Ascorbic acid and Gallic acid Mediated Green Synthesis of Copper Nanoparticles and Study of its Antioxidant Activity and Seed Germination Properties.

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CONTENTS

1.INTRODUCTION

A nano meter is one billionth (10^{-9}) of a meter and Nanoparticles are particles between 1 and 100 nano meters in size. Nanoparticle is defined as a small object that behaves as a whole unit with respect to its transport and properties. Nanotechnology is a field of science which deals with production, manipulation and use of materials ranging in nano meters. In nanotechnology, the prefix ‘nano’ is a Greek word which means 'dwarf'

Nanoparticles (NPs) are of great scientific interest as they are in effect, a bridge between bulk materials and atomic or molecular structures (IpsaSubhankari and Nayak, 2013). A bulk material should have constant physical properties regardless of its size. But nano particles exhibit unusual physical, chemical and biological properties. Thus, the properties of materials change as their size approaches the nano scale. These properties may differ in important ways from the properties of bulk materials and single atoms or molecules. They exhibit a distinct property of larger surface-area-to-volume ratio, which makes them better than their bulk counterparts in the sense of their activity. Nanoparticle research is currently an area of intense scientific interest due to a wide variety of potential application in variety of areas, including medicine, engineering, catalysis, and environmental remediation. (Taylor *et al,* 2013).

1.1. CLASSIFICATION OF NANOPARTICLES

Nanoparticles are classified according to diameter (Jain *et al,* 2011). Ultra-fine particles are the same as nanoparticles and between 1 and 100 nanometre in size, fine particles are sized between 100 and 2500 nanometre and coarse particles cover a range between 2500 and 10,000 nanometre. Nanoparticles can also be classified according to their dimensionality, morphology, composition, uniformity and agglomeration (Ahamed *et al,* 2011).

Based on their dimensionalities, nanomaterials are placed into four different classes (Kolahalam *et al,*2019).

Zero-dimensional nanomaterials (0-D): the nanomaterials in this class have all their three dimensions in the nanoscale range. Examples are quantum dots, fullerenes, and nanoparticles.

One-dimensional nanomaterials (1-D): the nanomaterials in this class have one dimension outside the nanoscale. Examples are nanotubes, nanofibers, nanorods, nanowires, and nanohorns.

Two-dimensional nanomaterials (2-D): the nanomaterials in this class have two dimensions outside the nanoscale. Examples are nanosheets, nanofilms, and nanolayers.

(4) Three-dimensional nanomaterials (3-D) or bulk nanomaterials: in this class the materials are not confined to the nanoscale in any dimension. This class contains bulk powders, dispersions of nanoparticles, arrays of nanowires and nanotubes, etc.

Nanoparticles can be spherical, tubular, irregularly shaped and can also exist in fused, aggregated or agglomerated form (Akhavan *et al*, 2012). If the size of NPs gets lower than 1 nm, the term atom clusters is usually preferred. NPs can be crystalline with single or multi-crystal solids, or amorphous. NPs can be either loose or agglomerated (Machado *et al*,2015).

Based on their composition, NPs are usually sited into three classes: organic, carbon-based, and inorganic (Ealia and Saravanakumar,2017).

Organic Nanoparticles: These are made of proteins, carbohydrates, lipids, polymers, or any other organic compounds (Pan and Zhong,2016). The examples of this class are dendrimers, liposomes, micelles, and protein complexes. They are generally non-toxic, bio-degradable, and are sensitive to thermal and electromagnetic radiation such as heat and light (Ealia and Saravanakumar,2017). Today, organic NPs are mostly used in the biomedical field in targeted drug delivery (Ealia and Saravanakumar,2017) and cancer therapy (Gujrati *et al*,2014).

Carbon-based Nanoparticles

These are made solely from carbon atoms and examples of this class includes fullerenes, carbon black NPs, and carbon quantum dots. Fullerenes are carbon molecules that are characterized by a symmetrical closed-cage structure, Carbon black NPs are grape-like aggregates of highly fused spherical particles and Carbon quantum dots consist of discrete, quasi-spherical carbon NPs with sizes below 10 nm (Lu *et al,*2016). Due to their unique electrical conductivity, high strength, electron affinity, optical, thermal, and sorption properties (Khan *et al*,2019), carbon-based NPs are used in a wide range of application such as drug delivery, energy storage (Liu *et al,*2018), bioimaging, photovoltaic devices, and environmental sensing applications to monitor microbial ecology or to detect microbial pathogens (Liu *et al,*2018).

Inorganic Nano particles

These class include metal, ceramic, and semiconductor NPs. Metal NPs are purely made of metal precursors, they can be monometallic, bimetallic, or polymetallic (Nascimento *et* al,2018). Bimetallic NPs can be made from alloys or formed in different layers (core–shell). Due to the localized surface plasmon resonance characteristics, these NPs possess unique optical and electricals properties (Khan *et al*,2019). In addition, some metal NPs also possess unique thermal, magnetic, and biological properties (Ealia and Saravanakumar,2017). This makes them increasingly important materials for the development of nanodevices that can be used in numerous physical, chemical, biological, biomedical, and pharmaceutical applications (Mody *et al*,2010). In present days, the size-, shape-, and facet-controlled synthesis of metal NPs is important for creating cutting-edge materials (Dreaden *et al*,2012).

1.2. SYNTHESIS OF NANOPARTICLES

Various conventional methods have been employed for the synthesis of Nanoparticles. Before adopting these methods for application in therapeutic purpose, it is important to evaluate if those methods are safe, eco-friendly, economic and non-time consuming.

Nanoparticles can be synthesized mainly by two process namely ‘Top down’ and ‘Bottom-up’ process (Fendler, 1998). Top-Down method refers to a set of fabrication technologies which fabricate by removing certain parts from a bulk material substrate. The removing methods can be mechanical, chemical, electrochemical etc., depending on the material of the base substrate and requirement of the feature sizes. The formed structures usually share the same material with the base substrate. There are a couple of manufacturing technologies in the conventional scale which can be categorized top-down. Milling is a representative example. In the milling process, material is selectively removed from the substrate, usually a metal sheet, forming a cavity with certain geometries. The dimensions of the cavity depend on the travel path of the mill, which can be precisely controlled with the help of computer assisted numerical systems. The milling technique, along with similar methods such as drilling and grinding, is the most widely used technique in conventional manufacturing industry.

As the opposite to top-down fabrication technologies, bottom-up methods refer to a set of technologies which fabricate by stacking materials on top of a base substrate. Bottom-up process is carried out by processes including pyrolysis, inert gas condensation, solvothermal reaction, sol gel fabrication etc. In pyrolysis, a vaporous precursor (liquid or gas) is forced through an orifice at high pressure and burned. Thermal plasma can deliver the energy necessary to cause vaporization of small micro meter- sized particles. The thermal plasma temperatures are in the order of 10,000 K, so that solid powder easily evaporates.

There are three methods for the preparation of nanoparticles. 1) Physical method 2) chemical method and 3) biological method.

