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GREEN SYNTHESIS OF COPPER OXIDE (CuO) NANOPARTICLES USING KHAT LEAF EXTRACT AND EVALUATION OF ITS ANTIBACTERIAL ACTIVIES

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BAHIR DAR UNIVERSITY
COLLEGE OF SCIENCE GRADUATE PROGRAM

DEPARTMENT OF CHEMISTRY

GREEN SYNTHESIS OF COPPER OXIDE (CuO) NANOPARTICLES USING KHAT LEAF EXTRACT AND EVALUATION OF ITS ANTIBACTERIAL ACTIVIES

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BAHIR DAR, ETHIOPIA
JUNE 2017

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GREEN SYNTHESIS OF COPPER OXIDE (CuO) NANOPARTICLES USING KHAT LEAF EXTRACTS AND EVALUATION OF ITS ANTIBACTERIAL ACTIVIES

A Thesis Submitted to the department of chemistry: Bahir Dar University, College of Sciencefor the partial fulfilment of the requirements for the degree of Master of Science in Chemistry (physical).

BY: KIFLOM G/MEDHN

ADVISOR: MULUKEN AKLILU (PhD)

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LETTER OF APPROVAL

As members of the board of examiners for the MSc thesis open defense examination, we certify that we have read and evaluated the thesis prepared by Kiflom G/medhn Kele and examined the candidate. We recommended the thesis to be accepted as fulfillment for the requirements of the Degree of Master of Science in chemistry.

Board of Examiners

External examiner	Signature	Date
Dr. ----------------------	______________	_________________
Internal examiner	Signature	Date
Dr. -----------------------	______________	_________________
Advisor	Signature	Date
Dr. Muluken Aklilu	______________	_________________

Declaration

I here by declare that the thesis entitled “Green synthesis of copper oxide (CuO) nanoparticles using khat leaf extracts and evaluation of its antibacterial activies”, that I submitted to the Department of Chemistry, Bahir Dar University in partial fulfillment of the Master Degree in Physical chemistry is a record of bonafied and original research work carried out by me under the guidance and supervision of Dr. Muluken Aklilu, Assistant Professor, Department of chemistry, Bahir Dar University. To the best of my knowledge no part of this thesis has been submitted to any other university or institution for the award of any degree or diploma.

Name: Kiflom G/medhn
Signature:
Place: Science Collage
Bahir Dar University
Date of Submission: June 2017

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Research is expected to be full of intense works which needs strong collaboration between people. That is exactly what I have experienced during whole of my work. That is why I am here to give credit to whom it deserve.

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LIST OF ABBREVIATIONS

Nps	Nanoparticle
NMs	Nanomaterials
EGCG	Epigallocatechin gallate
MOs	Microorganisms

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ABSTRACT

In the present investigation a fast, convenient and environment friendly method has been used for the synthesis of copper oxide nanoparticles by biologically reducing 1 mM$CuSO_4$ solution with extract of 2 % (m/v) of Khat (Catha edulis) under optimum conditions (pH of 11). The formation of copper oxide nanoparticles was indicated by the color changes from colorless to light green then to greenish yellow and finally, after days, to brown precipitate. Biosynthesized nanoparticles were also characterized by UV-Visible and FT-IR spectroscopies. The kinetics of the reaction was studied using UV–Visible spectrophotometer.The reduction process was simple and convenient to handle and was monitored by UV-Visible spectroscopy that showed surface plasmon resonance at 333 nm, which is very much different from 294 nm of the pure Khat extract.This has clearly revealed the formation of copper oxide nanoparticles. The presence of active flavanoids, phenolic groups, tannins, alkaloids, Glycosides and carbohydrates whichwere in the biomass of the Khat extract before and after reduction was identified using qualitative screening methods (observing the color changes) and FT-IR spectroscopy. These biologically synthesised copper oxide nanoparticles were tested for their antimicrobial activity against two human pathogens, i.e Salmonella typhimurium and Escheria Coli. They were found to have significant effect in controlling the growth of the human pathogens with an average zone of inhibition of 10 mm. Therefore, using Khat for the synthesis of the copper oxide nanoparticle, applied in different fields, is another possible way.

Keywords: CuO Nps, Khat, Green synthesis, FT-IR, UV-Vis, Antimicrobial activity

Introduction

1.1 Background of the study

CuO Nps is one of the useful metal oxides and which has many applications in different fields **[1]**. Among various metal oxide nanoparticles, CuO has attracted particular attention. It is the simplest member in the family of copper compounds, easy to prepare in pure form and shows a range of useful physical properties such as high temperature superconductivity, electron correlation effects, and spin dynamics **[2, 3]**.

Attention have been paid to copper oxide because of their potential commercial applications, their efficiency as nanofluids in heat transfer application and their mechanical and biological properties with up-to-date technologies viz. biocidal activity, magnetic phase transitions, gas sensors, catalysis, and superconductivity. Hence the applications of CuO nanoparticles include; antioxidant, antibacterial, anti fouling, anti-biotic, anti-fungal agent, catalysis and so on **[2 - 6]**. In the energy-saving area, energy transferring fluids filled with nano CuO particles can improve fluid viscosity and enhance thermal conductivity. CuO crystal structures possess a narrowband gap, giving useful photocatalytic or photovoltaic properties as well as photoconductive functionalities **[3]**.

Arrays of conventional methods have been employed in synthesis of nanoparticles. They can be categorized mainly under physical and chemical methods.

Pulsed laser ablation, vacuum vapor deposition, pulsed wire discharge, mechanical milling etc. are physical techniques. However, the quality of the material is not as high as chemically synthesized materials. while micro-emulsion method, arc submerged nanoparticle synthesis system flame based aerosol methods, sonochemical hydrothermal solid state techniques , thermal oxidation route, thermal decomposition, reverse microemulsion method, precipitation method, solution combustion method are chemical methods **[7-10]**.

All of the above methods employ toxic chemicals and energy intensive routes which make these choices eco-hazardous. Hence, the above listed different methods used for the preparation of copper oxide nanoparticles are not safe, where flammable or corrosive reducing agents like titanium tetrachloride and hydrazine have been used. This may pose potential environmental and biological risks. Because noble metal nanoparticles are widely applied to areas of human contact, and due to the high surface charge and high surface area of nanoparticles, harsh chemicals may remain adsorbed on to nanoparticles. Releasing these chemicals into the environment may cause adverse effects on organisms including microorganisms, plants as well as humans at various trophic levels. Thus, developing environment friendly protocols is the need of hour in nanomaterial synthesis. The interest in this field has then shifted toward ‘green’ chemistry and bio-processor approach **[9, 11]**.

Green chemistry is the practice of chemical science and manufacturing in a manner that is sustainable, safe, and non-polluting and that consumes minimum amounts of materials and energy while producing little or no waste material. This approach focuses on utilization of environmental-friendly, cost-effective, economical in nature and biocompatible reducing agents for synthesis of copper nanoparticles **[7, 12]**. In green methods, extract of living organisms such as plants, microorganisms and enzymes etc., are used. Green synthesized CuO Nps has similar physical and chemical properties as the one prepared by chemical methods **[13]**.

However using microorganism like fungi, bacteria, yeasts etc. require lots of maintenance; is complex and involves very complex procedures of maintaining microbial cultures **[7, 13]**. It is also not very suitable for industrial feasibility, because of its requirements of highly aseptic conditions and the difficulty in maintenance **[9]**.

Hence some specific plant parts or whole plant, favorablly angiospermic plants (flowering plants, approximately 80 percent of all the known green plants now living), are used for the green synthesis of nanoparticles in general. This is due to the reason that plants possess components that can act as reducing agent, capping agent, chelating agent and stabilizers for nanoparticles synthesis. The green synthesis of metal nanoparticles using biomolecules present in plant extracts such as enzymes, proteins, amino acids, vitamins and polysaccharides has been deeply studied in recent years **[14]**.

But none of the previous studies have used Khat leaf for the synthesis of CuO Nps. This thesis is the first to report such a work.

On the other hand, the research in the field of biosynthesis has been mainly focused on Ag and Au nanoparticles, and there have been very few reports on the synthesis of CuO nanoparticles. Till *August* 2014, the research in the field of biosynthesis mainly focused Ag nanoparticles (7,699 papers, 59%), Au nanoparticles followed by ZnO nanoparticles (4,640 papers, 36%) and finally CuO (690 papers, 5%) **[2, 15, 16]**. The mostly studied metallic nanoparticles, Au and Ag, are expensive as compared to CuO **[14]**. Hence, this thesis is believed to have its own contribution on increasing reports of green synthesis of CuO nanoparticles as well as in making nanoparticles easily prepared and cheaply available.

