

**GREEN SYNTHESIS OF COPPER NANOPARTICLES USING VERNONIA  
AMYGDALINA LEAF EXTRACT AND ITS ANTIMICROBIAL ACTIVITY**

**BY**

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## **CERTIFICATION**

This is to certify that this work was done by CHIZOBA BLESSING UMUNNA in the Department of Pharmaceutics and Pharmaceutical technology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria in partial fulfilment of the requirement for the award of the Doctor of Pharmacy degree (PHARM. D)

## **DEDICATION**

This work is dedicated to God almighty for the grace and unending love throughout my stay in this great citadel of learning.

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All thanks to God Almighty for His grace, mercy, love, and all his blessings throughout my years in this great university, University of Benin. It has been Him all along. My profound gratitude goes to my parents Mr. and Mrs. Chuka Umunna for their fervent prayers, support and love. Both of you will live long in good health to reap the fruits of your labours.

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## ABSTRACT

Pharmaceutical nanotechnology deals with the formation and development of substances of molecules and atoms ranging from size 0.1 – 100nm which can be further developed into special structures and devices with desired characteristics. This study employed green chemistry to synthesize copper nanoparticles (CuNPs) using fresh leaf extract of *Vernonia amygdalina*. The resultant CuNPs were investigated by UV-Vis spectroscopy. Fresh leaves were collected and identified, aqueous extraction was then carried out on the washed leaves using water at 60°C. The existence of flavonoids, tannins, glycosides and alkaloids in the plant extract acts as reducing, stabilizing and capping agents that reduce the metal ion to form metal nanomaterials. The UV-Vis absorption spectra show maximum absorption at 543nm. The synthesized nanoparticles (NPs) were tested for their in vitro antimicrobial activity against the bacterial strain *Staphylococcus aureus* using the agar well diffusion method and compared with the following test samples (copper salt solution, plant extract alone, mixture of copper salt solution and plant extract). From the result of the antimicrobial evaluation of the copper nanoparticles, the copper nanoparticles showed the highest zone of inhibition when compared to the other test samples. This is possible due to the nano-dimension of nanoparticles which increases the surface area of the particles, hence leading to increased antimicrobial activity.

The result proves that fresh leaves of *Vernonia amygdalina* could be used for the synthesis of copper nanoparticles using copper sulphate source as a source of copper and that the green synthesized copper nanoparticles have potent biological activity.

## CHAPTER ONE

### INTRODUCTION

Nanotechnology has emerged as a large multidisciplinary topic of study all over the globe for a few decades. The design, characterization, and use of nanoparticles have attracted interest owing to their particular physical and chemical characteristics (Arokiyaraj S, 2014).

Generally, nanoparticles are very tiny particles with sizes ranging from 0.1 to 100nm and display unique characteristics compared to those of bulk materials. They have a greater surface area to volume ratio, which introduces the variety in the other particular criteria like size, distribution, and shape. The greater surface area of the nanoparticle is responsible for their improved catalytic and biological characteristics (Kumar R, 2013).

The nanoparticles of noble metals including gold, silver, copper, and zinc are utilized in industrial and medicinal activities owing to their extraordinary physicochemical, optical, and biological characteristics (Siddiqi KS, 2018).

Pharmaceutical Nanotechnology deals with the synthesis and development of tiny structures like atoms, molecules, or compounds of size 0.1 to 100nm into structures that may be further developed into unique devices with desired traits and qualities (Fadel M, 2015).

The application of nanotechnology in pharmaceuticals aids in the design of more sophisticated drug delivery systems and therefore it is a vital and powerful instrument as an alternative to the traditional dosage form. Pharmaceutical nanotechnology is a specialist topic that will change the fate of the pharmaceutical business shortly. Pharmaceutical nanotechnology helps to fight against numerous illnesses by recognizing the antigen linked with diseases and also by detecting the microbes and viruses causing the diseases (Usta A, 2016).

There is a substantial worldwide evolution of multidrug resistance to bacteria, fungi, etc and this is driven by the rising usage of antibiotics. These bacteria along with other kinds of

microorganisms have developed antibiotic resistance which is a serious worldwide concern. To counteract multidrug resistance to bacteria owing to the increasing usage of antibiotics, nanotechnology has been applied in several ways to overcome multidrug resistance.

Pharmaceutical Nanotechnology has played a vital role to overcome numerous problems with traditional dosage forms such as pills, capsules, etc. The traditional forms faced problems such as inadequate bioavailability, poor patient compliance, damage to healthy cells, etc. which were corrected utilizing pharmaceutical nanotechnology (Olaso L et al., 2016).

### **History of nanotechnology**

Long before the period of nanotechnology, individuals were unwittingly coming across numerous nanosized things and performing nano-level procedures. In ancient Egypt, colouring hair in black was prevalent and was for a long time thought to be based on plant materials such as Henna (Tolochko NK et al., ). However, a new study on hair samples from ancient Egyptian burial sites demonstrated that hair was colored using paste from lime, lead oxide, and water (Walter P et al., 2006). In this dyeing procedure, galenite (lead sulfide, PbS) nanoparticles are generated. The ancient Egyptians were able to make the dyeing paste react with sulfur (part of hair keratin) and form minute PbS nanoparticles which produced even and constant colouring.

American physicist Richard Feynman is considered the father of nanotechnology. He outlined the theories and principles underpinning nanotech in a 1959 address titled 'There's Plenty of Room at the Bottom'. Feynman did not use the phrase 'nanotechnology', but envisioned a process in which scientists will be able to manage and control individual atoms and molecules.

Modern nanotechnology genuinely started in 1981, when the scanning tunneling microscope enabled scientists and engineers to view and control individual atoms. IBM scientists Gerd Binnig and Heinrich Rohrer earned the 1986 Nobel Prize in Physics for creating the scanning tunneling microscope. Modern nanotechnology may be very new, yet nanometer-scale materials have been utilized for decades.

### Pharmaceutical Nano Systems

*Polymeric Nanoparticles:* They have a size range of 10-1000nm and are Biocompatible and biodegradable offering total medication protection. Polymeric nanoparticles have been utilized as carriers for regulated and sustained administration of medications (Parteni O et al 2016).