Traditionally nanoparticles were produced only by physical and chemical methods. Some of the common methods are ion sputtering, solvothermal synthesis, reduction and sol gel technique. Several chemical methods have been developed for the synthesis of various nanoparticles including chemical reduction, aqueous solution chemical reduction, nonaqueous chemical reduction, the template method, electrochemical reduction, ultrasonic-assisted reduction, photo-induced or photo-catalytic reduction, microwave assisted synthesis, irradiation reduction, the microemulsion method, biochemical method etc., but these chemical methods have been reported along with various drawbacks, including the use of toxic solvents, generation of hazardous by-products, and high energy consumption, which pose potential risks to human health and to the environment . Though physical and chemical methods are more popular for nanoparticle synthesis, the use of toxic compounds limits their applications. And the physical and chemical processes for synthesis of nanoparticles are very costly.

To overcome this limitation, researchers have found the cheapest way, the biological method, using microorganisms and plant extracts for nanoparticle synthesis process. Bottom-up approach is commonly used for the biological synthesis of nanoparticles. The use of microorganisms in biological method has been widely established and it is a kind of bottom up approach where oxidation/reduction is the main reaction (Gericke and Pinches, 2006). As compared to chemically/physically synthesized nanoparticles with biosynthesized nanoparticles, it is found that biosynthesized nanoparticles are eco-friendly and biocompatible in medical applications.

1.3. GREEN CHEMISTRY OF NANOPARTICLE

Green chemistry has been an eye catching area of interest since the past few years. Biological methods using plant extracts and microorganisms have been proposed as alternative environment friendly methods in the synthesis of metallic nanoparticles. Biomolecules can function as both reducing and capping agents, eliminating the use and generation of substances hazardous to human health and environment. While exploring the natural secrets for the biological synthesis of nanoparticles by microbes and plants, they are regarded as potent eco-friendly green nano factories.

Green chemistry aims at preventing waste, minimizing energy use, employing renewable materials, and applying methods that minimize risk. So green chemistry is environment friendly way to synthesize nanomaterials that are used in green chemical applications such as environmental remediation, decomposition of hazardous wastes, dye degradation, and other areas. Environmental friendly synthesis methods are becoming more and more popular in chemistry and chemical technologies. This trend has several origins, including the need for greener methods counteracting the higher costs and higher energy requirements of physical and chemical processes. For this reason, scientists search for cheaper methods of synthesis. In order to reduce the environmental impact of nanoparticle synthesis, greener routes have been investigated for over a decade.

1.4. PLANT BASED NANOPARTICLE SYNTHESIS

The most interesting biochemical and yield specific source for synthesis of nanoparticles is the plant biodiversity. Plants with highly rich genetic variability possess a number of interesting biomolecules in the form of coenzyme, vitamin-based intermediates, and so many others which can reduce metal ions to nanoparticles in a single step (Kaviya *et al*, 2012). Moreover, these methods can be easily conducted at room temperatures and pressure, without any hard and fast technical requirements. Furthermore, plant-based nanoparticles synthesis approaches are easy to scale up and are traditionally also favoured because of their environment friendliness. Plant metabolite materials serve as excellent reducing agents, which includes phenolic compounds, alkaloids and sterols. Additional advantage is that it is a green synthesis and, along with the use of plant extracts, live plants can also be used for synthesis of nanoparticle. The thrust behind plant mediated nanoparticle synthesis attracting significant boost is due to the fact that this route of nanoparticle synthesis enables the products which can be exploited for multiple applications such as those of nanomedicine-based innovations (Malik and Goyal, 2011).

One significant advantage of plant mediated nanoparticle synthesis is that they are easy and inexpensive as compared to microorganisms (Mubarak Ali *et al*, 2011). Using of plant extract instead of whole plant for making nanoparticle have given significant results (Lee *et al*, 2011).

1.5. PROPERTIES OF NANOPARTICLES

Materials reduced to nano scale can show different properties compared to what they exhibit on a macro scale, enabling unique applications. The interesting and sometimes unexpected properties of nanoparticles are therefore largely due to the large surface area of the materials, which dominates the contributions made by the small bulk of the material. For instance, opaque substances can become transparent (copper); stable materials can turn combustible (aluminum); insoluble materials may become soluble (gold). A material such as gold, which is chemically inert at normal scales, can serve as potent chemical catalyst at nanoscales (Lubick and Kellyn, 2008).

Nanoparticles have an array of advantage over bulk materials due to their surface plasmon resonance (SPR), enhanced Rayleigh scattering and surface enhanced Raman scattering (SERS) in metal nanoparticles, quantum size effect in semiconductors and super magnetism in magnetic nanomaterials. Therefore, nanoparticles are considered as building blocks of the next generation of optoelectronics, electronics, and various chemical and biochemical sensors (Wong and Schwaneberg, 2003).

The term metal nanoparticle is used to describe nano-sized metals with dimensions (length, width or thickness) within the size range 1-100 nm. Several metallic nano particles including Gold (Au), Silver (Ag), Copper (Cu), Platinum (Pt), Palladium (Pd) and Magnetite (Fe_3O_4) have many uses in industries and medical fields. Catalysts, cancer treatments, cosmetics and electronics are just a few examples of application of metallic nano particles.

1.6. CHARACTERISATION OF NANO PARTICLES

Different methods and techniques are used for the analysis and characterization of the various physicochemical properties of NPs.

1.6.1 Techniques used for the characterization of Morphology and topography of NPs

The morphological and topographical features of NPs influence most of the properties of NPs. The features include the size, shape, dispersity, localization, agglomeration/aggregation, surface morphology, surface area, and porosity of the NPs.

Electron microscopy (EM)

Scanning electron microscopy (SEM), scanning tunneling microscopy (STM), and transmission electron microscopy (TEM) are frequently employed for the analysis of NP size, shape, and surface. SEM provides different information about the NPs such as size, shape, aggregation, and dispersion (Vladar and Hodoroaba,2020). Similarly, TEM provides information about the size, shape, localization, dispersity, and aggregation of NPs in two-dimensional images . STM is based on the phenomenon of quantum tunneling, and is mainly used to characterize the topography of NPs.

1.6.2. Techniques used for the characterization of Structural and chemical features of NPs

The structural characterization of NPs and the study of their composition strongly influence certain parameters on the physicochemical properties. The following techniques are commonly used for the analysis of NP composition, phase, crystallinity, functionalization, chemical state (oxidation), surface charge, polarity, bonding, and electrochemical properties.

X-ray diffraction analysis (XRD)

This technique is based on irradiating a material with incident X-rays and then measuring the intensities and scattering angles of the X-rays that leave the material (Epp,2016). This technique is widely used for the analysis of NP phase and crystallinity.

Energy-dispersive X-ray spectroscopy (EDX)

This technique is based on the irradiation of the sample with an electron beam. Electrons of the electron beam when incident on the sample surface eject inner shell electrons, the transition of outer shell electrons to fill up the vacancy in the inner shell produces X-rays. Each element produces a characteristic X-ray emission pattern due to its unique atomic structure, and therefore can be used to perform compositional analysis (Groarke *et al*,2021)

Fourier-transform infrared spectroscopy (FTIR)

This technique is based on irradiating a material with infrared light, where the absorbed or transmitted radiation is recorded. The resulting spectrum represents a unique fingerprint of samples, where information about the nature of the sample can be obtained such as the bonds involved, polarity, and oxidation state of the sample (Deepty *et al*,2019). This technique is mainly used for the characterization of organic materials such as the surface chemical composition or functionalization of NPs. It is also used for the identification of contaminants when high purity is sought (Chevali and Kandare,2016)

1.6.3. Characterization of optical properties

These techniques give information about the absorption, reflectance, fluorescence, luminescence, electronic state, bandgap, photoactivity, and electrical conductance properties of NPs.