1.2 Statement of the problem

These days different researches on synthesis of metallic /metallic oxide nanoparticles have been reported in many areas of applications. The nanoparticles are mostly prepared using chemical and physical methods. However, the qualities of the synthesized materials by physical methods are not as high as chemically synthesized materials **[4]**. The problem associated with chemical methods is that they use very hazardous organic solvents, expensive reagents, and have low productivity, non-eco friendly, produce toxic byproducts and need longer time. Hence there arises a growing need to develop green synthesis routes for the nanoparticles. Still, using microorganisms as a green method of synthesis requires lots of maintenance and involves very complex procedures of maintaining microbial cultures **[9, 13]**. It is also slow which is not very suitable for industrial feasibility because of its requirements of highly aseptic conditions.These problems drive the need of green synthesis to rise. The other driving force is the number of works or literatures on the Copper Oxide nanoparticles is relatively few as compared to other inorganic or metalic nano particles. The most studied metallic nanoparticles, Ag and Au, are very much expensive as compared to CuO nanoparticles. On the other hand, microbes are becoming resistant to the usual antimicrobials and so more consistent way of protecting or killing microbes (such as bacteria) is becoming a more focused area of study.

1.3 Significance of the study

This work is expected to have its own contribution on the green synthesis of the nanoparticles using locally available plants and safe methods so that there can be many possible ways for the green synthesis of metallic oxide nanoparticles, as they have applications in many fields. It was also hoped that, it could be used as one possible means of synthesizing copper oxide nanoparticles of desired quality with low cost and convenient methods of preparation so that the toxicity or hazardous nature of the chemical methods of synthesizing copper oxide nanoparticle can be avoided. It was believed that this work will have a positive impact on increasing the number of available research works dealing with CuO Nps synthesis and its antibacterial activity.

Having easily available CuO nanoparticles was also expected to make the protection and /or removal of bacterial microbes easy and effective.

1.3 Objective of the study

1.1.1 General objective

The main purpose of this study was synthesizing the CuO nanoparticles using Khat leaves extract and applying it for its antibacterial activity.

1.1.2 Specific objectives

To achieve the above general objective there were some specific tasks to be performed.

- Testing the existence of different phytochemicals in the Khat leaf extract using different standard solutions as well as FT-IR spectroscopy.
- Synthesizing the CuO Nps from the Khat extract and $CuSO_4.5H_2O$
- Optimizing the conditions (concentration of the copper sulphate solution and Khat extract,, pH and reaction time)
- Characterizing the CuO Nps using visual observation, UV-Vis, FT-IR,
- Assesing the antibacterial activity of the CuO Nps by measuring its zone of inhibition over different bacterial species.

2. Review literature

2.1 Historical aspects and growth of Nanotechnology and Nanoparticles

The term “Nano” comes from the Greek word *dwarf* which generally elaborates the particle of size roughly in the range of 1to100 nanometers. The theoretical concept of nanotechnology was first begun with lecture delivered by Richard Feynman in 1959. He gave a lecture titled “*There's Plenty of Room at the Bottom*”, suggesting the possibility of manipulating things at atomic level. He speculated on the possibility and potential of nanosized materials **[16, 17]**. The term "Nanotechnology" was first defined by Norio Taniguchiof the Tokyo Science Universityin 1974 and Nanotechnology was shortened to “Nanotech”. However, the real burst of nanotechnology didn’t come until the early 1990s. In the early 1990s Huffman and Kraetschmer, discovered how to synthesize and purify large quantities of fullerenes (molecules composed entirely of carbon, in the form of a hollow sphere, ellipsoid, or tube are similar in structure to graphite, which is composed of a sheet of linked hexagonal rings, but they contain pentagonal (or sometimes heptagonal) rings that prevent the sheet from being planar). Shortly after a meeting of the Materials Research Society in 1992, Dr. T. Ebbesen described to a spellbound audience his discovery and characterization of carbon nanotubes. Using the same or similar tools as those used by Huffman and Kraetschmer, hundreds of researchers further developed the field of nanotechnology. No one knows how many products on the market today contain nanoparticles or are manufactured with the help of nanotechnologies **[16]**.

Nanotechnology is a field of science which deals with design, production, manipulation and use of materials ranging in nanometers called nanomaterials **[9]**. It is the application of science to control matter at the molecular level **[16]**. It is also the study and application of small object which can be used across all fields such as chemistry, biology, physics, material science and engineering **[18]**. It has appealed many researchers from the above listed fields **[19]**. Generally, nanotechnology deals with structures sized between 1 to 100 nm, nanoparticles. It involves developing materials or devices within that size range **[16]**.

Tremendous growth in nanotechnology has opened up novel fundamental and applied frontiers in materials science and engineering, such as nanobiotechnology, bionanotechnology, quantum dots, Surface-enhanced Raman Scattering (SERS) and applied microbiology **[18]**. The branch of nanoscience and technology is truly multi-disciplinary and is an emerging technology with full of promises to have an impact on virtually every spectrum of civilization including communications, computing, textiles, cosmetics, sports, therapy, automotives, environmental monitoring, fuel cells and energy devices, water purification, food and beverage industry, etc **[16, 20]**.

Nanoscience and nanotechnologies are revolutionizing our understanding of matter and are likely to have profound implications for all sectorsof the economy, including; agriculture and food, energy production and efficiency, the automotive industry, cosmetics, medical appliances and drugs, household appliances, computers, and weapons. Nanotechnology has the capacity to improve our ability to prevent, detect, and remove environmental contaminants in air, water, and soil in a cost effective and environmentally friendly manner **[16]**.

2.1 Nanoparticles (Nps) / Nanomaterials (NMs)

Nanomaterials (NMs) are defined as materials with at least one external dimensionlies in the size range of approximately 1-100 nm.While Nanoparticles (Nps) are solid particles with all three external dimensions at the nano scale that can drastically modify physico-chemical properties compared to the bulk material **[5]**. Bulk materials possess relatively constant physical properties regardless of their size, but at the nanoscale this is often not the case. The nanoparticles possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired applications **[20]**. They exhibit unique electronic, magnetic, optical, catalytic and medicinal properties as compared with the traditional and commercial bulk materials. This is due to its quantum size effect, large surface to volume ratio and their electronic energy states become discrete **[13, 20]**. As particles are reduced from a micrometer to a nanometer size, the resultant properties can change dramatically. For example, electrical conductivity, hardness, active surface area, chemical reactivity and biological activity are all known to be altered **[3]**. Because; as the material becomes smaller the percentage of atoms at the surface increases relative to the total number of atoms of the material bulk. This can lead to unexpected properties of nanoparticles which are partly due to the surface of the material dominating over the bulk properties. It is also known that with the decrease in the dimensions of the materials to the atomic level, their properties change.

All nanoparticles, regardless of their chemical constituents, have surface area to volume ratios that are extremely high. This causes nanoparticles physical properties to be dominated by the effect of the surface atoms and capping agents on the nanoparticles surface. A particle with a high surface area has a greater number of reaction sites than a particle with low surface area, and thus, results in higher chemical reactivity. High surface area to volume ratio is important for applications such as catalysis.

This increased reactivity due to surface area to volume ratio is widely taken advantage of in nature; one biological example is the body's digestive system. Within the small intestine, there are millions of folds and sub folds that increase the surface area of the inner lining of the digestive tract. These folds allow more nutrients and chemicals to be absorbed at the same time, greatly increasing our body's efficiency and the rate at which we digest food. Another example, a cube of sugar reacting with water as the water dissolves the outside of the sugar. The same size of sugar cube is cut into many little pieces. Each cut makes new outer surfaces for the water to dissolve. The smaller particles of sugar have same volume but have much more surface area.

Almost all properties of nanoparticles are due to their small sizes. Nanoparticles, due to their extremely small size and large surface area, possess many interesting properties. Due to this they find novel applications in various areas of electronics, optoelectronic, magnetic, information storage, recording media, sensing devices, catalysis, chemistry, environment, energy, agriculture, medicine and drug delivery, communication technology, aircraft technology, heavy industry and consumer goods etc **[10]**.

In recent years the interest in nanomaterials has increased dramatically due to their unique chemical and physical properties **[8]**. It is also obvious that research interest in nanomaterials has increased exponentially thanks to their unique chemical and physical features, different from those of their bulk materials, but not limited to diffusivity, electrical resistivity, electrical conductivity, strength and hardness, chemical reactivity and diverse and versatile biological activity **[21]**. Nanoparticles are of great interest; because they act as bridge gap between the atomic/molecular structure to the material in bulk as they exhibit completely new or improved properties based on specific characteristics such as size, shape, distribution, ionic strength, capping agent and morphology **[10]**. And also on account of their potential applications which are strongly influenced by their size, morphology and structure **[22]**.

Nanoparticles have been influencing material science considerably. It seems this dominance will continue in the future years, because of fundamental and technological importance with implementing incessant researches in this field. Nanoparticles are considered as building blocks for the next generation of technology with applications in many industrial sectors **[7]**.

The nanoparticles are broadly grouped into organic and inorganic nanoparticles. The latter have gained significant importance due to their ability to withstand adverse processing conditions.