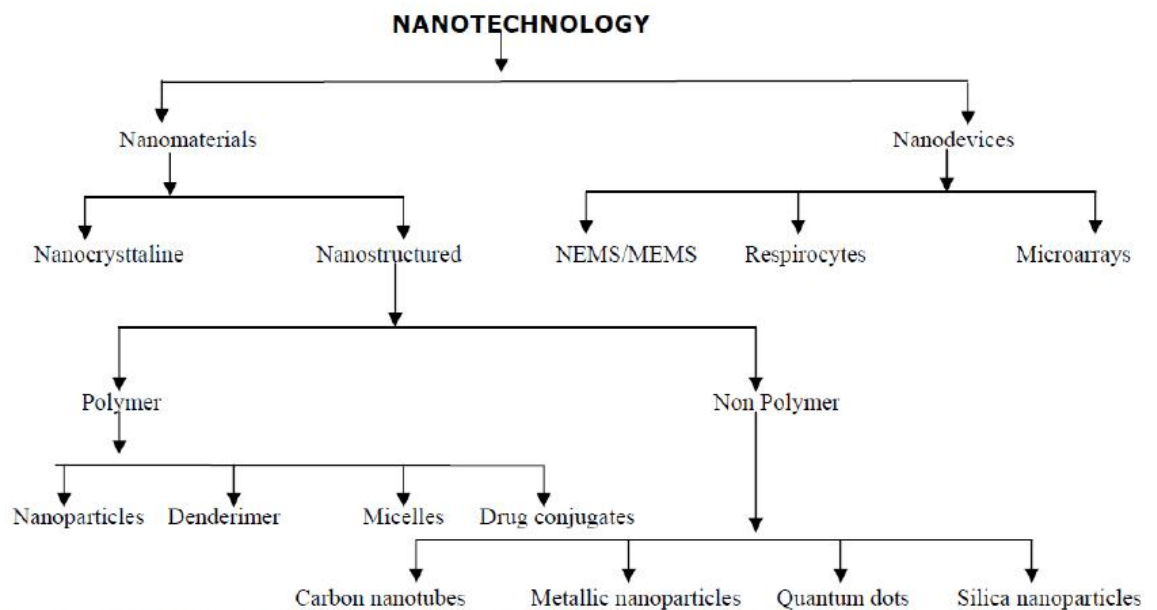
*Dendrimers:* Dendrimers have a size of <10nm and are created through controlled polymerization. These are extremely branching mono-dispersed polymeric structures. Dendrimers are employed for the regulated administration of medications and targeted delivery of pharmaceuticals to macrophages and the liver (Solomon AO, et al 2015).

*Metallic nanoparticles:* Metallic nanoparticles are gold and silver colloids with a size of <100nm. They are extremely tiny in size resulting in greater surface area and have better bioavailability and stability which is the ideal features of medicine. These are employed for medication and gene delivery and are used in sensitive diagnostic tests, thermal ablation, and radiation enhancement (Koteswari P, et al 2015).

*Polymeric micelles*: They have a size range of 10-100nm with strong drug-trapping nature and biostability. These are of significant diagnostic value and are employed for active and passive targeted medication administration (Kaliappan I, et al 2015).

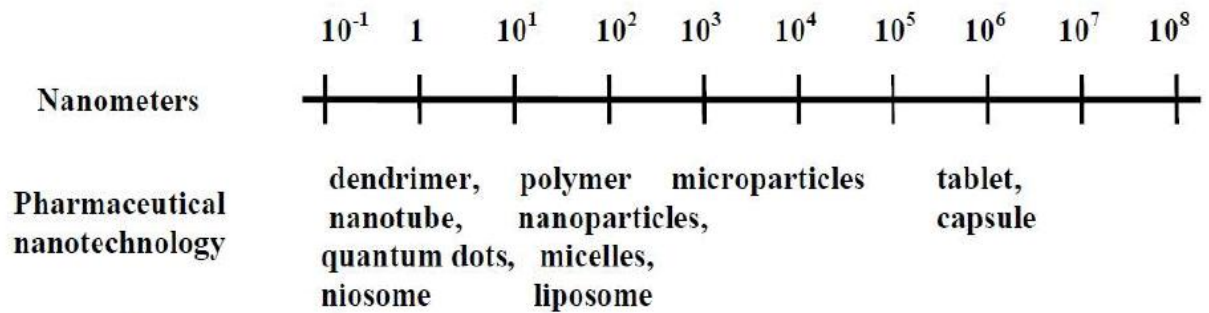
*Liposomes*: These are phospholipid vesicles with a size range of 50 – 100nm with properties of high biocompatibility and entrapment efficiency. These are utilized for the passive and active administration of genes, proteins, and peptides (Panchangam RBS 2015).

The various systems under nanotechnology are shown in Figure 1.1



**Figure 1:** Different systems in nanotechnology.

The various dimensions of particles in Nanotechnology are shown below



**Figure 2:** Particles and their dimensions in nanotechnology.

### Physical Characterization of Nanoparticles

The unique properties define the potential and use of a nanoparticle. The nanoparticle characterization is carried out by numerous measuring methods which include

#### Size

The size is one of the most fundamental and crucial measurements for nanoparticle characterization. It influences the size and dispersion of the particle and whether it falls within the nano or micro scale (K. W. Powers, 2007). The particle size and distribution are most typically studied using an electron microscope. The pictures of the Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) are utilized for the measurement of particles and clusters while laser diffraction techniques are employed for assessing bulk materials in the solid phase.

#### Surface area

The surface area is also a major aspect of nanoparticle characterization. The surface area volume ratio of a nanoparticle has a tremendous impact on its performance and attributes. The surface area is most typically assessed using BET analysis. Firstly, materials made up of

nanoparticles have a relatively higher surface area when compared to the same volume of material made up of bigger particles. With the reduction in the size of nanoparticles, surface area rises which generates a dose-dependent increment in oxidation and DNA damaging capacities of these nanomaterials (L. Risom, 2005) considerably greater than bigger particles with the same mass dosage (K. Donaldson and V. Stone 2013).

### **Composition**

The chemical or elemental composition influences the purity and performance of the nanoparticle. The presence of increased secondary or undesirable components in the nanoparticle may impair its effectiveness and also lead to secondary reactions and contamination in the process. The composition measurement is commonly carried out using X-ray photoelectron spectroscopy (XPS).

### **Surface morphology**

The nanoparticles feature diverse forms and surface structures that play a significant role in utilizing their capabilities. Some of the forms include spherical, flat, cylindrical, tubular, conical, and irregular shapes with a surface like crystalline or amorphous with consistent imperfections on the surface. The surface is often determined using electron microscopy imaging methods like scanning electron microscopy(SEM) and transmission electron microscopy(TEM) (Burrows, 2013).

### **Crystallography**

Crystallography is the study of atoms and molecules' organization in crystal solids. The crystallography of nanoparticles is carried out using a powder X-ray, and electron or neutron diffraction to identify the structural arrangement. It was revealed that nanosilver and nano copper with their soluble forms induced toxicity in all examined species, however, TiO<sub>2</sub> of the same dimensions did not create any toxicity concerns (R. J. Griffitt 2008)

### **Applications of Pharmaceutical Nanotechnology**

Drug delivery systems: Conventional drug delivery systems have many restrictions of lack of specificity, increased rate of drug metabolism, cytotoxicity, high dosage need, poor patient compliance, etc and these may be solved by drug delivery systems designed employing the principles of pharmaceutical nanotechnology. This is performed via encapsulation or adsorption of a medication, a therapeutic foreign gene molecule on its surface, and also coupling of a particular targeting molecule to the surface of the nano-carrier, molecular transport to certain organelles, and other techniques.