Ultraviolet–visible spectroscopy (UV–vis)

UV–vis spectroscopy uses visible and UV light to measure the absorption or reflectance of a sample. It is a is a simple and common technique that is used for the characterization of the optical properties of NPs.

1.7. APPLICATIONS OF NANOPARTICLES

Nanotechnology and nanoparticles based products and applications have increased now a days due to biological effectiveness. Nano-particle usually forms the core of nano-biomaterial. The core particle is often protected by several monolayer of inert material and can be used as a convenient surface for molecular assembly, and may be composed of inorganic or polymeric materials. More often an additional layer of linker molecule is required to proceed with further functionalization. The linear linker molecule has reactive group at both the ends. One group is aimed at attaching the linker to the Nanoparticle surface and the other is used to bind various moieties like biocompatible (dextran), antibodies, fluorophores etc., depending on the function required by the application.

Bio-nanotechnology is the use of biomolecules for applications in nanotechnology, including use of viruses and lipid assemblies (Mashaghi *et al*, 2013). Nanoparticles made of semi conducting material may also be labelled quantum dots if they are small enough (typically sub 10nm) so that quantization of electronic energy level occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents. A prototype Nanoparticle of semi-solid nature is the liposome. Various type of liposome nanoparticles are currently used clinically as delivery systems for anti-cancer drugs and vaccines.

Recent application of nano materials includes a range of biomedical applications, such as tissue engineering and biosensors (Cho *et al*, 2015). Researchers are attempted to mimic the nano scale feature of cells microenvironment to direct its differentiation down a suitable lineage (Cassidy, 2014). Few examples of application of metallic nano particles are listed below:

Targeted drug delivery is an important potential application of NPs. ZnO and Fe3O4 NPs were efficiently used for targeted drug delivery and selective destruction of tumor cells (Rasmussen *et al*,2010). Gold nanoparticles with a phthalocyanine- antibody complex proved to be a promising way of targeting and killing breast cancer cells (Husseiny *et al,* 2007). NPs have been also been successfully used in different medical applications such as cellular imaging, or in biosensors for DNA, carbohydrates, proteins, and heavy metal ions, determination of blood glucose levels (Saha *et al,*2012), etc.

Silver nanoparticle has antibacterial and therapeutic potential. Silver nano particle is used in similar way as gold for targeting cancer cell (Santos *et al*, 2014). They have also sparked interest in other biomedical uses as well due to their high versatility in different areas of research. They have powerful antimicrobial effects (Zain *et al*, 2014). They also have ability to create reactive oxygen sp. which cause irreversible damage to bacteria and also have a strong affinity in binding to DNA or RNA which interferes with microbial replication process (Santos *et al*, 2014).Copper nanoparticle is also used as antibacterial agent. Cu and Ag nanoparticle fused together creates metallic nanoparticle with increased anti- bacterial effects (Zain *et al*, 2014). For instance, Au NPs were conjugated with SARS-CoV-2 antigens to rapidly detect the presence of SARS-CoV-2 IgM/IgA antibodies in blood samples within 10–15 min . Platinum nanoparticles possess superior catalytic activity (Chen *et al*, 2009).

2.REVIEW OF LITERATURE

Copper (Cu) of block D and period 4 of the periodic table is a microelement required for the development of plant. It exists as Cu^{2+} and Cu^{+} under physiological condition. Concentration required for normal development of plant is from 10-14 to 10-16 M (Harold *et al*, 2007) below which deficiency occurs. However higher concentration than optimum showed toxicity in uptake of nutrients. It acts as a structural element in protein regulation, participates in photosynthetic electron transport, mitochondrial respiration, cell wall metabolism, hormone signalling, oxidative stress response, cofactor for many enzymatic reactions carried out by enzymes such as polyphenol oxidase, amino oxidase, plastocyanin, laccase, super oxide dismutase. At the cellular level, it plays an important role in oxidative phosphorylation, signal trafficking machinery and ion mobilization. Cu can disrupt plant growth and development by interfering with key physiological processes in either deficiency or excess. The Cu deficiency in plants is expressed as curled leaves; petioles bent downwards and light chlorosis along with permanent loss of turgor in the young leaves. Chronic Cu deficiency develops a rosette form of growth. Diagnosis of Cu deficiency in plants is an important as it results in yield losses, with little evidence of the characteristic symptoms (Jian *et al*, 2006). Cu deficiency may become more prevalent in coming future. Higher concentration of Cu leads to toxicity, growth inhibition, photosynthesis interferences, photo respiration and increases oxidative stress. Photosynthetic electron transport is disrupted under both Cu deficiency and excess Cu conditions. In order to maintain healthy plant growth and development, Cu must be extracted from the soil, transported throughout the plant, distributed, and compartmentalized in various tissues, and its levels in various cells and organelles must be meticulously controlled (Yruela,2009).

2.1. COPPER NANO PARTICLES

Amongst other metals, copper nano particles are of great interest due to its low cost and easy availability with the property like other metal nanoparticles. Synthesis of nanostructure copper particles is studied with different methods such as, chemical reduction in aqueous solution, colloidal synthesis with reduction and extraction steps, evaporation and condensation of metal vapour on a cold surface etc. The synthesis of stable, mono disperses and a uniformly shaped copper nanoparticle has proven difficult because of the tendency of copper to rapidly oxidize (Mott *et al*, 2007). Therefore, it needs capping agent or stabilizing agent to prevent the agglomeration and oxidation of Cu-NPs to occur. Nanoparticles of copper and its alloys have been applied more often as catalyst due to their high surface to volume ratio and less cost compared to noble metals. Copper is a promising alternative material due to its high conductivity and lower cost. Copper and copper-based compounds are efficient biocidal properties, which are generally used in pesticidal formulations (Borkow and Gabbay, 2010) and several health-related applications.

2.2. PLANT BASED GREEN SYNTHESIS OF COPPER NANOPARTICLES

Even though conventional method could produce nanoparticles in high production rate, however, due to the high cost and complicated process, green synthesis is much more preferred nowadays. It is a fast reaction, easy to control method and less waste production (Yew *et al*,2018). In particular, as the production of nanoparticles using green synthesis started from the metal atom and forming clusters to produce nanoparticles, so this method is classified under bottom- up approach. The synthesis of nanoparticles using plants offers advantages, such as the utilization of safer solvents, decreased use of dangerous reagents, milder response conditions, feasibility, and their adaptability in use for medicinal, surgical, and pharmaceutical applications (Chandran *et al*., 2006). The use of plants for nanoparticle synthesis offers a wide range of benefits over other biological synthesis methods because it does not require the maintenance of cell cultures and incorporates support for the large-scale synthesis of nanoparticles. Extracellular nanoparticle synthesis, which utilizes extracts from individual, leaves rather than entire plants, may prove to be more inexpensive due to easier downstream processing.