2.3 Noble metal nanoparticles / Metal Oxide nano particles

Noble metal nanoparticles have been the subject of focused research, due to their unique optical, electronic, mechanical and chemical properties that are significantly different from those of bulk materials. For this reason, metallic nanoparticles have found uses in many applications in different fields, such as catalysis, photonics, and electronics **[5, 9]**.
As the metal particles are reduced in size, bulk properties of the particles disappear to be substituted to that of quantum dot, following quantum mechanical rules. It can thus be easily understood that metal nanoparticles chemistry is different from that of the bulk materials **[23]**. Many kinds of nanoparticles, including metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles, and even composite nanoparticles, have been widely used in electrochemical sensors and biosensors. Metal oxide nano particles are also important because of their superior electronic, electrochemical, paint/ink materials, catalytic and magnetic properties **[5, 24]**. Metal oxide nanoparticles, such as copper oxide (CuO), have attracted attention mostly because of their antimicrobial and biocide properties and they may be used in many biomedical applications **[24]**. They have been receiving considerable attention for their potential applications in optoelectronics, nanodevices, nanoelectronics, nanosensors, information storage, and catalysis **[5, 22, 25]**.

Most of relevant recent studies on conductive inks have focused on noble metals exempt from significant oxidation, such as silver and gold nanoparticles (silver slightly oxidizes but its oxide is still a good conductor). In particular, silver with its high conductivity is of great interest and has led to much development with commercially available products. However, these noble metals are too expensive to be used in large quantities. In this context, copper is a good candidate material because it is highly conductive but significantly cheaper than Au and Ag. Copper nanoparticles synthesized in ambient atmospheric temperature and pressure inevitably have surface oxide layers because the CuO phases are thermodynamically more stable than pure copper **[25]**.

Hence, among various metal oxide nanoparticles, CuO Nps has attracted particular attention. Because, it is the simplest member of the family of copper compounds, easy to prepare in pure form and shows a range of useful physical properties such as high temperature superconductivity, electron correlation effects, and spin dynamics **[26, 27]**.

2.4 CuO nanoparticle: properties and uses

2.4.1 Comparision with the bulk Copper Oxides

Copper as a red-brown metal, is in high demand with a usage of more than 20 million tons per year as a bulk.The electronic configuration of copper is $[Ar]3d^{10}4s^1$*and not* $[Ar]3d^9 4s^2$, because a fully–filled 3d sub–shell seems to be a little lower in energy, and marginally more stable **[28]**. Hence copper is a d-block and period 4 element, while oxygen is a p-block and period 2 element. Cu_2O (Cuprous oxide) and CuO (Cupric Oxide) are two important oxide compounds of copper.

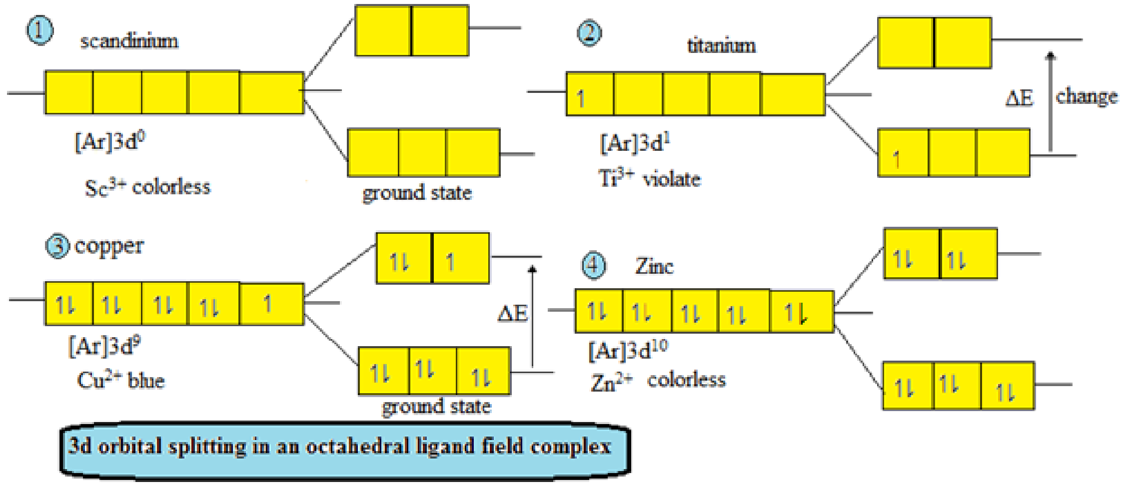
Copper(II) oxide occurs naturally as Tenorite. This black crystalline solid can be obtained by the pyrolysis of the nitrate, hydroxide or carbonate salts. It is also formed when powdered copper is heated in air or oxygen. It has similar color with the CuO Nps.

Table 2.1 Some properties of copper oxides

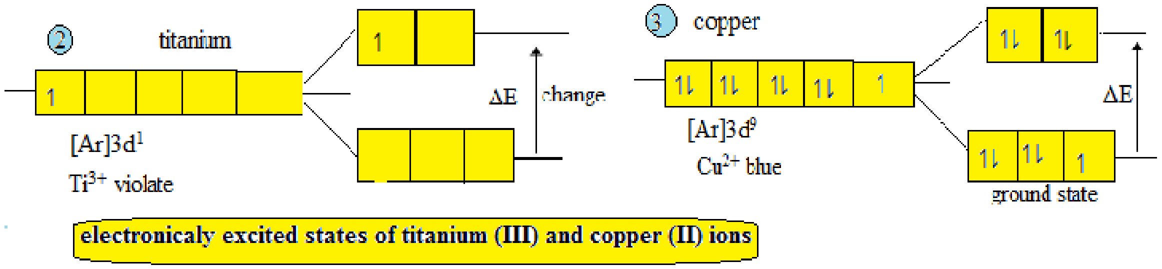
Copper oxides			
Formula	Colour	Oxidation State	Melting Point
CuO	black	Cu^{2+}	1026 ^{o}C
Cu_2O	red	Cu^{+}	1230 ^{o}C

Table 2.1 shows that the two oxides have different surface Plasmon resonance which results in different color. Due to the difference in oxidation state and strength of the ionic bond they form, they have varied melting points **[29]**.

When Copper (II) salts are dissolved in water, the blue tetraaqua copper(II) ion or the hexa aqua copper(II) ion is formed. The scope for a variety of colored compounds arises from the fundamental electronic configuration of the Cu^{2+} ion, namely $[Ar]3d^9$, giving an incompletely filled 3d sub-shellwhich is a criteria for being a true transition metal. That means there is at least one electron that can be promoted to a higher level when the 3d sub-shell is split and at the same time the central metal ion interacts with the ligands. Visible light photons absorbed and color results! The $Cu^{2+}$and Ti^{3+} ions having at least one electron to excite to the higher energy level form colored compounds. On the other hand, metlaic ions like Sc^{3+} and Zn^{2+} which either do not have an electron at the valence orbital or all their orbitals are fully occupied do not show colors in their compounds, as illustrated at fig. 2.1 **[30]**.



Bottom left shows the ground state of copper(II) ion



On the right the diagram shows the excited state of the copper(II) ion

Fig. 2.1 The excitation of an electron from d-orbital of scandium, titanium, copper, and zinc ions

Table 2.2 reveals that copper forms compounds which are solube, slightly solule and insoluble in water. It is helpful to select the salt used as a precursor for the synthesis of copper oxide nanoparticle. Definetly the soluble ones like copper(II)nitrate and copper sulfate pentahydrate salts are better choices.

Copper is also able to form different complexes such as: tetrachlorocuprate(II) $[CuCl_4]^{2-}$ ion(olive-green or yellow), tetraamminediaquacopper(II) ion, $[Cu(NH_3)_4(H_2O)_2]^{2+}$ (deep blue), hexaaqua copprate(II) $[Cu\ (H_2O)_6]^{2+}$ ion (blue).Copper compounds in bulk forms are used as catalysts in the chemical industry. CuO is used in paints. Copper and copper oxide(s) are both standard materials for the production of electrical cables and coins. Copper oxide is used for its anti-microbial properties **[30]**.

Table 2.2 Partial list of solubility of the most commonly used copper salts in water.