### **Diagnosis**

Molecular imaging is the discipline of describing and measuring biological processes in animals which include gene expression, protein-protein interaction, signal transduction, cellular metabolism, and both intracellular and intercellular transport. Nanodevices has found relevance in this area.

### **Drug discovery**

Pharmaceutical nanotechnology plays an important role in drug discovery and development as it helps in improving the characteristics such as solubility, bioavailability, etc of the potent drugs and excipients into pharmaceuticals which helps in the formulation of more advanced drug delivery systems and so it is an important and powerful tool as an alternative to the conventional dosage form.

### **Cosmetics and Sunscreens**

The typical ultraviolet (UV) protection sunscreen lacks long-term stability during use. The sunscreen comprises nanoparticles such as titanium oxide and zinc oxide nanoparticles since they are transparent to visible light as well as absorb and reflect UV rays that made their way to be employed in certain sunscreens. Some lipsticks employ iron oxide nanoparticles as a pigment.

### **Food**

The enhancement in the manufacturing, processing, protection, and packaging of food is done by applying nanotechnology. For example, a nanocomposite coating in a food packaging process might directly introduce anti-microbial chemicals on the coated film surface (Land M and Jatti V K S et al., 2016). One of the instances is the canola oil manufacturing business incorporates nano drops, an ingredient meant to transmit the vitamins and minerals in the meal.

### **Green Nanotechnology: risk assessment and management**

The newness of nanotechnology applications in green fields, coupled with concerns about the possible influence of NMs on the health and safety of employees, urgently needs scientific, technical, and governmental initiatives to actively control hazards for the workforce. This is

to identify real dangers generated from NM exposure in the workplace (risk assessment) to plan implement control measures (risk management) and to explain the strategy.

### **Risk assessment of nanomaterials(NMs)**

Risk assessment of NMs notably comprises the same processes used for the risk assessment of other kinds of chemicals. These are hazard identification; hazard characterization with a focus on establishing important target organs and dose-response relationships; evaluation of exposure for various scenarios and a synthesizing step risk characterization (Savolainen K et al., 2010). Unfortunately, the NM risk assessment procedure currently suffers from a lack of toxicological data on a broad range of NMs. Therefore, future studies should be focused on systematically increasing the knowledge of metrics, such as size, surface area, functionalization, or particle number concentration that may be responsible for NM toxicity.

### **Risk management of nanomaterials**

The ultimate purpose of risk assessment is to offer quantitative forecasts of given hazards allowing their evidence-based management. However, tremendous ambiguity regarding hazards, exposures, and dangers in the nascent green nanotechnology area make it important to adopt a dynamic-precautionary management strategy before all of the data is finished. This implies that risk management techniques and recommendations will be evolving and regularly examined, enhanced, and confirmed as risk information gets more significant (Schulte PA 2013). To successfully manage possible green nanotechnology-associated hazards, a risk management strategy incorporating a hierarchy of controls should be emphasized(Engeman CD, 2013). The first stage in designing such a strategy is to discover

which employees may have possible exposures, assess these exposures and analyze how the exposure may change based on the job activity. Potential worker exposures should be managed using the hierarchy of controls, which starts with eliminating the hazard, adopting green chemistry by substituting with a non-hazardous or less hazardous alternative (such as modifying the molecule if possible), and introducing engineering controls like enclosed systems, local exhaust ventilation, engineering hoods, or pressure differentials (Schulte PA, 2013). Administrative controls, such as training programs through which businesses inform employees of information sufficient for them to comprehend the nature and routes of potential NM workplace exposure, potential risks, appropriate job procedures, preventive and protective measures, and policies adopted, should be implemented (Eastlake A, 2012).

## **BIOLOGICAL SYNTHESIS OF NANOPARTICLES**

‘Green synthesis’ is a technique of synthesis and assembly of nanoparticles and has been employed for a range of specific industrial processes. This technique benefits from the development of clean, non-toxic, and ecologically acceptable processes which utilize organisms ranging from bacteria to fungi and even plants.

Metal nanoparticles including silver, zinc, and gold have been employed as therapeutic agents in medical establishments for some years. Transitional metal oxides such as CuO, TiO<sub>2</sub>, and FeO NPs have demonstrated successful uses as advanced nano-substances in energy, biomedical and environmental areas of research. The excellent adsorption capacity displayed by these NPs considerably boost their performance and applications. CuNPs exhibit exceptional anticancer, antibacterial, and antioxidant effects which offer them a viable tool for biological applications.

Various methods have been employed for the synthesis of nanoparticles including chemical, physical, electrochemical, photochemical, and biological techniques. Although most of the

approaches are effective in manufacturing pure and clearly defined nanoparticles, they are highly costly or possibly toxic to the environment.

Synthesis of nanoparticles using the aid of green approaches has obtained tremendous interest in recent years. Plant systems extensively scattered within the ecological limits, are conveniently accessible and safe to handle. Such investigations might show to have a large influence shortly if plant tissue culture and downstream processing processes are utilized to generate metallic as well as oxide nanoparticles on an industrial scale.

As metal nanoparticles are extensively employed in the regions of human interaction, the demand to create eco-friendly technologies for nanoparticle production that do not use harmful chemicals has been steadily expanding. Green production of copper nanoparticles is of tremendous interest because of numerous advantages: copper is very conductive and much cheaper than silver and gold. Human people have been employing copper (Cu) and copper complexes for diverse reasons for ages such as water purifiers, algaecides, fungicides, and as antibacterial and antifouling agents. Copper-based compounds have efficient biocidal characteristics, which are commonly employed in numerous health-related applications.

### **Bacteria-Mediated Synthesis of Nanoparticles**

Pure gold nanoparticles were created by bacteria, *Delftia acidovorans* (Johnston et al, 2013), Delftibactin is a tiny non-ribosomal peptide and is deemed suitable for the manufacture of gold nanoparticles since it is known to promote resistance against harmful gold ions. The transition metal, gold did not exert toxicity against bacteria owing to the creation of inert gold nanoparticles (AuNPs) attached to delftibactin (Pantidos and Horsfall, 2014). A substitutive technique for gold nanoparticle production by the bacterium *Rhodopseudomonas capsulate* was found to synthesize extracellular gold nanoparticles ranging in size from 10 to 20nm through NADH-Dependent Reductase (He et al, 2007). Green products may operate as a

stabilizing and reducing agent for AuNPs production and these formulations demonstrate therapeutic uses (Lee et al, 2020). Green production of AgNPs utilizing lactic acid bacteria was achieved by Sintubin et al. (2009). *Lactobacillus* spp., *Pediococcus pentasaceus*, *Enterococcus faecium*, and *Lactococcus garvieae* was demonstrated to produce the nanoparticles by various microorganisms. The technique of AgNP production was hypothesized to be a two-step approach. The biosorption of Ag ions on the cell wall was followed by a reduction of these ions resulting in AgNP production (Sintubin et al., 2009). Additionally, the cell wall may be believed to be a capping agent, ensuring their stability by halting their aggregation.