Plant crude extracts contain secondary metabolites such as phenolic acid, flavonoids, alkaloids, and terpenoids, which are mainly responsible for the reduction of ionic metal into bulk metallic nanoparticles (Raveendran, 2003).Primary and secondary metabolites are constantly involved in redox reactions required to synthesize eco-friendly nanoparticles. Biosynthesis reactions can be modulated to transform the shape and size of nanoparticles by using different metal concentrations and amounts of plant extract in the reaction medium. The source of the plant extract is known to influence the characteristics of the nanoparticles (Kumar and Yadav, 2009). This is because different extracts contain different concentrations and combinations of organic reducing agents (Mukunthan and Balaji, 2012).

In the production of nanoparticles from the plant extracts, the plant extract is simply mixed with a solution of metal salt at room temperatures. The reaction is completed within few minutes and, as a result of biochemical reduction, the metals are converted from their mono or divalent oxidation states to zero-valent states. This marks the formation of nanoparticles, which is physically indicated through the colour change observed in the culture medium vessel. Synthesis of copper, gold, silver, and a number of other metal-based nanoparticles have been reported in this manner (Safaepour *et al*, 2009).

Copper being a transition metal, have applications in magnetic storage media, solar energy transformation, electronics, gas sensors and catalysis (Ahmad *et al*, 2011). Water-soluble cupric oxide nanoparticles and Copper oxide nanoparticles are stable over a wide range of pH and temperature. This excellent stability in the form of aqueous colloidal suspensions makes the application of the water-soluble Copper oxide nanoparticles easier in aqueous systems. Various studies showed that plant-mediated CuO NPs are relatively safe on normal human cell lines with different particle sizes; 32 nm, 10–30 nm (Hemmati, *et al*,2020) and 20–50 nm (Sulaiman *et al*,2018). It was reported that the human body could already digest Cu NPs (Crisan *et al*,2021).

Plant-derived metabolites have been utilised for green synthesis of metallic or metallic oxide nanoparticles (NPs). These metabolites play role as both reducing agents and stabilisers in the process of NPs synthesis. Many experiments have been dedicated to develop efficient green synthesis techniques for producing metal NPs. Using metabolites helps in producing simple, profitable, and cost-effective strategy to synthesise metal NPs. Among these, flavonoids and phenolic acids have been extensively under attention for their applications in nanomedicine. Very recently, Sathishkumar et al. has reviewed the roles of flavonoids as mediators in metal NPs synthesis. As a phytochemical for metal NPs synthesis, phenolic acids are antioxidants that represent many health benefits.

Ascorbic acid (Vitamin C): Vitamin C is a water-soluble vitamin found in citrus and other fruits and vegetables, Synthesis of iron nanoparticles using ascorbic acid (Vitamin C) have been studied by Nadagouda *et a*l. (2007}. Core-shell Fe and Cu nanoparticles have been produced by using aqueous ascorbic acid (Vitamin C) which reduced the transition metal salts into their respective nanostructures. Likewise Savasari *et al*. (2015) used ascorbic acid to produce stabilised zero valent iron nanoparticles assembled in a chain in which individual particles were round in shape with a diameter of 20 to 75 nm. Moreover, ascorbic acid has been used as functionalizing and stabilizing agent for the synthesis of nanoparticles.

Gallic acid **:** It is a trihydroxy benzoic acid with the formula $C_6H_2(OH)_3CO_2H$. Gallic acid (GA) are natural polyphenolic compounds belonging to the family of tannins, a subset of secondary metabolites that stands out due to their biological activity, including anticancer, antioxidant, antimicrobial and antiviral activities. Gallic acid and its derivatives are present in nearly every part of the plant, such as bark, wood, leaf, fruit, root, and seed. Gallic acid also has been used as functionalizing and stabilizing agent for the synthesis of nanoparticles. It has been widely used to synthesise different metal or metal oxide NPs. Gallic acid is a natural antioxidant representing antibacterial, antiviral, and anticancer (Chuang *et al*,2010*)* properties. Gallic acid has been used as either reducing or stabiliser agent for synthesising gold, silver, and selenium NPs.

2.3. AGRONOMICAL EFFECT OF COPPER NANOPARTICLES

Metallic NPs offer a wide range of applications and have much potential in nanotechnology. Consequently, it is essential to understand these NPs' environmental fate and long-term impacts. Plants can absorb metal NPs in different ways. There are two ways to enter the plant's system: nanoparticles (NPs) or oxidized metal ions in soil solution, which are then reduced in the plant system (Perea Vélez *et al*,2021). The hydrophobicity or hydrophilicity of NPs affects their interaction with plant cell membranes. Hydrophilic NPs tend to adsorb on bilayer membrane surfaces, allowing them to bind to intracellular vesicles, whereas hydrophobic nanomaterials can implant into the membrane's hydrophobic core without causing harm or leakage (Kurczynska *et al*,2021).

The propensity of the nanoparticles to cross cell barriers and its interactions with intracellular structures owing to its small size and high surface reactivity, contribute to potential phytotoxicity, genotoxicity, as well as cytotoxic effect (Kovacic and Somanathan, 2010). In spite of increasing research on the toxicity of various nanoparticles, limited studies are available in higher plants. Very few nanoparticles and plant species have been studied, mainly at its very early growth stages of the plants and dried biomass. NPs can have both negative and positive effects on the growth and development of a plant, and their influence on plants depends on the size, shape, and properties of both plant species and NPs (Ma *et al* ,2012). Literature survey on the toxicity of Copper nanoparticle available is very less. The toxicity of Copper nanoparticle depends on the combination of several factors such as concentration of nanoparticles, pH, temperature and aeration. The higher temperature, aeration, low pH decreases the agglomeration and provide more surface area for interaction with bacterial membranes for solubilization of copper ions that leads to toxicity. Lee *et al*. (2008) analyzed toxicity and bioavailability of Copper nanoparticle of the plants *Phaseolus radiates* (mung bean) and *Triticuma estivum* (Wheat). The bioavailability of copper was found to be 8 mg/kg for mung bean and 32 mg/kg for wheat plants treated with 1000 mg copper nano particle per Litre. Copper nanoparticles tested on *Cucurbita pepo and zucchini* plants showed the reduced emerging root length (Stampoulis *et al*, 2009). In most cases, Copper toxicity considerably reduces the uptake of Iron by tomato plant, thereby imposing visible symptoms similar to those of Fe deficiency. Exposure to 0.1 to 0.4 g/L CuO nanoparticles for 48h, induced strong inhibition of photosynthetic processes resulting in a decrease of plant growth in *Lemnagibba* has also been reported. The radishes and the two ryegrasses exposed to Copper nanoparticle and large sized cupric oxide particles (100nm) showed many DNA lesions (Atha *et al*, 2012). The cellular uptake of Copper nanoparticle was significantly greater than the larger particles when plants were treated with nanoparticles. The cellular uptake resulted in difference in DNA damage profiles of ryegrasses from the radish, indicating the damage is dependent on the plant species and on the nanoparticle concentration. Copper nanoparticle, having a core of Copper nanoparticle and a shell of polyacrylic acid (PAA), were found to be very toxic to unicellular algae, causing inhibition of PSII electron transport capacity in *Chlamydomonas reinhardtii* cultures (Saison *et al,*2009).