Salt	Solubility	%Cu	Color
Copper Metal	Insoluble	100.00	Reddish brown
Copper(I) and (II) Oxide	Insoluble	79.88	Yellow or red
Copper Oxychloride	Insoluble	59.51	Green
Copper Carbonate	Insoluble	57.47	Pale blue
Copper Acetoarsenite	Insoluble	25.07	Garis green
Copper(II) oxalate	Insoluble	41.72	Blue
Copper (II) hydroxide	Insoluble	64.95	Pale blue
Copper(II) sulphide	Insoluble	49.60	Black
Copper(I) Chloride	Very Slightly Sol.	64.18	White
Copper(II) Fluoride	Slightly Soluble	62.58	White
Copper(II) Benzoate Dihydrate	Slightly Soluble	20.78	Bright red
Copper Sulfate Pentahydrate	Very Soluble	39.81	Deep glue
Copper(I)Iodide	Very Slightly Sol.	66.84	White
Copper(II) nitrate	Very Soluble	39.38	Blue

2.4.2 Copper Oxide nanoparticles, CuO Nps: properties and uses

CuO has a monoclinic structure and has p-type semiconductor behavior with a band gap of 1.21-2.51ev **[2, 31]**. It is an important transition metal oxide semiconductor with a narrow band gap, which has attracted considerable interest because of its inexpensiveness and high stability. CuO is also a high dielectric constant semiconductor which is used to make materials of high efficiency solar cells, because their band gap is close to the ideal energy gap for solar cells **[32]**. Water soluble oxide nanoparticles are stable over a wide range of pH and temperature. This excellent stability of the colloidal suspensions makes the application of the water soluble CuO nanoparticles easier in aqueous systems **[5]**. Nanoparticles should be water-dispersible and stable in aqueous media for prolonged periods, to be useful in biological applications.

Oxides of copper have been investigated for decades due to their unique semiconductor and optical properties. It can be reduced to metallic copper when meets hydrogen or carbon monoxide under high temperature. Nano copper oxide attracts more and more people's attention, and become one of the most extensively used inorganic materials. Attention have also been paid to copper oxide because of their potential commercial applications, their efficiency as nanofluids in heat transfer application and their mechanical and biological properties with up-to-date technologies viz. biocidal activity, magnetic phase transitions, gas sensors, catalysis, and superconductivity **[2, 3, 6, 33]**. The wide different applications are according to the physical and chemical properties, such as superconductivity, photovoltaic properties, relatively stable, low cost and the antimicrobial activity. As oxides of transition metals do, the biomedical applications of CuO Nps, in a wider sense, include; antioxidant, antibacterial, anti fouling, anti-biotic, anti-fungal agent, catalysis and so on **[34]**. Are generally used in pesticidal formulationsand several health related applications. In contrast to silver nanoparticles, which have been studied extensively for antibacterial application **[35]**, copper is an essential element for living organisms and may be suitable for biomedical applications. It has been reported that 4% addition of CuO improves the thermal conductivity of water by 20% **[36]**.

For technological applications the detailed understanding of size, morphology controlled emergence of different properties are important **[37 - 39]**. Compared with ordinary copper oxide powder, the nano particles of CuO show superior catalytic activity and selectivity. It has been used as heterogeneous catalysts in many important chemical processes, such as degradation of nitrous oxide with ammonia and oxidation of carbon monoxide, hydrocarbon and phenol in supercritical water **[40]**. The CuO-CeO_2 nanocomposite is a highly efficient recyclable catalyst for the green synthesis of 1,8-dioxooctahydroxanthenes in water.

Even though CuO Nps have proved their use in biomedical applications; the major disadvantage for their use on the medical field is their potentially toxic effects **[41]**. CuO Nps may be toxic for mammalian cells as well as for vertebrates and invertebrates. Some features that can be modified to influence the toxicity of CuO Nps are:

(a) Size: small nanoparticles are more toxic than larger ones.
(b) Surface charge: the toxicity of nanoparticles is enhanced by a positive charge. This positive charge facilitates interactions between cells and nanoparticles.

(c) Dissolution: the dissolution of CuO Nps depends on the temperature and pH of the solution and this has a major influence on their toxicity.

The properties of the CuO Nps depend on the synthesis method selected and it is very important for their applications in various areas, such as biomedical research, which is the most predominant. The most important feature is the size of the nanoparticles (which may be controlled during the synthesis), because it allows the tailored modeling of their optical, catalytic, electrical, and biological properties. Therefore the applied synthesis method, the modulation of the reaction parameters and the composition of bulk material represent key aspects in the direct control of size and direct or indirect control of other important physical, chemical and biological properties. Therefore the synthesis procedure plays crucial role in controlling the size, shape of the nanostructure and hence detecting different properties of the material **[37]**. Recent investigations revealed that properties and potential applications of nanoparticles varied with the phases, sizes and morphologies of these particles. Thus, controlled synthesis of nanomaterial with novel morphologies has gained much attention **[42]**.

2.4.3 Synthesis methods of copper oxide nanoparticles

The design and synthesis of nanostructured materials have attracted much scientific attention due to their interesting size-dependent chemical and physical properties and also the potential technological application **[37, 43]**. The synthesis approach significantly impacts the properties of such nanoparticles and these properties in turn have a significant impact on their biomedical applications.

Methods employed for the synthesis of nanoparticles are broadly classified under two processes. Such as; “Top-down” process and “Bottom-up” process (Fig. 2.2).

Top-down approach: Bulk material is broken down into particles at nanoscale with various lithographic techniques e.g.: grinding, milling etc. Physical techniques such as lithography, laser ablation, sputtering deposition, pulsed electrochemical etching and vapor deposition are among the most common ones.

Bottom-up approach: Atoms self-assemble to new nuclei which grow into aparticle of nanoscale. It involves the synthesis of material, atom by atom, molecule by molecule, or cluster by cluster. **[44]**.

It includes; sol-gel processing, chemical vapor deposition, plasma or flame spraying synthesis, laser pyrolysis, micro emulsion and the green synthesis.

2.4.3.1 Physical and chemical methods

Arrays of conventional methods have been employed in synthesis of nanoparticles. They can be categorized mainly under physical and chemical methods.

Pulsed laser ablation, vacuum vapor deposition and mechanical milling are examples of physical techniques. The absence of solvent contamination in the prepared thin films and the uniformity of nanoparticles distribution are the advantages of the physical approaches in comparison with chemical processes. However the quality of the material is not as high as chemically synthesized materials. It consumes a great amount of energy while raising the environmental temperature around the source material, and requires a great deal of time to achieve thermal stability.

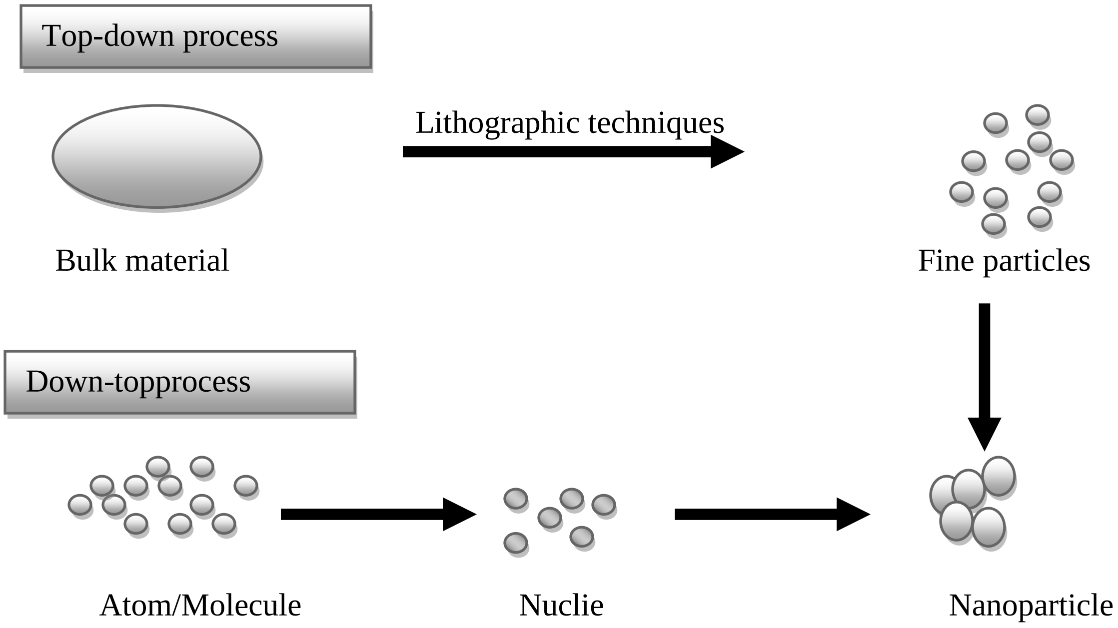


Fig. 2.2 Methods employed for the synthesis of nanoparticles

While micro-emulsion method, arc submerged nanoparticle synthesis system, flame based aerosol methods, sonochemical, hydrothermal, solid state techniques, thermal oxidation route, thermal decomposition, reverse microemulsion method, precipitation method, solution combustion method are chemical methods **[7-10, 19]**.