The biosynthesis of Ag and AuNPs has been a main topic of investigation because of their antibacterial characteristics. Comprehensive investigations were undertaken to synthesize the metallic nanoparticles employing *Bacillus* species because of their metal accumulating abilities (Pollmann et al., 2006; Kalimuthu et al., 2008; Pugazhenthiran concentrations of Al, Cd, Cu, Pb, and U. The Uranium bioremediation from the aqueous environment was linked to the S-layer proteins of *B. sphaericus*. Copper (Cu) is not considered stable and is oxidized swiftly to copper oxide (CuO). Therefore Cu nanoparticles need to be stabilized as soon as they are formed. The manufacture of Cu nanoparticles utilizing *Morganella morganii* is shown with the aid of intracellular absorption of Cu ions accompanied by the means of binding of ions to a metallic ion reductase or an analogous protein assuring in the reduction of the ion to metallic Cu<sup>0</sup> (Baco-Carles et al., 2008). The Cu nanoparticles produced AgNPs (Parikh et al., 2008). The Cu nanoparticles production utilizing *M. morganii* may be owing to an Ag resistance mechanism to supply elemental Cu nanoparticles via silE homolog to copper-binding protein from various microorganisms (Ramanathan et al., 2013).

## **Nanoparticle Synthesis Using Fungi**

The production of AgNPs utilizing fungus has been the focal point of inquiry because of its uses in several sectors such as antimicrobials and electronics (Rai et al., 2008; Ummartyotin et al., 2012). The capacity of the fungus *Fusarium oxysporum* to synthesis AgNPs has been proven with diameters ranging from 5 to 15nm which have been capped by fungal proteins to lead them to become stable. *Fusarium oxysporum* could also synthesize nanoparticles extracellularly as compared to earlier studies in which intracellular production of Ag and AuNPs, lead sulfide (PbS), cadmium sulfide (Cds), molybdenum sulfide (MoS), and zinc sulfide (ZnS) nanoparticles intracellular production of Ag and AuNPs, cadmium sulfide (Cds), lead sulfide (PbS), zinc sulfide (ZnS), and molybdenum sulfide (MoS) had been reported (Ahmed et al., 2002, 2003a).

*Aspergillus fumigatus* is employed to generate extracellular silver nanoparticles of greater sizes ranging from 5 to 25 nm as compared to *Fusarium oxysporum*, with the drawback of difficulties in predicting the catalytic activity with the size variation in every batch (Bhainsa and D'Souza, 2006). However, the bioproduction of AgNPs utilizing *A. fumigatus* is an appealing potential since the organism converts Ag ions into nanoparticles after 10 min of contact (Bhainsa and D'Souza, 2006). Fungus *Trichoderma reesei* might potentially be employed for the extracellular generation of AgNPs with a size range of 5–50 nm nanoparticles. It took 72 h to synthesize AgNPs which was substantially slower than *A. fumigatus* and *Fusarium oxysporium* (Ahmad et al., 2002, 2005; Bhainsa and D'Souza, 2006). Furthermore, the use of *T. reesei* has an advantage over the use of other fungi since it has been an extensively-studied organism that may be manipulated for the production of an excessive quantity of enzymes (Roy et al., 2008; Vahabi et al., 2011) and may help increase the rate of production of nanoparticles. However, the nanoparticles were not as homogeneous as those which were formed by *A. Fumigates* (Bhainsa and D'Souza, 2006) and *F.*

oxysporum (Ahmad et al., 2002). The fungal characteristic to make intracellular nanoparticles is beneficial in getting rid of the fungus and its accumulated metallic contamination. A white-rot fungus (*Coriolus Versicolor*) is indicated to supply and accumulate AgNPs extra and intracellularly by altering reaction circumstances (Sanghi and Verma, 2008). Only a few fungi are thought to have the capacity to synthesize gold nanoparticles despite the rising demand in numerous areas. The tiny size of gold nanoparticles leads them to become more reactive and suited as opposed to the bulk form to be employed as precursors for electronics applications and catalysts (Mukherjee et al., 2001; Eustis and El-Sayed, 2006). The manufacture of AuNPs utilizing *Verticillium* sp. by the biological reduction of AuCl<sub>4</sub> localized on the surface of the mycelia

### **Nanoparticle Synthesis Using Yeast**

Yeasts may absorb and collect a high number of harmful metals from their neighboring regions owing to their vast surfaces (Bhattacharya and Gupta, 2005; Mandal et al., 2006). Yeast has several detoxification methods to adapt to harmful metals such as bio-precipitation, chelation, extracellular sequestration, and bio-sorption. These methods developed via yeast cells are exploited during nanoparticle synthesis to produce and enhance the endurance of nanoparticles, giving rise to diversity in particle size, particle characteristics, and location (Hulkoti & Taranath, 2014). The intracellular production of CdS quantum dots turned into proven through *Candida glabrata* when exposed to cadmium salts (Dameron et al., 1989). The development phase of yeast *Schizosaccharomyces pombe* cells and the generation of CdS quantum dots are connected (Kowshik et al., 2002). *Torulopsis* sp. synthesizes PbS quantum dots when exposed to Pb<sup>2+</sup> ions while *Pichia jadinii* synthesizes Au nanoparticles intracellularly. The size range of these nanoparticles extends from a few nanometers to about

100 nm. The morphological properties of these nanoparticles were simply done by monitoring the cellular activities and development of *P. jadinii* throughout the production of the nanoparticle (Gericke and Pinches, 2006). The usage of metallic nanoparticles has become crucial owing to their safety and possible uses.