2.4. ANTI OXIDANT EFFECT OF NANO PARTICLES

Free radicals play a key role in oxygen dependent (aerobic) living systems. They are part of cell respiration and other important cellular processes but are also involved in aging and disease development (Harrman,1992). Free radicals are highly reactive species having unpaired electrons in their outermost shell. They are highly reactive because the free electrons always attempt to bond with other electrons and form covalent pairs. In this process, the free radicals remove the electrons from other molecules. Beyond affecting regulation of cells, it can also damage molecules such as carbohydrates, fats, proteins and nucleic acids (Valko *et al*,2007). In aerobic organisms, free radicals are produced during normal metabolic processes. They react with the polyunsaturated lipids (lipid peroxidation) of cell membranes along with the normal RNA and DNA, the genetic material of the cell. In order to prevent intracellular damage by free radicals, cells have built up intracellular antioxidant system. This process converts free electrons into a non-reactive form by proteins. An effective antioxidant complex has various types of radical catching antioxidant sites that seek and destroy free radicals at many cellular sites. Antioxidants control oxidative reactions by inhibiting, delaying or hindering the oxidation of the biomolecules (Kumar,2011). The key antioxidant enzymes possess certain elements that shield and protect proteins (Trombino *et al*,2004). Non enzymatic antioxidants can also neutralize radicals for example water soluble substances such as Vitamin C, glutathione or fat-soluble substances such as Vitamin E, β-carotene (Trombino *et al*,2004).

Thus, the search for effective, non-toxic, natural compounds with antioxidative activity has been increased in recent years. the strong antioxidant property exhibited by some nanomaterials is opening exciting potential to develop new regimens with enhanced and targeted actions. Studies on the potential effects and benefits of NPs in diseases involving oxidative stress are receiving growing attention. Cardiovascular risk factors such as hypercholesterolemia or hypertension promote the generation of reactive oxygen species (ROS), which leads to the oxidative stress seen in inflammatory diseases such as atherosclerosis. Therefore, the maintenance and optimization of antioxidant defences can minimize side effects. In this sense, nanoparticles are of great interest, because of their antioxidant properties and easy internalization by the cells For example, gold, silver and selenium nanoparticles have been shown to possess ability to reduce oxidative stress due to their efficient redox-active radical-scavenging properties (Sood and Chopra,2017). Liposomes are also used for delivering the antioxidant agents to the target site. The amphiphilic and biocompatible nature of these liposomes allows them to load both hydrophilic and lipophilic compounds and favour the encapsulation of the water-soluble and water-insoluble antioxidant enzymes. Pu et al. (2014) reported the encapsulation of curcumin antioxidant compounds within the nanocarrier and regulation of release of antioxidant compounds, by changing the pH and oxidative stress of inflamed tissues to increase the overproduction of RNS/ROS synthesized by lipopolysaccharide (LPS)-stimulated macrophage (Du *et al*,2014).

Among different antioxidant metal oxide nanoparticles, CuO and Cu2O NPs have attracted significant attention due to their low cost, abundant availability of copper salt, and high antioxidant activity of CuO and Cu2O NPs. Antioxidant properties of CuNPs depend on their nature, polymorphism, crystal structure, chemical composition, surface charge, particle size, surface-to-volume ratio, surface coating, and dispersion state (Vanathi *et al*,2016). Rajeshkumar and his colleagues (2019), reported a radical scavenging property of copper nanoparticles synthesized using leaf extract of *Cissus arnotiana*, when compared with the standard ascorbic acid.

2.5. SIGNIFICANCE OF THE STUDY

Applying bioactive natural compound instead of whole or part of plant extract is a new approach for green synthesis of metal NPs .The diversity of these natural compound, alongside their metal ions bio reduction capability, represent these phytochemicals as an ideal choice for preparation of biocompatible metal NPs with numerous biomedical applications. Many phyto compounds have also been investigated as either reducers or stabilisers for metal NPs synthesis. However, limited studies have been dedicated to the synthesis and characterisation of NPs produced by these compounds.

3.AIMS AND OBJECTIVES

3.1. Aim of the study

The aim of the present study was to synthesis copper nanoparticle using plant metabolites such as Ascorbic acid and gallic acid, followed by UV/Visible characterization, anti-oxidant assay and seed germination studies.

3.2. Objectives of the study

- Synthesis of copper nanoparticles using Ascorbic acid and gallic acid.
- Initial characterization of copper nanoparticles by UV-Vis spectrophotometer.
- Screen the antioxidant activity of the copper nanoparticles.
- Study of the effect of synthesised copper nanoparticles on the germination of green gram.

4.MATERIALS AND METHODS

4.1. SYNTHESIS OF COPPER NANO PARTICLES

Ascorbic acid and Gallic acid work both as reducing and protecting agent, which makes the process economical, nontoxic and environment friendly.

A 500 mL of 0.01 millimolar Copper (II) Sulphate solution was prepared by dissolving copper salt in de-ionized water. Solutions of 0.5M L-Ascorbic Acid (AR grade) and Gallic acid (AR grade) were also prepared in de-ionized water. Ascorbic acid/Gallic acid was introduced drop wise into the Copper sulphate solution in three different ratios; 1. Copper Sulphate: Ascorbic acid/Gallic acid (1:1) ,2. Copper Sulphate :Ascorbic acid/Gallic acid (2:1) ,3. Copper Sulphate :Ascorbic acid/Gallic acid (1:2). These mixtures were heated continuously at 60^{o}C in a water bath for 30 min. Within the particular time, the change in the colour of the reactants or the appearance of a colloidal nature in the solution indicates the formation of copper nanoparticle.

4.2. CHARACTERIZATION OF SYNTHESISED COPPER NANO PARTICLES

a) **Visual characterization:** A change in the colour to light orange, or red indicated the formation of copper nano particles, which can be identified visually.

b) UV-Vis Spectra Analysis:

The reduction of pure Cu to nanoparticle was monitored by measuring the UV-Vis Spectrum, the most confirmatory tool for the detection of surface Plasmon resonance property (SPR) of Copper nanoparticles. UV-Vis spectral analysis was done by using UV-Vis spectrophotometer Systronics 118 at the range of 300-600nm.

4.3. ASSAY OF ANTIOXIDANT ACTIVITY

a. DPPH Radical scavenging activity

The DPPH free radical scavenging assay was carried out for the evaluation of the antioxidant activity. This assay measures the free radical scavenging capacity of the investigated extract. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour typical for free DPPH radical decays and the absorbance change is measured at ʎ=517 nm. The antiradical activity of the plant extract was examined based on the scavenging effect of the stable DPPH free radical activity.