Currently developed synthesis methods for CuO Nps include; chemical reduction, thermal decomposition, polyol, laser ablation and electron beam irradiation. Among these methods, chemical reduction is the most preferred. Because this method is simple and economical, and it can realize better size and size distribution control by optimizing the experimental parameters, such as the molar ratio of the capping agent with the precursor salt and the ratio of reducing agent with the precursor salt. A chemical reduction method usually involves the reduction of metal salts in some type of solvent and a separate reducing agent **[14]**. Most of the chemical methods use copper(II)sulfate pentahydrate **[45]**, copper(II)chloridedihydrate **[2]**, copper nitrate, **[1, 38]**, coppernitratehydrated **[37]**, copper acetate monohydrate and glacial acetic acid **[43]** as precursors. While sodiumborohydride, Urea andsodium hydroxide solutionshave been used as the main reducing agents **[2, 37, 43, 46 - 48]**.

2.4.3.2 The drawbacks of the physical and chemical techniques

The drawback of the physical technique is that; resultant nanoparticles have defective surface formation, low production rate, high cost of manufacturing and large energy requirement, it raises the environmental temperature around the source material, and requires a great deal of time to achieve thermal stability

Almost all of the possible chemical methods employ toxic chemicals and energy intensive routes which make these choices eco-hazardous and preclude their applications in biology, medicine and clinical applications. They are not safe, where flammable or corrosive reducing agents such as; titanium tetrachloride and hydrazine have been used. Some wet chemical methods employ the toxic organic reactant such as ethylene glycol, while certain methods use additional reducing agent such as sodium. They also involve toxic chemicals and it’s dangerous by products, concentrated reducing agents, high level of radiation, and contamination from precursor chemicals. Hence reproducibility and stability of the nanoparticles with controlled size are very difficult to achieve by popular chemical reduction methods which involve expensive reagent, hazardous reaction condition and need longer time as well as tedious process to isolate nanoparticles which is alarming threat in every aspect of flora, fauna and human health **[4 - 6, 8-14, 16, 33]**. This may pose potential environmental and biological risks in a broader sense. Which is due to the high surface charge as well as high surface area of nanoparticles, and harsh chemicals may also remain adsorbed on to nanoparticles. Releasing these chemicals into the environment may cause adverse effects on organisms including microorganisms, plants, invertebrates and vertebrates including humans at various trophic levels. Chemical synthesis leads to the presence of some toxic chemicals absorbed on the surface that may cause adverse effects in medical applications. Since noble metal nanoparticles are also widely applied to areas of human contact, there is a growing need to develop environmentally friendly processes for the nanoparticle synthesis that do not use toxic chemicals **[9, 11]**. Thus, developing environment friendly protocols is much needed in nanomaterial synthesis.

Therefore, development of clean, biocompatible, nontoxic, eco-friendly, cheap and sustainable methods for nanoparticles, in general and CuO in specific, synthesis deserves merit. As a result the interest in this field has shifted toward ‘green’ chemistry and bio-processor approach **[7, 12]**.

2.4.3.3 Green synthesis of CuO nanoparticles

In order to overcome the negative impacts of the chemical methods, a new science for the research and commitmenthas been coined by Paul T. Anastas in the year 1991as “Green Chemistry”. The concept of Green Chemistry has been defined as the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances and was developed in principles to guide the chemists in their search towards greenness. Thus, it is the practice of chemical science and manufacturing in a manner that is sustainable, safe, as well as non-polluting and consumes minimum amounts of materials and energy while producing little or no waste material. It begins with recognition of the production, processing, usage, and eventual disposal of chemical products that may harm when performed in an incorrect manner. In order to achieve its objectives, Green Chemistry and green chemical engineering may modify or totally redesign chemical products and processes with the objective of minimizing wastes and the use or generation of particularly dangerous materials. It does so in a manner that is economically feasible and cost effective. It is found that, it is the most efficient possible practice of Chemistry and the least costly when all of the costs of the practice of Chemistry, including hazards and potential environmental damage are taken into account.This approach focuses on utilization of environmental-friendly, cost-effective, economical in nature and biocompatible reducing agents for synthesis of nanoparticles, such as; CuO Nps **[7, 12]**. Biosynthesis of metal/metal oxide nanoparticles using environmentally friendly methods without the use of harsh, toxic reducing agents (eg, hydrazine hydrate, sodium borohydride, dimethylformamide, ethylene glycol, and so on), and expensive chemicals are the main principle of green chemistry. It has opened up a new era of safe nanotechnology. Integrating green chemistry principles into nanotechnology has led to the identification of environmentally friendly reagents that are multifunctional, in that they can serve as a reducing agent as well as a capping agent. Moreover, nanoparticles synthesized using biological methods are more compatible for medical use as compared to chemical and physical methods where toxic material may adsorb on the surface of the nanoparticles that may have adverse effect when used for medicinal purpose.

In synthesis of metal oxide nanoparticles by reduction of the corresponding metal ions, there are three areas of opportunity to engage in green chemistry: (i) Choice of solvent (ii) the reducing agent employed, and (iii) the capping agent used as cited in **[22]**.

In green methods, extract of living organisms such as plants, microorganisms and enzymes etc., are used. CuO Nps such synthesized have similar physical as well as chemical properties as prepared by chemical methods **[13]**.

Fig. 2.3 compares conventional synthesis methods such as, physical and chemical methods with those of green synthesis of nanoparticles.

However, the exact mechanism for the synthesis of nanoparticles (including CuO Nps) using biological agents has not been devised yet. But it is suggested that different biomolecules are responsible for the synthesis of nanoparticles. Nevertheless, the ”green” synthesis of nanoparticles of metals with lower reduction potentials still presents a high challenge for scientists in the coming years. Harvesting of endangered species used for the synthesis may pose a risk towards plant kingdom which forms a major challenge. These are considered to be the limitations; otherwise biological methods of synthesizing the Nanoparticles are becoming very popular, effective and acceptable in the nanotechnology world **[48]**.

The research in the field of biosynthesis has been mainly focused on Ag and Au nanoparticles, and there have been very few reports on the synthesis of Cu/CuO nanoparticles. Till *August* 2014, the research in the field of biosynthesis mainly focused Ag Nps [7,699 papers, 59 %], Au Nps followed by ZnO [4,640 papers, 36 %] and finally CuO Nps [690 papers, 5 %]. The few papers in the literature on synthesis of copper have been able to synthesize them in their oxide form **[49]**. This is probably because copper is well known to be susceptible to oxidation and the most successful chemical synthesis of metallic copper Nps is either carried out in organic phases or requires elaborate setups for aqueous phase synthesis to avoid potential oxidation of copper into its oxide forms. The synthesis of pure metallic copper nanoparticles in aqueous phase is therefore still an open challenge for bionanotechnologists **[50]**. But this work was aimed at synthesizing CuO Nps.

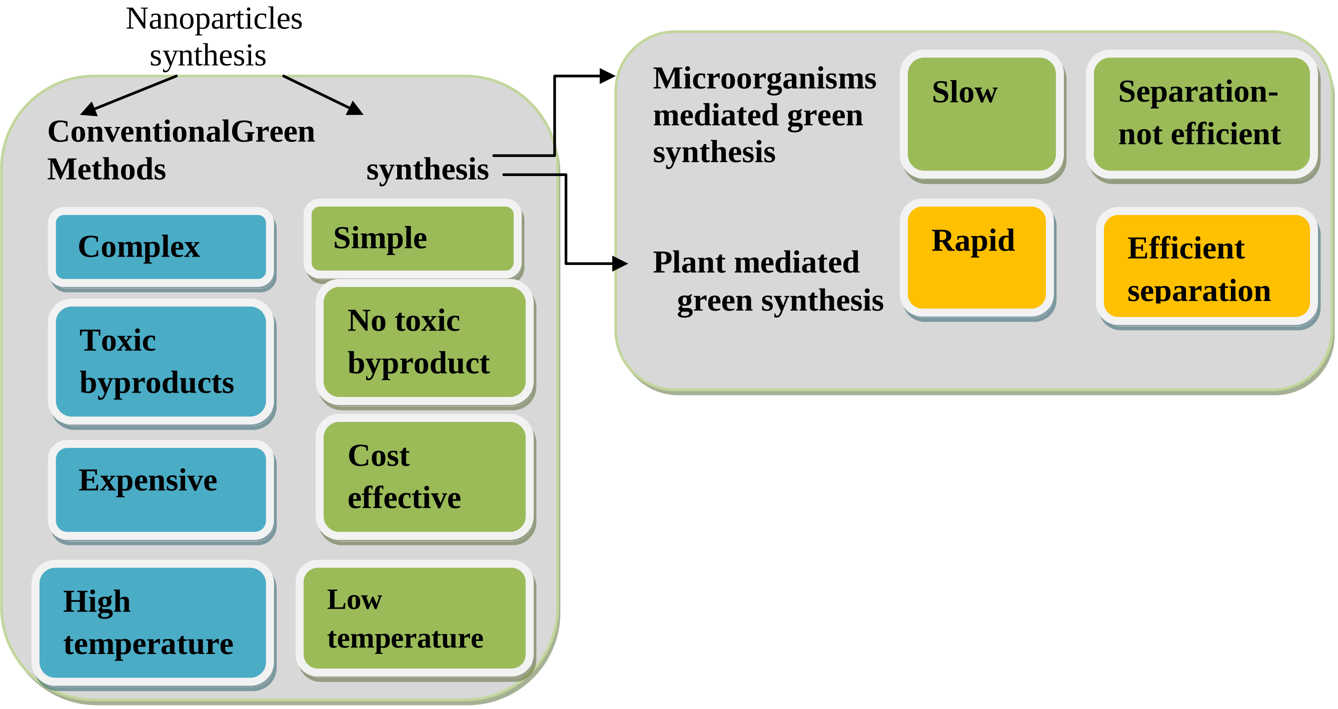


Fig. 2.3 Comparison of conventional methods and Green synthesis methods of nanoparticle