### **Nanoparticle Synthesis Using Plants**

The plants are believed to be more suited compared to bacteria for green synthesis of nanoparticles since they are nonpathogenic and several routes are fully investigated (Figure 4). A broad variety of metal nanoparticles has been created utilizing various plants (Narayanan and Sakthivel, 2011; Irvani and Zolfaghari, 2013; Mittal et al., 2013; Das et al., 2017). These nanoparticles exhibit distinct optical, thermal, magnetic, physical, chemical, and electrical characteristics compared to their corresponding bulk material with multiple applications in numerous sectors of human interest (Husseiny et al., 2007; Duran and Seabra, 2012). Several biological entities are exploited for AgNPs production (Keat et al., 2015). *Jatropha curcas* extract resulted in the generation of homogeneous (10–20 nm) AgNPs from AgNO<sub>3</sub> salt in 4 h (Bar et al., 2009). The leaf extracts of *Acalypha indica* have shown the aptitude to produce AgNPs. The size of the AgNPs formed became broadly homogenous and varied from 20 to 30 nm (Krishnaraj et al., 2009). In another investigation, *Medicago sativa* seed exudates were employed for the production of AgNPs. The reduction of Ag<sup>+</sup> occurred fairly quickly as nanoparticles had been identified within a minute of metal salt contact and 90% of Ag<sup>+</sup> was reduced at 30°C in <50 min. The resultant nanoparticles were flowerlike and/or triangular and spherical with a size range of 5–108 nm and exhibited a heterogeneous size distribution (Lukman et al., 2010). The leaf extract of *Ocimum sanctum* may also decrease Ag<sup>+</sup> resulting in the AgNPs of 3–20 nm in size production. The particles were spherical and stabilized by the manner of a component of the leaf broth (Koduru et al., 2011). *Terminalia chebula* fruit extract has been employed to swiftly manufacture Ag nanoparticles

(Jebakumar Immanuel Edison and Sethuraman, 2012). *Eucalyptus macrocarpa* leaf extract produced Ag nanoparticles of cubic shape ranging in size from 50 to 200 nm (Poinern et al., 2013); spherical gold nanoparticles of around 20 nm by *Nyctanthes arbor tristis* (night jasmine) flower extract (Das et al., 2011); leaf extract from *Coriandrum sativum* (coriander) leaf extract produce Ag and Au nanoparticles of 7–58 nm (Mittal et al., 2013)

## **FACTORS AFFECTING BIOLOGICAL SYNTHESIS OF METAL NANOPARTICLES**

The morphological properties of nanoparticles may be modified using several factors viz. reaction time, reactant concentrations, pH, and temperature. Such characteristics are critical to understanding the influence of environmental conditions on the synthesis of NP as they may play a key role during the optimization of metallic NPs production by biological methods.

### **pH**

The reaction medium pH has a crucial function in the production of nanoparticles (Gardea-Torresdey et al., 1999). The size and form of nanoparticles change with the pH of the medium, and big-sized nanoparticles are created in acidic pH (Dubey et al., 2010; Sathishkumar et al., 2010). The rod-shaped gold nanoparticles were manufactured by employing biomass of *Avena sativa* (Oat) resulting in the size range from 25 to 85 nm at pH 2 which was considerably smaller (5–20 nm) at pH 3 and 4 (Armendariz et al., 2004). Further, the accessibility of functional groups for particle nucleation in the extract was greater at pH 3 or 4 as opposed to pH 2 as fewer functional groups were accessible spurring particle aggregation to produce bigger Au nanoparticles. An enhanced quantity of spherical Ag nanoparticles was generated in *Cinnamom zeylanicum* bark extract at higher pH (pH >5). A modest increase was found in particle size with higher pH when *Cinnamom zeylanicum* bark

extract was employed for the manufacture of palladium (Pd) nanoparticles, and particle size was calculated from 15 to 20 nm at pH <5, and 20-25 nm at the higher pH (Kumar and Yadav, 2009).

### **Reactant Concentration**

The creation of metallic nanoparticles is regulated by the concentration of biomolecules present in the extract. The morphology of the biosynthesized Au and Ag nanoparticles by employing the sun-dried *Cinnamomum camphora* (camphor) leaf extract changed by the quantity of biomass in the reaction media (Huang et al., 2007). Exposure of the precursor chloroauric acid to rising quantities of the extract resulted in the creation of spherical nanoparticles instead of triangular. A shift in the ratio of spherical nanoparticles to triangular plates in the reaction medium bearing chloroauric ions owing to the presence of carbonyl compounds in the extract was detected when treated with varying doses of *Aloe vera* leaf extract. Nanoparticle size may be adjusted between 50 and 350 nm by employing varied extract concentrations (Chandran et al., 2006). Spherical, triangular, hexagonal, and decahedral forms of AgNPs were generated by changing the quantity of *Plectranthusamboinicus* leaf extract in the reaction media (Narayanan and Sakthivel, 2010). An increase in the diversity of Ag nanoparticles was detected with increasing concentrations of *Cinnamomumzeylanicum* bark extract (Kumar and Yadav, 2009). The extracellular (Agnihotri et al., 2009) and intracellular production (Pimprikar et al., 2009) of Au salt concentration generated both nanoscale spheres and plates. In another work, a silver-tolerant yeast strain MKY3 produced spherical Ag nanoparticles extra-cellularly with a size ranging from 2 to 5nm (Kowshik et al., 2003).

### **Reaction Time**

The reaction time plays a crucial role in synthesizing nanoparticles. A quick color change was seen within 2 min when *Anana scomosus* (Pineapple) extract was employed for AgNPs production and aqueous AgNO<sub>3</sub> solution was rapidly lowered, generating nanoparticles within 2 min. The response persisted for up to 5 min and then there was a small color shift. The form of produced nanoparticles was spherical with a mean size of 12 nm (Ahmad, 2012). *Chenopodium album* leaf extract was employed for the biogenic synthesis of Ag and Au nanoparticles. The nanoparticles were generated after 15 min of the reaction and the reaction proceeded for a period of 2 h and relatively few nanoparticles with greater size were created (Dwivedi and Gopal, 2010). Change in the particle size (range 10–35 nm) was seen when the reaction time was extended from 30 min to 4 h using *Azadirachta indica* leaf extract and AgNO<sub>3</sub> (Tc et al., 2011). Reaction Temperature The reaction temperature is a vital component that plays a major role in regulating the form, size, and yield of synthesized nanoparticles utilizing plants (Song et al., 2009a; Sathishkumar et al., 2010). The peel extract of *Citrus sinensis* (sweet orange) generated particles with an average size of roughly 35nm at 25°C. The average size of the nanoparticles dropped to 10 nm with the increase in the reaction temperature to 60°C (Kaviya et al., 2011). The stable Ag nanoparticles were generated by *Diospyros kaki* (persimmon) leaf extract at the reaction temperature varied from 25 to 95°C (Song et al., 2009). The alteration in the temperature of reaction conditions for the synthesis of Au nanoparticles using *Avena sativa* (oat) biomass concluded in variations in the form and size of the nanoparticles generated (Armendariz et al., 2004). A greater temperature promotes an enhanced rate of production of Au nanoparticles. The spherical Au nanoparticles were largely generated at the lower temperature but at higher temperatures, rod-like and platelike nanoparticles were created (Gericke and Pinches, 2006). The reaction rate and particle production rate increased with the rise in the reaction temperature. The particle conversion rate continuously rose and average particle size observed a drop with the rise in

the reaction temperature to 60°C. The extracellularly synthesized platinum nanoparticles quantity was reported to be 5.66 mg l<sup>-1</sup>, with the fluctuation in the temperature that impacts the production rates of the pumps. The minor shift in pH from the standard limits plant nanoparticle production (Riddin et al., 2006).