The reaction mixture consists of 3 ml each of nano suspension and 1 ml of methanol solution of DPPH [0.1 mmol/l]. The mixture was kept in the dark at a room temperature for 30 minutes and the absorbance was measured at 517 nm against a blank. The following equation was used to determine the percentage of the radical scavenging activity of each extract.

Percentage of radical activity = {[OD control-OD sample]/OD control} × 100

4.4. STUDY ON SEED GERMINATION

The effect of nanoparticles on seed germination was carried out by immersing the seeds into 20 ml DM water in dark at 25°C for 4 h, the swelling period. Swollen seeds were then transferred on to double layer of sterilized filter papers in a petri dish, with 6 seeds per dish and 1 cm or larger distance between each seed (Kikui *et al*, 2005). Seeds were then soaked with different concentrations of nanoparticle suspension. The covers of the petri dishes were closed and they were incubated at 25°C for 5 days. DM water and 10mM Copper sulphate solution were used as control. The germination rate was recorded every 24 h. A 1-mm radicle emergence from seeds was considered seed germination. Each test was carried out in three replicates (Yang and Watts, 2005).

The parameters adopted to evaluate the conditions of seed germination are the following:

$$\text{Relative Shoot elongation} = \frac{\text{Mean Shoot length in test sample} \times 100}{\text{Mean Shoot length in control}}$$

$$\text{Relative Root elongation} = \frac{\text{Mean Root length in test sample} \times 100}{\text{Mean Root length in control}}$$

$$\text{Relative germination rate} = \frac{\text{Seeds germinated in test sample} \times 100}{\text{Seed germinated in control}}$$

$$\text{Germination Index} = \frac{\text{Relative germination rate} \times \text{Relative Root elongation}}{100}$$

5. RESULTS AND DISCUSSION

5.1. CHARACTERIZATION OF BIOSYNTHESIZED COPPER NANO PARTICLES

5.1.1. Visual Characterization

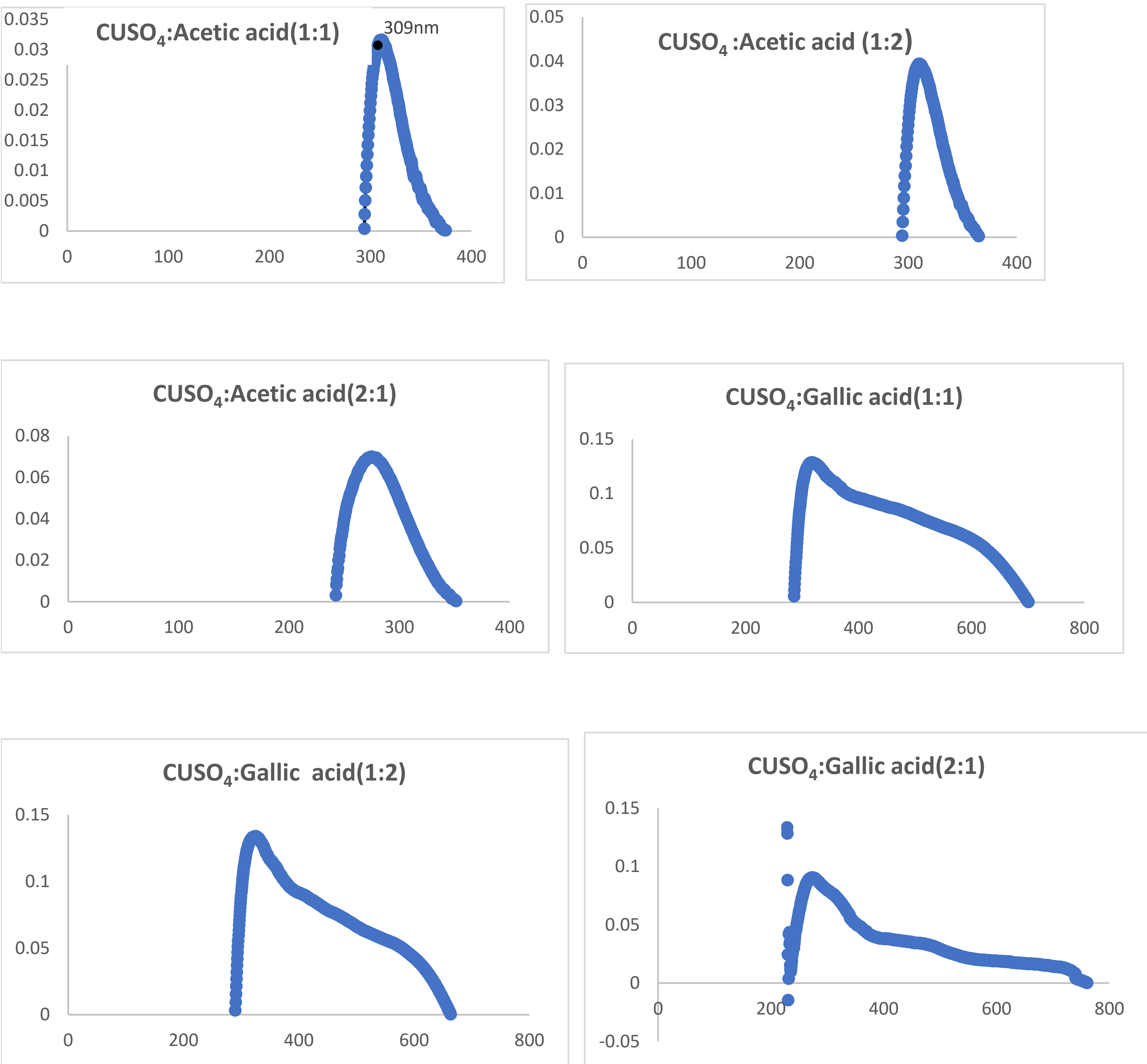
During the biosynthesis, formation of nanoparticles were indicated by the change in olour of the mixture into light orange and red within half an hour of the reaction.

5.2.2. UV/Visible Spectrophotometer Analysis

UV-visible spectra were recorded using a Shimadzu UV-Vis1800 Spectrophotometer, (Research & PG Dept. Physics, Bishop Moore College, Mavelikara) for the confirmation of nanoparticle formation. The characterization of copper nano particles (CuNps) by UV/VIS spectrophotometer was done in the range 200-700 nm. It is the most important method to detect the Surface Plasmon Resonance property of CuNPs. The results obtained from UV-Visible spectroscopy analysis of the colloidal suspensions are presented in Fig 1. The UV-Visible absorbance spectroscopy of the synthesized CuNps showed peak at around 270-320nm (Fig 1).

Visual characterization is the preliminary step for the identification of nano particles. The colour change indicates the reduction of copper sulphate and formation of copper nanoparticles. UV/VIS spectroscopic characterization is the technique to confirm the presence of nano particles. In the present study the UV-Visible absorbance spectroscopy of the synthesized copper nano particle showed peak at around 270-320nm. This may due to the formation of CuO (Copper Oxide) nano particles which shows a characteristic peak near 220-280nm range. However, it is known that, the plasmon resonance peak of nano sized copper particles are observed at approximately 580 nm (Mallick *et al*, 2011). The exact position of the band may shift depending on the individual particle properties including size, shape, solvent used and capping agent employed (Mott *et al*,2007).