2.4.3.3.1 Microorganism mediated synthesis of CuO nanoparticles

In green nanotechnology, different microorganisms (MOs) produce inorganic materials with properties similar tochemically synthesized materials **[50]**, because of their antioxidant or reducing properties typically responsible for the reduction of metal compounds in their respective nanoparticles. It has been well established that when microbes were kept in harsh toxic metal environment, they evolve mechanism of bioremediation to survive in stringent conditions by transforming toxic metal ions into their corresponding non-toxic forms like metal sulfides or oxides. But the details of the mechanisms involved in nano scale transformation are yet to be established. It is believed that the presence of proteins in the microorganisms' biomass plays an important role in nanoparticle synthesis and stabilization. Besides, it was suggested that some of these proteins are capable of hydrolyzing metal precursors to form metal oxides **[24]**. There has been an important observation that molecules possessing thiol groups are produced by the bacteria in response to oxidative stress. These molecules have been associated with playing the role of capping agents in bacteria-mediated synthesis of nanoparticles where they prevent the oxidation of metal nanoparticles **[49]**. Biosynthesises of CuO Nps using various species of microorganisms have been reported so far. Some of them are; *Penicillium aurantiogriseum*, *Penicillium citrinum* and *Penicillium waksmanii*fungi specieses **[51]**, *Enterococcus faecalis*bacterial species **[52]** and *Phormidium cyanobacterium* bacterial species **[24]**.

However using MOs like fungi, bacteria, yeasts etc. requires lots of maintenance and highly aseptic conditions; involve very complex procedures of maintaining microbial cultures. Due to this it is not very suitable for industrial feasibility. Plants are then relatively better choices **[5, 9, 13]**.

2.4.3.3.2 Plant mediated synthesis of CuO nanoparticles

Synthesis of nanoparticles using plant extracts is a quite novel method and leads to true green Chemistry at a very affordable cost. Nanoparticles produced by plants are more stable with various sizes and shapes, and the rate of production is faster than in the case of microorganisms. It has drawn great attention as an alternative to conventional methods, since plants are more abundant in nature. Some specific plant parts or whole plant especially angiospermic plants (flowering and seed-bearing vascular plants) are used for the great synthesis of nano particle. Maintaining product integrity is the main challenge to the preparation of nanoparticles via green approach. Plants possess components that can act as reducing agent, capping agent, chelating agent and stabilizers for nanoparticles production. Noble metals nanoparticles with high reduction potentials have been successfully synthesized by using different plants extracts.

The green reduction of metal nanoparticles using biomolecules present in plant extracts such as enzymes, proteins, amino acids, vitamins, polysaccharides, saponins and aromatic compounds has been deeply studied in recent years. The phytochemicals (non-nutritive plant chemicals that have protective or disease preventive properties) responsible colud be identified as terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids by IR spectroscopic studies. The polyol and the water soluble heterocyclic phytochemicals components; flavones, organic acids and quinones are responsible for immediate reductionas well as stabilization of nanoparticles **[14]**. Flavonone and terpenoid components of leaf broth are being predicted to stabilize the formation of nanoparticles. Proteins are the ones responsible for capping and stabilizing of nanoparticles. The coordination of carboxylic acids in the protein of plants leaf extract with nanoparticles play a major role on dispersing, stabilizing and capping of nanoparticles **[8]**. Therefore, the reason why plants are selected for the biosynthesis of nanoparticles is that they contain important reducing agents like Citric acid, Ascorbic acids, Flavonoids, Alkaloids, reductases etc.,

Sometimes the synthesis of nanoparticles using plants or parts of plants can prove advantageous over other biological processes by eliminating the elaborate processes of maintaining microbial cultures, valuable alternative for the large-scale production of metal nanoparticles, can overcome the time consuming process of employing microbes, provides a single step technique for the biosynthesis process, used to produce more stable, to produce Nps of various sizes and shapes, faster rate of production and less biohazardness. It is one of the best plat forms for the synthesis of nanoparticles as it is free from toxic chemicals and provides natural capping agents for the stabilization of copper nanoparticles. Hence these days plant mediated synthesis of metal nanoparticles is receiving lots of attention **[11, 7]**.

Recently, several researchers exploited inactivated plant tissue, plant extracts, exudates, gums and other parts of plants for the synthesis of CuO Nps. Biosynthesis of CuO Nps by various plants have been reported sofar, such as; *Acalypha indica* **[53]**, *Phyllanthus Amarus* Leaf **[54]**, *T.arjuna* bark extract **[55]**, *Calotropis gigantean* leaf extract **[56]**, *Malva sylvestris* leaf extract **[8]**, aqueous extract of flowers of *Cassia alata* **[5]**, *Ocimum Sanctum* leaf Extract **[57]**, *Gloriosa superba L.* **[58]**, *Carica papaya* **[4]**, *Tabernaemontana divaricate* leaf extract **[59]**, *Aloe barbadensis* **[60]**, *Tinospora cordifolia* **[61]** are few of them.

As explained before, compared to other metal nanoparticles such as Silver, Gold, Zinc nanopartilces etc, CuO Nps has been biosynthesized less. Hence green synthesis of CuO Nps provides more CuO Nps to be available cheaply so as to be used in different fields of applications. One way of doing this is using more local and easily available plants. Therefore Khat has been selected for such a purpose. No literature so far has reported using Khat leaf extract for the synthesis of CuO Nps. This work is the first to report the synthesis of CuO Nps using Khat (*Catha edulis Forsk*) leaf extract. Khat, for years, has been chewed by people around different parts of the world forits stimulating activity.

Fig. 2.4 Fresh Bahir Dar Khat leaf

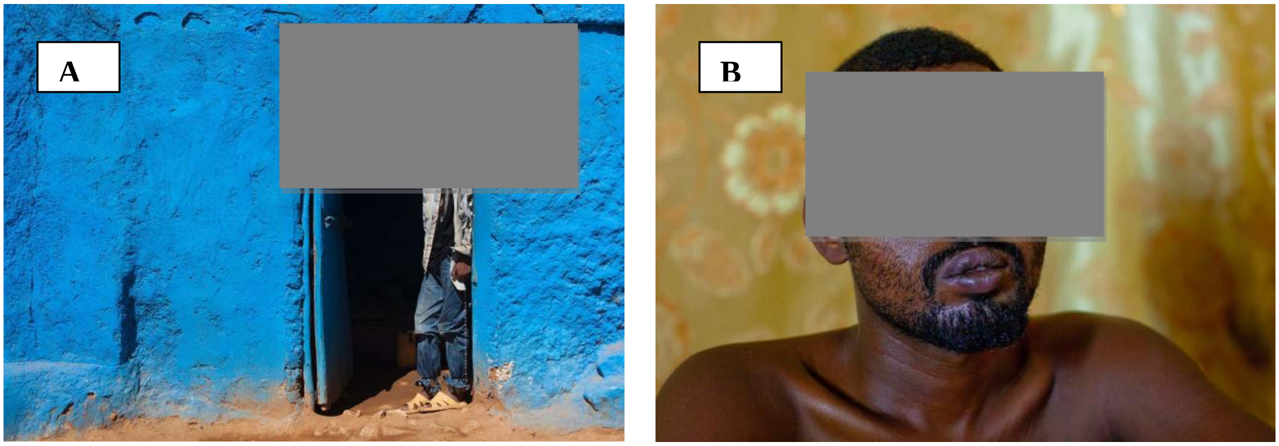
Khat is an evergreen plant grown by grafting and cultivated as a bush or small tree. It is mainly found in Ethiopia, Yemen, Somali, Sudan, Madagascar and South Africa, but it is also seen in Turkestan and Afghanistan. It grows at altitudes of 1500 - 2500 meters and it usually reaches a height of 3 - 8 meters. Under optimal conditions the tree may reach 15 - 20 meters. The tree requires about 10 years for attaining maturity. But the leaves and shoots are already harvested after 3 - 4 years. Khat is harvested throughout the year. A tree gives two crops a year. *Catha edulis*, the Arabic Khat, the Amharic or Ethiopic Tschat, or the English Qat, was probably grown and used as a food stimulant in the present area of the Ethiopian highlands around Harar from the earliest times.