### **Effects of Copper Nanoparticles**

The nanoparticles of Cu showed anti-oxidant, anti-bacterial, and antimicrobial activities against common pathogenic strains such as *Escherichia coli* and *Staphylococcus aureus* and are proven to have significant application potential (Heinlaan et al., 2008 Das et al., 2012; Padil and Cernik, 2013). Cu nanoparticles show antibacterial properties against common pathogenic bacteria *Escherichia coli* (Lee et al., 2011).

They show effective decontaminating characteristics against numerous pathogenic microbes with the potential to be employed as bactericidal material Akhavan and Ghaderi, 2010; Hassan et al., 2012; Subhankari and Nayak, 2013). The Cu nanoparticles generated by the stem latex of *Euphorbia nivulia* were shown hazardous to human lung cancer cells (Valodkar et al., 2011c) revealing its potential applicability in the area of cancer treatment.

#### **Nano copper toxicity**

The LD50 values of nano copper (23.5nm) are 413mg/kg of body weight and are considered moderately toxic materials. Nano-copper toxicity may be related to in vivo ionization (Chen Z., 2006). Therefore, compared to the particles of micro-copper, nano-copper (same mass), they are more likely to collide with the bio-substances in vivo. For nanometer sized copper particles, a large surface leads to ultra-high reactivity. NP copper reacts drastically with H<sup>+</sup> in the gastric juice and can lead to massive formation of HCO<sub>3</sub>, which is extremely excreted by

the kidney due to kidney due to kidney disorders. The excess of  $\text{HCO}_3^-$  in vivo becomes the main cause of metabolic alkalosis, which becomes the origin of the symptom hypopnea and the electrolyte pathologies produce tremors observed in mice exposed to copper NP. In vivo homeostasis continues to be copper ions metabolized in the liver and in the evacuated kidney((Chen Z., 2006, Bertinato J, 2004, Tao TY, 2003). The toxic classes of NP copper are class 3 (moderately toxic). One study suggests that ionic copper and nano copper have different effects on fish. They enter the body of the fish probably with different shapes, generating different effects with different shapes, generating different effects with different sizes in the plasma of copper, ceruloplasmin, iron and ions. Copper, sodium and iron chloride in plasma suggest that Gill is the main site for absorption of ionic copper, while nano-copper can be absorbed through the intestine (Hedayati A., 2016)

### **Role of *Vernonia amygdalina***

*Vernonia amygdalina* leaf extract is an annual tiny plant that has various folklore applications in the traditional system of remedies. This plant is known to exhibit numerous pharmacological qualities including antioxidant, antiviral, antibacterial, and anticancer activity in the experimental research that could be related to the presence of alkaloids, flavonoids, tannins, lipids, sterol, and lignins. In the current work, we have studied a simple and green synthetic technique for the manufacture of copper nanoparticles by decreasing copper ions accessible in the copper sulphate solution using *Vernonia amygdalina* leaf extract.

### **AIM AND OBJECTIVES**

- The aim of this study is to evaluate the antimicrobial property of green synthesized copper nanoparticles.
- This study is also to synthesize nanoparticles using copper sulfate and fresh leaves of *Vernonia amygdalina*

## **CHAPTER 2**

### **MATERIALS AND METHODS**

The materials used in this study include:

1. Fresh Plant leaves (5gms)
2. Distilled water
3. Whatman No. 1 Filter paper.
4. 250ml Erlenmeyer flask
5. Petri dish
6. Solid nutrient agar
7. Cork borer
8. Swab stick
9. Test tubes
10. Pasteur pipette
11. Bunsen burner
12. Microorganism
  - *Staphylococcus aureus*

### **Preparation of plant extract**

Fresh leaves of *Vernonia amygdalina* were collected from a plant located near Department of Biochemistry, University of Benin, Benin city. Approximately 2g of dried *Vernonia amygdalina* leaves were added to 30ml of distilled water and heated in a water bath for about 30mins at a temp of 80°C. The solution was filtered to obtain a clear solution of *Vernonia amygdalina* extract.

### **Synthesis of Copper Nanoparticles(CuNPs)**

The copper sulphate in freshly prepared distilled water was utilized as a source of copper metal ions. For the reduction of copper metal to copper ions, the various concentrations of copper sulphate solution was prepared (50µg/ml, 100µg/ml, 200 µg/ml) which was mixed with a constant concentration of plant extract slowly dropwise with constant stirring at 80°C for 60 minutes. The color change was checked periodically (after 30 minutes and 60 minutes). The change in color from blue to light green visually indicates the formation of Cu NPs, and then, the solution was centrifuged for 15 min at 1000rpm. The obtained green copper nanoparticles were washed with deionized water to remove any impurities. Thereafter, the NPs were allowed to dry so as to be used for further analysis.