Fig.1 : UV Spectra of Copper nanoparticles



5.2. ANTI OXIDANT ACTIVITY OF SYNTHESISED NANOPARTICLES

- **DPPH radical scavenging assay**

Table1: % Radical scavenging activity of biosynthesized nanoparticles using Copper Sulphate and Ascorbic acid

Samples	500 µg	250 µg
1:1	47	20.2
2:1	11.33	5.62
1:2	35.8	12.5

Table 2: % Radical scavenging activity of biosynthesized nanoparticles using Copper Sulphate and Gallic acid

Samples	500 µg	250 µg
1:1	28.56	20.9
2:1	42.4	18.47
1:2	46.51	15.75

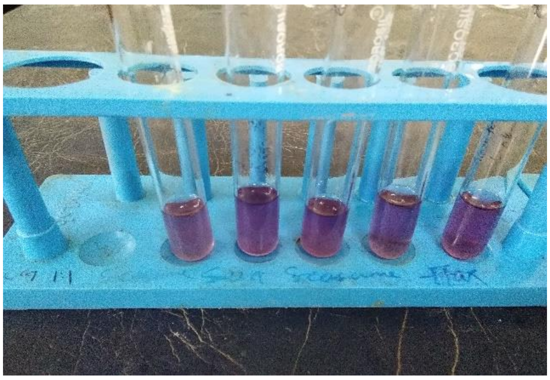
The DPPH radical scavenging activity of the different concentrations of synthesized nanoparticles were presented in Table 1& 2. All the samples exhibited radical scavenging activity to an extent depending on the concentrations. Among ascorbic acid treated samples, (1:1) exhibited highest and (2:1) exhibited lowest activity. In the case of gallic acid treated nanosuspension (2:1) and (1:2) exhibited similar activity.

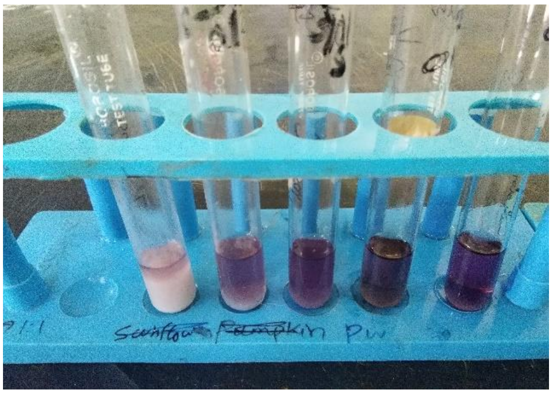
Molecules that are capable of preventing the oxidation of other molecules are known as antioxidants. The role of these molecules is very important in the treatment of various diseases. The basic function of these molecules is to help in preventing the oxidative stresses and to help in protecting the cells by scavenging the free radicals (Mohamed Imran, et al., 2011).

DPPH is stable free radical at room temperature and accepts an electron / hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 5l7 nm, induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the anti oxidative activity.

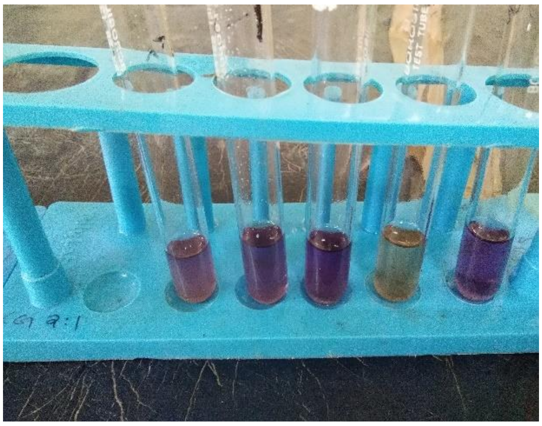
Plate 1 : Antioxidant activity of synthesized nanoparticles

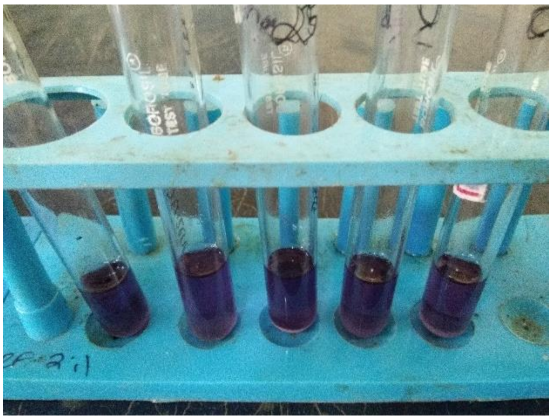
Copper: gallic acid **copper: ascorbic acid**



