weight
Ag	75	80.25	468.11
O	15	2.4	93.62
K	5	1.95	31.21
Ca	0.5	0.2	3.12
Na	0.9	0.198	5.62
Mg	1	0.24	6.24
S	0.9	0.288	5.62
Si	0.5	0.3	3.12
C	1.2	0.144	7.49

EDAX of CuNPs

Element	Weight %	Atomic %	Net. Weight
Cu	60.04	38.15	359.12
O	18.98	3.03	113.52
C	9.51	1.14	56.88
S	4.11	1.31	24.58
Cl	3.32	1.17	19.85
Ca	2.63	1.05	15.73
Mg	0.79	0.19	4.72
Na	0.4	0.09	2.39
Si	0.22	0.06	1.31

TEM Analysis of silver nanoparticles

TEM micrograph shown in below Figures revealed that the particles are spherical, oval, and well dispersed. The particle size of synthesized AgNPs is in the range of 100 nm and 50–100 nm, respectively. Figure 4.11 shows the Histogram of AgNPs of *Pithecellobium dulce*. It is evident that there is variation in particle sizes.





Histogram of AgNPs of *Pithecellobium dulce*. It is evident that there is variation in particle sizes.

Antioxidant activity

Total Antioxidant activity by Phosphomolybdenum method

$$\% \text{ Antioxidant activity} = (1 - (\text{Abs Std} - \text{Abs sample} / \text{Abs Std})) * 100$$

Total antioxidant capacity of silver nanoparticles from enzyme

Sample	Concentration $\mu\text{g/ml}$	OD @ 695 nm	% Antioxidant activity
Ascorbic acid	1000	1.500	
K-Ag enzyme	200	0.140	9.33
	400	0.295	19.67
	600	0.590	39.33
	800	0.750	50
	1000	1.124	74.93

Medicinal plants are a source for a wide variety of natural products, such as the phenolic acids, flavanoids, terpenoids and proteins which are very interesting for their antioxidant properties. The total antioxidant activity of the extracts was measured spectrophotometrically. From the absorbance value, the potential of antioxidant activity was determined. The results presented in Table 4.5 was found to be good potential of antioxidant activities in silver nanoparticles synthesized from *Pithecellobium dulce* enzyme extract.

Conclusion

Silver and Copper nanoparticles were successfully synthesised using eco-friendly, rapid, simple and low-cost method. Synthesised nanoparticles were studied using visual observation, XRD, FTIR, AFM, TEM, UV-Vis and SEM techniques. Triangular shapes of different sizes were seen in the topography of AFM image of silver and copper nanoparticles. Some elongated oval shaped particles were present in the topography.

X-ray diffraction investigations of the synthesized AgNPs and CuNPs were crystalline nature. The SEM micrographs clearly show well aggregates of silver nanoparticles of *Pithecellobium dulce* with average particle sizes ranging from 20 to 65 nm and copper nanoparticles with 30–65 nm in size. TEM micrograph revealed that the particles are spherical, oval, and well dispersed. The particle size of synthesized AgNPs is in the range of 100 nm and 50–100 nm, respectively.

The silver nanoparticles synthesized from enzyme *Pithecellobium dulce* FTIR spectra indicated the presence of phytochemicals, which may be *Pithecellobium dulce*.

leaf extract showed the greater potential of antioxidant activities. Hence, we conclude that the leaf extract of *Pithecellobium dulce* can be used by various food and pharmaceutical companies.

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