It was first classified by Forskal, the botanist who started with the Niebuhr mission into Yemen in 1762. He described the plant under the name of *Catha edulis* and classified it along with an additional species then designated as *spinosa.* Forskal noted its cultivation along with *Coffea* and reported that the Arabs in Yemen ate the green leaves and ascribed medicinal virtues to the plant material. Present-day classification places the genus *Catha* in the family *Celastraceae* and recognizes but one species, *Catha edulis,* Forsk. Quartin-Dillondesignated the plant material as the des *Abyssins or Abybsiniantea*and it has also been called African or Arabian Tea, ascited at **[62]**.

The impacts of Khat are the observed when tender leaves and shoots are chewed. Fresh leaves are preferred as the psychoactive properties decrease after harvesting, in particular during drying of the plant material. This is caused by the decomposition of cathinone into an inactive ‘dimer’. Khat is harvested in the early morning hours and sold at markets in late morning. To preserve its freshness, Khat is wrapped in banana leaves and sold as bundles (30 - 40 cm) of twigs, stems and leaves **[63]**.

The major effects include those on the gastro-intestinal system and on the nervous system. Constipation, urine retention and acute cardiovascular effects may be regarded as autonomic (peripheral) nervous system effects; increased alertness, dependence, tolerance and psychiatric symptoms as effects on the central nervous system. As cathinone, and to a lesser extent cathine, are held responsible for the effects of Khat on the nervous system, the effects of the many other constituents of the Khat plant are frequently overlooked.

Khat chewing also induces a state of euphoria and elation with feelings of increased alertness and arousal. This is followed by a stage of vivid discussions, loquacity and an excited mood. Thinking is characterized by a flight of ideas but without the ability to concentrate. However, at the end of a Khat session the user may experience depressive mood, irritability, anorexia and difficulty to sleep **[64]**. Lethargy and a sleepy state follow the next morning. The major toxic effects include increased blood pressure, tachycardia, insomnia, anorexia, constipation, general malaise, migraine and impaired sexual potency in men. Mild depressive reactions have been reported during Khat withdrawal or at the end of a Khat session. As can be observed at fig. 2.6, frequent use of high doses may evoke psychotic reactions which might lead to mental illness. As can be observed from fig. 2.5, it can also result in different types of mouth diseases **[64, 65]**.



[redacted]

[redacted]

Fig. 2.5 A. A man from Harar in serious mental illness after chewing too much kept in chain

B. A Somali man suffering from mouth disease because of Khat

The environment and climate conditions determine the chemical profile of Khat leaves. In the Yemen Arab Republic, about 44 different types of Khat exist originating from different geographic areas of the country. In Ethiopia depending upon geographical location, various chemotypes of Khat are cultivated and these chemotypes differ in color, height and size of the young shoots of the leaves and the plant as a whole. They are marketed under different names such as;Wondo Chat from Wondo, Gelemso and Aweday from Harar, Bahir Dar Khat from Bahir Dar, Sebeta from Sebeta ... etc **[66]**. Its taste varies from one kind to another and depends on the tannic acid content. Khat leaves have an astringent taste and have an aromatic odour. The young leaves are slightly sweet.

Many different compounds are found in Khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals **[63, 64]**. The phenylalkylamines and the cathedulins are the major alkaloids. The cathedulins are based on a poly-hydroxylated sesquiterpene skeleton and are basically polyesters of euonyminol. Recently, 62 different cathedulins from fresh Khat leaves were characterized. The Khat phenylalkylamines comprise cathinone and cathine and norephedrine (Fig. 2.6). Cathinone is mainly found in the young leaves and shoots. During maturation, cathinone is metabolized to cathine. Other phenylalkylamine alkaloids found in Khat leaves are the phenylpentenylamines merucathinone, pseudomerucathine and merucathine. These seem to contribute less to the stimulant effects of Khat **[64]**.

Cathinone is unstable and undergoes decomposition reactions after harvesting and during drying or extraction of the plant material. Decomposition leads to a 'dimer' (3,6-dimethyl-2,5-diphenylpyrazine) and possibly to smaller fragments. As cathinone is presumably the main psychoactive component of Khat, this explains why fresh leaves are preferred and why Khat is wrapped up in banana leaves to preserve freshness. The phenylalkylamine content of Khat leaves varies within wide limits. Fresh Khat from different origin contained on the average 36 mg cathinone, 120 mg cathine, and 8 mg norephedrine per 100 gram of leaves **[67 - 70]**.

Cathinone $C_9H_{11}NO$ cathine and norephedrine: $C_9H_{13}NO$

Fig. 2.6 The two phenylalkylamines alkaloids

The phenolic acids and flavonoids function as reducing agents.Water-soluble flavonoids are specifically responsible for bio-reduction and stabilization of nanoparticles. Flavonoids and phenolic acids are secondary metabolites of plants (organic compounds tha are not directly involved in the growth, development or reproduction of the plant) with polyphenolic structure. Thus, flavonoid groups of poly phenolic compounds have low toxicity in mammals and are widely distributed in plant kingdom **[71]**. Major dietary sources of flavonoids in the form of flavonols, flavones, isoflavones, flavonones are; tea, red wine, apple, tomato, cherry, onion, thyme, parsley, soya beans, and other legumes, grape fruit, orange, lemon, ginkgo, neem etc. Flavonoids contain C_{15} atoms in their basic nucleus and C_{15} atoms composed of two aromatic rings linked through a heterocyclic pyrane ring (Fig. 2.7). All flavonoids share the basic C_6- C_3- C_6 Structural skeleton, consisting of two aromatic C_6 rings and a heterocyclic ring that contains one oxygen atom.

Phenolic compounds confer unique taste, flavor, and health-promoting properties found in vegetables and fruits. The term "phenolic acids", in general, designates phenols that do possess one carboxylic acid functional group, more over the reason for including phenolic acids in the family of plant poly phenols lies in the fact that they are bio-precursors of poly phenols and, more importantly, they are metabolites of poly phenols.

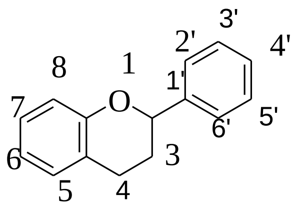


Fig. 2.7 Basic structure of flavonoids

The reduction activity of phenolic and flavonoid compounds depends on the number of free hydroxyl groups in the molecular structure, which would be strengthened by steric hindrance **[72]**. Naturally occurring phenolic acids contain two distinctive carbon frame works; the hydroxycinnamic and hydroxybenzoic structures.

From the many Khat types cultivated in Ethiopia, “Bahir Dar Khat” was the one selected (Fig. 2.4). It is known for its highly stimulating activity. It was expected to have higher amounts of the photochemicals. It was then believed that it is a favorable Khat type to reduce the copper ions of the salt solution to copper metal for the synthesis of the CuO Nps.

Considering the above health effects of the Khat, using the plant for the synthesis of the CuO Nps will be one of the ways of making it less harmful and even more advantageous. Even though it is difficult to say that Khat is a cheap plant; it is much available in most part of our country. Therefore, its availability is also another factor taken in to account while selecting it for such a purpose. Khat leaves posses biomolecules such as phenolic acids, flavonoids, and alkaloids which could be used as reducing, stabilizing, chelating and capping agents to react with copper ions and as scaffolds to direct the formation of CuO Nps in solution.

Although the exact mechanism for the synthesis of nanoparticles using plant extracts such as Khat has not been devised yet, it was suggested that different polyol components are responsible for the synthesis of the nanoparticles.The FT-IR spectrum is usually used to confirm the formation of CuO nanoparticles in the presence of plant extracts. It was suggested, in one work, that well dispersed copper nanoparticles may have obtained through the reduction of Cu^{2+} using tea leaf and coffee extracts which contains epigallocatechin gallate (EGCG). It belongs to the flavonoids found in green and black tea and in red wine. The lone pair of electrons in the polar groups of EGCG might have occupied the two sp orbits of the copper ion to form a complex compound. Thus, EGCG was capped with the copper ions to synthesize Cu(0) nanoparticles through reduction of Cu^{2+} inside the nanoscopic templates (Scheme 2.1). In the presence of nanoscopic templates, small copper nanoparticles were initially formed which on further oxidation gave copper oxide nanoparticles **[73]**.This definitely reveals that flavonoids, poly phenols and other phytochemicals found in plant extracts are the ones responsible for the synthesis of nanoparticles like copper oxide by firstly donating electrons to the metallic cation and stabilizing the newly formed nanoparticles. Since Khat is one of the angiosperm plants which is composed of phytochemicals like phenolic acids, flavonoids, and alkaloids, it was expected to be helpful in synthesizing the CuO Nps by donating electrons to the copper(II) cation of the copper salt solution.

$$Cu^{2+} + 2e^{-} \rightarrow Cu^{0} + \text{Air}(O_2) \rightarrow CuO$$

Scheme 2.1 The possible reaction mechanism for the formation of CuO Nps using plant extract

2.4.4 Antibacterial activity of the CuO nanoparticles

Nanoparticles with one dimension of 100 nm or less in size are now being increasingly utilized for their medical applications and are of great interest as an alternative approach to control infectious agents. Recently, much attention has been paid towards the use of nanoparticles as an alternative to antibiotics due to their distinct advantages over conventional antimicrobial agents. Focus on microbicidal properties of nanomaterials have also increased because microorganisms are developing more resistance towards existing antibiotics **[73]**. Current advances in the field of nanobiotechnology, particularly the ability to prepare metal oxide nanomaterials of specific size and shape, are likely to lead to the development of new antibacterial agents.