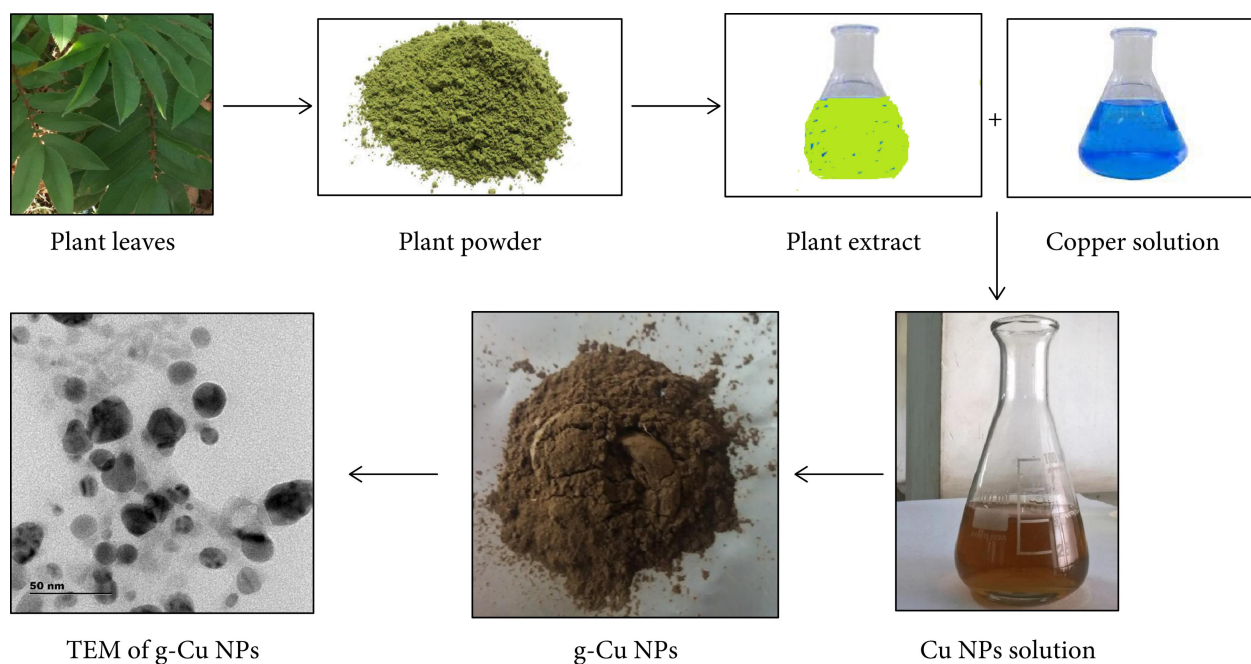


Fig 2.1 Steps of copper nanoparticles synthesis

### Characterization Techniques

The UV-visible absorbance and reflectance spectra of the samples were recorded in the range of 200 – 800nm using UV visible spectrophotometer. UV-visible spectra were measured by transmission. The Cu nanoparticle solution was prepared by mixing the freshly prepared plant extract solution with Cu solution after appropriate dilution. An absorbance spectrum was recorded by allowing the instrument to scan wavelengths ranging between 200 and 800 nm and the absorbance of the nanoparticle solution against blank extract solution was collected.

### Method of Antimicrobial Evaluation

The in vitro antibacterial activity of green copper NPs was evaluated using an Agar well-diffusion method against selected one gram-positive bacterium (*Staphylococcus aureus*).

Microbial strains were obtained from the pharmaceutical microbiology laboratory, University of Benin, Benin city

Fresh cultures of bacteria (adjusted to the standard 0.5 McFarland turbidity) were swabbed on the surface of MHA media. In the agar well diffusion method, in all Petri plates, four wells were formed with the help of a sterile cork borer (6mm wells). For the antibacterial activity test at a concentration of 50µg/ml, three sets of Petri plates were prepared for all bacteria. Each of the plate wells contain copper oxide nanoparticles, *Vernonia amygdalina* leaves extract, copper sulphate salt solution and a mixture of copper sulphate salt solution and plant extract.(one well per sample). In the first set of plates, the well concentration of the sample was 50µg/ml, in the second set of plates, wells contain a concentration of sample 100µg/ml, and the third set of plates contain a concentration of 200µg/ml. The quantity of sample used was 50µl approximately. All the Petri plates were then placed in incubation for 24 hours at 37°C. The zones of inhibition were measured (Khan S., Shahid S., Jameel M., 2016).

## **Chapter 3**

### **Results and Discussion**

#### **Green copper nanoparticles**

The green copper nanoparticles were made by employing copper sulphate as a precursor and plant leaf extract as a reducing and capping agent. The shift in hue from blue to light green visibly reflects the development of copper nanoparticles. The green copper nanoparticles were cleaned with deionized water and ethanol to eliminate any undesirable particles.

Thereafter, green copper nanoparticles were dried and crushed and afterward exposed to several characterization procedures. *Vernonia amygdalina* leaf extract has been found to include phenolic compounds, tannins, and various glycosides. Bioactive substances such as phenolic compounds serve as a ligand and bind to metal ions and reduce them and cap them to produce nanoparticles. These ligands also serve as particle size regulators as described by the previous researcher (Khatami M, 2017).

Primary components of *Vernonia amygdalina* extract include phenolic compounds, tannins, anthraquinone glycosides, and cardiac glycoside (Wolde T., Bizuayehu B., 2016). The antioxidant effects of polyphenolic compounds are mostly owing to their significant predisposition towards chelating the metals. Phenolic compounds include hydroxyl and carboxylic groups which have a high propensity to bind metal ions. Metal ions in solution interact with polyphenolic substance and assists in the nucleation and production of Copper nanoparticles (Nazar N., Bibi I., Kamal S., et al., 2018)

From the result of the experiment

## **Characterization of Copper Nanoparticles**

### *UV-Visible Spectrum*

The UV-visible absorbance spectra measured for Cu NPs displayed  $\lambda_{\text{max}}$  of 563 as shown in fig 3.1 This absorption band is essentially due to the surface plasmon resonance of Cu NPs. A similar result was found following the study of produced Cu NPs utilizing the *Ocimum sanctum* leaf extract (Jain S. and Mehata M. S, 2017). The surface plasmon absorbance largely relies on the size of NPs, and consequently, every study reports a different  $\lambda_{\text{max}}$  value for NPs manufactured using various plant extracts.

## **Antimicrobial Activity**

The green copper nanoparticles produced by utilizing the leaf extract of *Vernonia amygdalina* demonstrated antibacterial activity against *Staphylococcus aureus* with a broad range of zone of inhibition at varying doses (Table 3.1). The copper nanoparticles have demonstrated a record of having a high antibacterial activity (Din M., Arshad F., Hussain Z. and Mukhtar M., 2017). The antibacterial action of nanoparticles may be linked to the presence of bioactive chemicals on the surface of NPs as capping and stabilizing agents. In this respect, the activity was pronounceable against *S. aureus* compared to the controls utilized. The largest zone of inhibition (mm) observed with copper nanoparticles against *S. aureus* bacteria was 43mm and the lowest zone of inhibition (mm) recorded was 30mm at the lowest concentration (Fig 3.2), consequently, it is concentration dependant. The extensive zone of inhibitions in Fig 1 supports their tremendous promise as a cure for infectious disorders produced by the investigated bacterial pathogens.

The copper salt solution also demonstrated an inherent antibacterial activity against *Staphylococcus aureus* but is not in use owing to its toxicity impact (Gupta D., Kerai S., and Mohd S., 2018).

*Vernonia amygdalina* extract has established antibacterial action which may be ascribed to the presence of several kinds of phytoconstituents. Thus, it can be concluded that the synergistic impact of copper nanoparticles associated with bioactive components such as tannins and glycoside of *Vernonia amygdalina* leaf extract was demonstrated to be effective against infections. Many mechanisms of antibacterial activity have been documented by previous researchers consequently, the action of green copper nanoparticles on the bacteria and interaction with the electronegative components inside the cell membrane. This leads to failure metabolism consequently leading to interference and disruption of transcription in

bacteria and hence produces antibacterial action of copper nanoparticles. It is also thought that the synergistic action of copper nanoparticles with bioactive chemicals of extract would have played an important influence to prevent the activity of harmful bacteria as reported by the latest study.

It is potentially understood that the helical structure of DNA molecules would have been disturbed by the activity of copper nanoparticles. In addition, the electrochemical potential across the cell membrane reduces depending on the interaction with the released copper metal ion by copper nanoparticles influencing the integrity of the membrane.