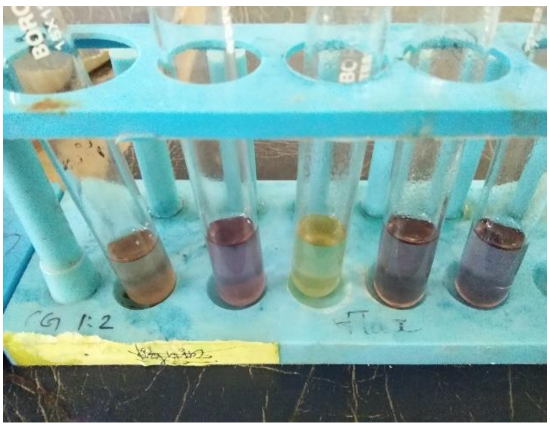


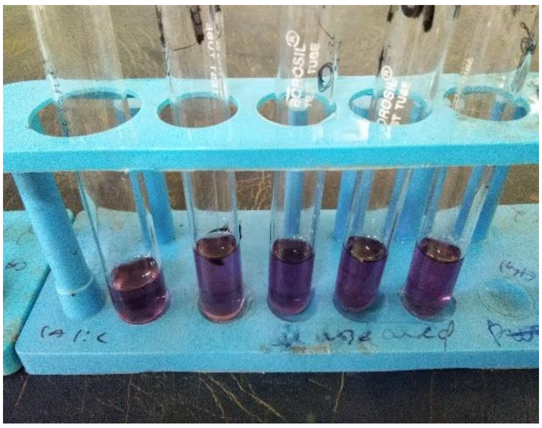
1:1 **1:1**





2:1 **2:1**





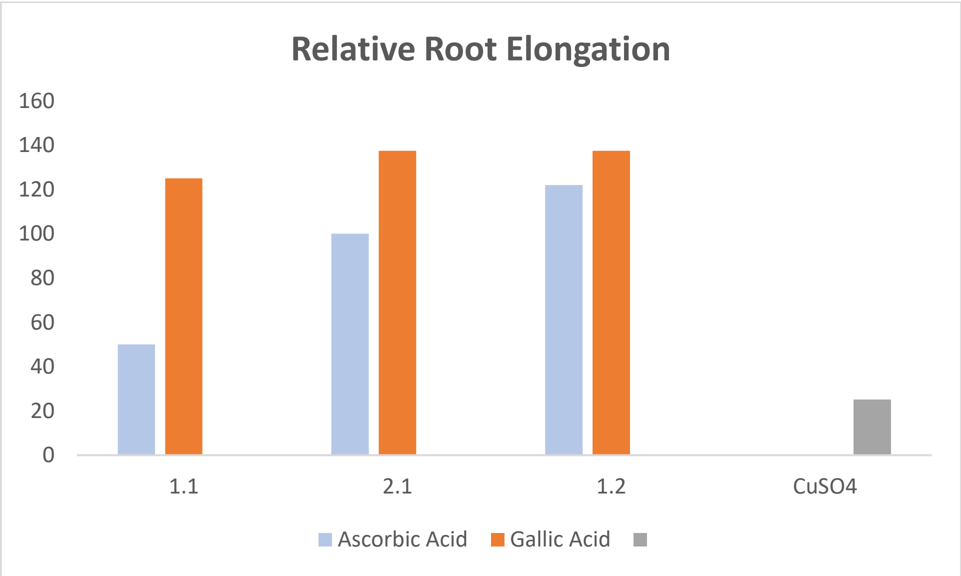
1:2 **1:2**

5.3. SEED GERMINATION STUDIES

The effect of copper nanoparticles on seed germination was studied by using green gram. Relative root and shoot elongation, relative germination rate and germination index were calculated. The seeds were treated using different nano suspensions. The control used was DM water. All treatments led to successful germination of green gram.

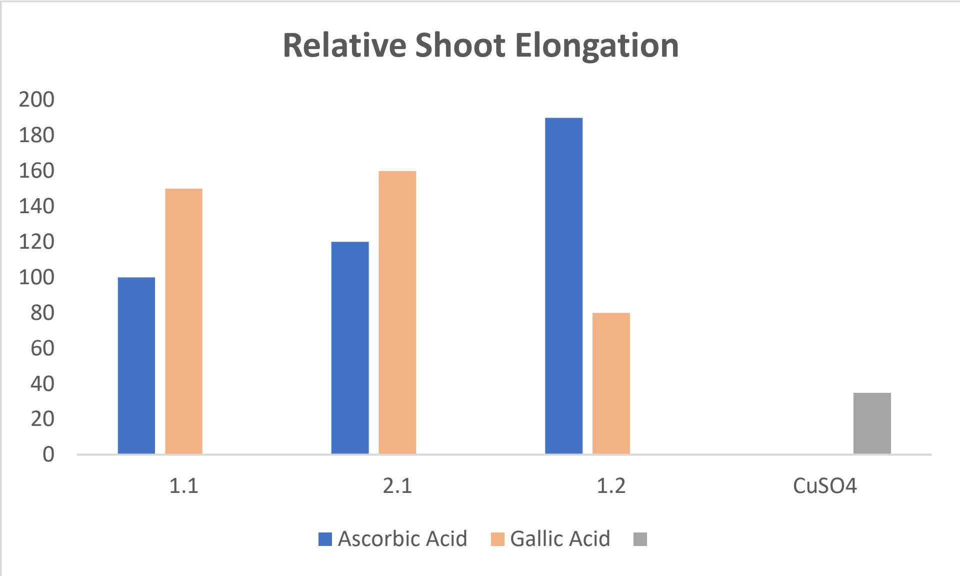
The phytotoxicity of nanoparticles was evaluated by the seed germination technique. Seed germination and root elongation are rapid and widely used acute phytotoxicity test for unstable chemicals or samples (Wang *et al*, 2001). In the present investigation, seeds showing emergence of radical or cotyledon coming out of the seed coat were recorded as being germinated.

Fig 2: Effect of copper nanosuspensions on relative root elongation in Green gram.



The effect of nanoparticles on root and shoot elongations were presented in Figure 2&3. From the study, it was observed that maximum root elongation obtained for 1:2 ratio for both ascorbic acid and gallic acid samples. Highest shoot elongation was also attained by 1:2 ratio, for both samples. In both cases nanoparticle treated samples exhibited more relative elongation than CuSO4 treated samples.

Fig 3 : Effect of copper nanosuspensions on relative shoot elongation in Green gram



The relative germination rate of green gram on nanoparticle treatment was obtained 100, indicating that nanoparticle treatment did not affect overall growth of the seed.

Table 3: Effect of copper nano suspensions on Germination Index in Green gram

Treatment	**Germination Index**	**Nano suspension**
1:1	50	Copper/ Ascorbic Acid
2:1	100	
1:2	122	
1:1	125	Copper/ Gallic Acid
2:1	137.5	
1:2	137.5	
CuSO4	35	

The phytotoxicity of nanoparticles was evaluated by the seed germination technique. Visible consequences caused by Copper Nanoparticle exposure include prolonged root and shoot development, but rate of germination and growth showed a positive effect. the treatment with Copper nanoparticles (CuNPs) showed a significantly high germination rate and seedling growth compared to untreated seeds. But the overall germination of Green gram was not affected by the Copper nanoparticles. Phytotoxicity in higher plants should be investigated in order to develop a comprehensive toxicity profile for nano particles (Munzuroglu and Geckil, 2002). They reported that, Copper nanoparticles show positive effect on germination, but are phytotoxic at seedling stage. Exposure of plants to elevated levels of Cu-NPs increases bioavailability. Consequently, massive accumulation of nanoparticles in roots and shoots occurs leading to phytotoxicity. Hence, increase in copper nanoparticle concentration may affect the shoot and root growth as well as germination rate.

In this study, there were variation in root and shoot length and germination index among the nano suspensions. This may be due to the toxicity in plants which are influenced by several factors like concentration of nano particles, particle size and specific surface area, physiochemical properties of nano particles, plant species, plant age/life cycle stage, NP stability, and diluting agents (Munzuroglu and Geckil, 2002).

Plate 2: Seed germination

control 1:1 2:1 1:2

Shoot elongation

Development of leaf

6. SUMMARY AND CONCLUSION

Nanotechnology plays a very important role in modern research. Nanoparticle's research is currently an area of intense scientific interest due to wide variety of potential applications in bio medical, optical, pharmaceutical and chemical fields. Nanoparticles can be produced by many methods of which green synthesis is gaining popularity, because it is cost effective, eco-friendly and less toxic compared to other methods. Major studies are been carried out of producing copper nanoparticles due to low cost of copper from other metals.

The present study focused on the synthesis of copper nanoparticles using plant metabolites such as Ascorbic acids and Gallic acid. The presence of copper nanoparticles was first indicated by colour change in the mixture, followed by UV/VIS spectroscopy. The UV/VIS analysis confirms the presence of copper nano particle as a result of the characteristic absorption peak at 270 -320 nm. This may due to the formation of Copper Oxide (CUO)nanoparticles.

The study also shown that the percentage of scavenging effect of CuNps on the DPPH radical was increased with the increase in the concentration of CuNps from 250 to 500 µg. All the samples exhibited radical scavenging activity except 2:1 sample, for both nano suspensions and 1:1 combination of both nano suspensions exhibited the highest activity. The seed germination assay designated that all the treatments showed germination, indicating that copper nanoparticles have less toxicity on germination. Root elongation and shoot elongation rate varied in different nanoparticles treatment. Maximum root elongation and shoot elongation obtained for 1:2 ratio for both ascorbic acid and gallic acid treated samples.

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