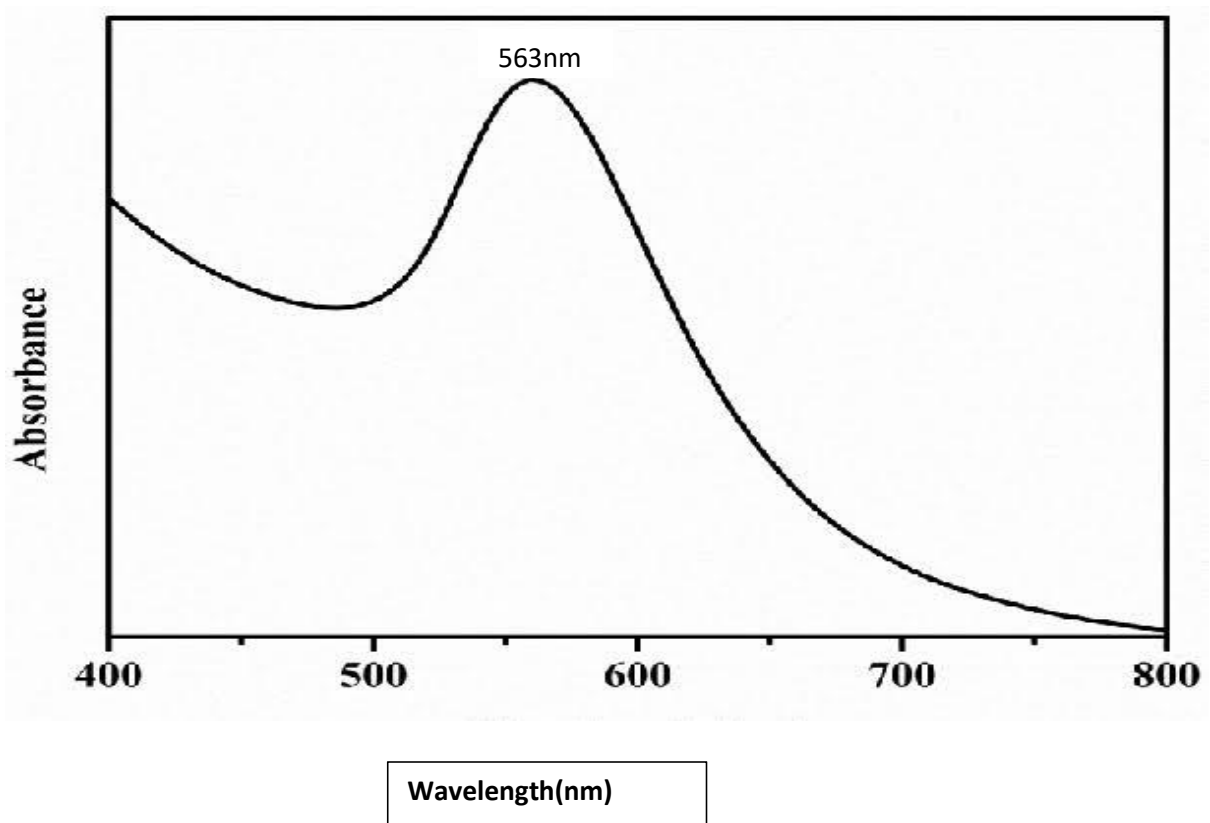


Fig 3.1: UV-visible absorbance spectra of Cu NPs



Fig 3.2: A picture of zones of inhibition of copper nanoparticles with test samples(50µg/ml) on staphylococcus aureus

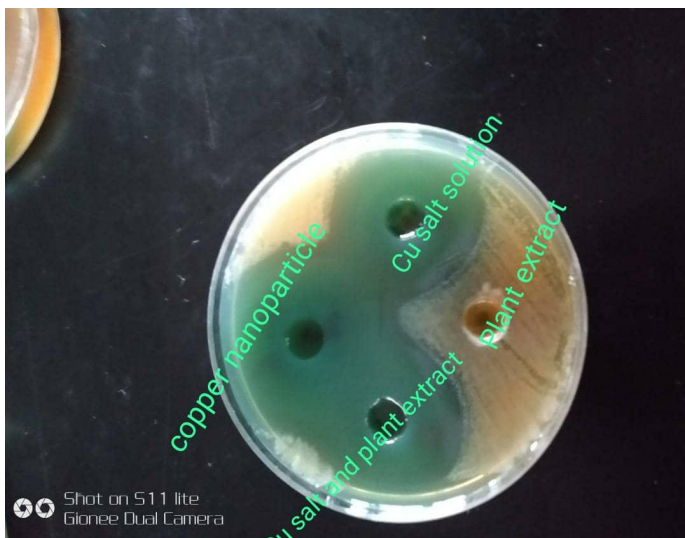


Fig 3.3: A picture of zones of inhibition of copper nanoparticles with test samples(200µg/ml) on staphylococcus aureus



Fig 3.4: A picture of zones of inhibition of copper nanoparticles with test samples(200 $\mu$ g/ml) on staphylococcus aureus

Table 3.1: Staphylococcus aureus zones of inhibition produced by different concentration of test samples

Test Samples	Zone of Inhibition(mm)		
	50 $\mu$ g/ml	100 $\mu$ g/ml	200 $\mu$ g/ml
Copper nanoparticles solution	30	40	43
Copper salt and plant extract solution	29	34	36
Copper salt solution	25	34	37
Plant extract solution	16	18	22

## Chapter 4

## CONCLUSION

From the result of the study, the green copper nanoparticles were successfully synthesized by using medicinal plant *Vernonia amygdalina* leaf extract. The presence of phytoconstituents such as polyphenols of tannins played roles in reducing and capping agents during the formation of green copper nanoparticles. The UV-visible absorbance and reflectance spectra showing  $\lambda_{\text{max}}$  of 563nm confirmed the formation of green copper nanoparticles. The wide zone of inhibition of green copper nanoparticles against pathogens confirms their great potential as a remedy for infectious diseases caused by the tested bacterial pathogens. Finally, it can be concluded that the synergistic effect of bioactive compounds from medicinal plant coupled with copper nanoparticles has been proved to be beneficial against pathogens.

Green synthesis technology provides a clean, non-toxic and eco-friendly approach for the synthesis of metallic nanoparticles and is of significant interest owing to economic prospects and practicality. However, procedures need to be changed further for making these approaches cost-effective and competitive with established methods for the large-scale synthesis of nanoparticles. Improvement of dependable and eco-friendly procedures for the production of metallic nanoparticles is a key breakthrough in the area of applied nanotechnology. Further, most of these solutions are still in the developmental stage and obstacles need to be taken care of. These involve the stability and aggregation of nanoparticles, regulating the crystal development, shape, and size. The separation and purification of nanoparticles is another crucial characteristic that has to be researched further. Metal nanoparticles created by plants and/or plant extracts are more stable as compared to those produced by various species. Genetically modified organisms (GMOs) have significant power to optimize for creating a bigger amount of proteins, enzymes, and biomolecules

essential for the biosynthesis and stability of nanoparticles. We think genetic alteration to boost the metal tolerance and accumulation ability is the future method to enhance the production of metal nanoparticles by adopting the “green synthesis” strategy.

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