Metal based nanoparticles are demonstrated to be excellent antimicrobial agent and exhibit broad spectrum antimicrobial activity against bacteria, fungi and viruses. With recent advances in nanotechnology, various types of metal and metal oxide nanoparticles with antimicrobial (microbiocidal or growth-inhibiting) activity have been synthesized **[74]**. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface area to volume ratio, which enable them to interact closely with microbial membranes **[6]**. Metal nanoparticles with bactericidal activity can be immobilized and coated on to surfaces, which may find application in various fields, i.e., medical instruments and devices, water treatment and food processing. Metal nanoparticles may be combined with polymers to form composites for better utilization of their antimicrobial activity.

Highly ionic nanoparticulate metal oxides, such as CuO, may be particularly valuable antimicrobial agents as they can be prepared with extremely high surface areas and unusual crystal morphologies. Metal nanoparticles containing magnesium oxide, copper, silver, iron, zinc oxide, and nickel oxide do exhibit antimicrobial properties. The antimicrobial activity has been observed to vary as a function of surface area in contact with the microbe; therefore nanoparticles with large surface area ensure a broad range of reactions with the bacterial surface. Owing to their high antibacterial properties, nanoparticles of silver, oxides of Zinc, titanium, copper, and iron are the most commonly used nanoparticles in antimicrobial studies. Furthermore, these nanoparticles have been used to deliver other antimicrobial drugs to the site of pathological process. Silver nanoparticles are known to possess high antimicrobial activity but it is also one of the costy metals. Therefore cost effective materials with equivalent activity have to be identified. Therefore copper can be an excellent candidate for production of antimicrobial textiles. Furthermore CuO Nps demonstrated superior antibacterial activity compared to the silver nanoparticles **[75]**.

Limited information on the possible antimicrobial activity of nano CuO is available. Copper used as an antimicrobial agent for decades has revealed a strong antibacterial activity and was able to decrease the MOs concentration by 99.9%. Copper /copper oxide Nps are known to exhibit wide range of antibacterial activity against different strains of gram positive (thick peptidoglycan layer) and gram negative bacteria (thin peptidoglycan layer) **[76]**. CuO Nps were effective in killing a range of bacterial pathogens involved in hospital-acquired infections. The antibacterial activity of CuO Nps seems to be different depending on the particularities of bacteria cells. For examples, their cellular walls seem to impact the antimicrobial effect of CuO Nps, Gram character being a key aspect. It was reported that 100% of *Escherichia coli* cells, which are Gram negative, were killed when a concentration of CuO Nps higher than 9.5% was used, while for the Gram positive species *Staphylococcus aureus* the killing ability was lower. It was also reported that the antimicrobial properties depend on the surface properties and size of nanoparticles. It seems that small particles with a large surface area have better antibacterial activity, as compared with larger ones **[77]**.

Although only a few studies have reported on the antibacterial properties of CuO Nps, they have shown CuO Nps have a significant promise as bactericidal agent **[78]**. CuO Nps acts as potential antimicrobial agent against infectious organisms such as *Escherichia coli*, *Bacillus subtilis*, *Vibria cholera*, *Pseudomonas aeruginosa*, *Syphillis typhus*, and *Staphylococcus aureus* **[79]**.

The CuO Nps synthesized using plant extract of *Magnolia*, *Syzygium aromaticum*, *Tridax procumbens* were tested against *Escherichia coli*, and they showed higher antibacterial activity on cells after 24 h growth **[80]**. Therefore, it was clear that green synthesized CuO Nps could be used as an antibacterial agent. And hence the Khat synthesized CuO Nps was expected to be effective in killing bacterias.

2.4.4.1 The possible mechanism of antimicrobial activity of CuO Nps

According to different researchers the exact mechanism behind is not known and needs to be further studied. But there are many hypothesiszed ways of expainining it. The antimicrobial activity of CuO Nps is linked with ions that are released from nanoparticles. The activity is further enhanced by its small size and high surface area to volume ratio that it allows them to interact closely with microbial membranes. Typically, antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism, there by inhibiting the synthesis of functional biomolecules or impeding normal cellular activities. Nanoparticles attached to the microbial surfaces can decrease both cell mobility and nutrient flow between the cell's exterior and interior compartments **[81]**. Differentiating copper from other trace metals, results in the production of hydroxyl radicals that subsequently bind with DNA molecules and lead to disorder of the helical (zigzag) structure by cross-linking within and between the nucleic acid strands and damage essential proteins by binding to the sulfhydryl amino and carboxyl groups of amino acids. This denatures the protein and makes the enzymes ineffective. It inactivates cell surface proteins necessary for transport of materials across cell membranes, thus affecting membrane integrity and membrane lipids. Copper ions inside bacterial cells also disrupt biochemical processes. Based on all of prevous studies, the denaturing effect of copper ion on proteins and enzymes in microbes gives CuO its antimicrobial characteristics. The mechanism of toxicity of CuO Nps in the cellular membrane was suggested to follow a Trojan horse-type mechanism (Fig. 2.8). If these nanoparticles are soluble they can penetrate the membrane cancelling the barrier function of the membrane. After entering the cells, nanoparticles are able to dissolve at acidic intracellular pH (4.5), and metal ions produce pores in the membrane **[82]**.

It is also assumed that the high affinity of metallic nanoparticles towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, metallic nanoparticles (such as CuO Nps) can react with sulfur-containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. The general understanding is that metallic nanoparticle of typically less than 20 nm diameters get attached to sulfur-containing proteins of bacterial cell membranes leading to greater permeability of the membrane, which causes the death of the bacteria.

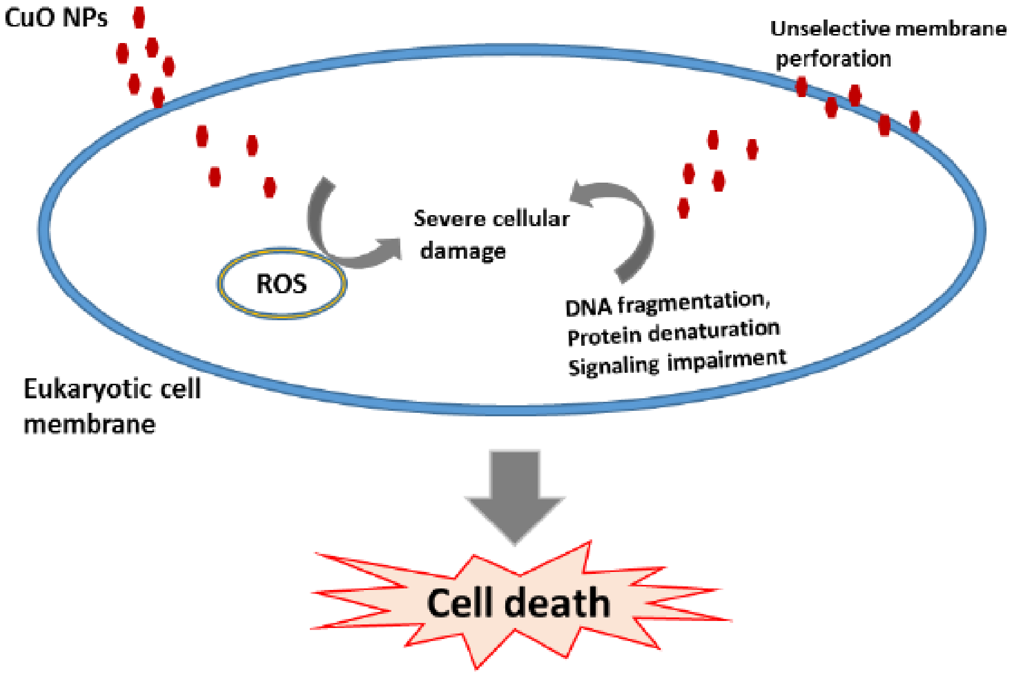


Fig. 2.8 Toxicity mechanism of CuO Nps in eukaryotic cell

In this work antibacterial activity of CuO Nps was examined by disc diffusion method for only two bacterial specieses. This was quantitatively done by measuring the clear zone of inhibition. Generally, both Gram-positive and Gram-negative bacterial strains are thought to present a major public health problem **[82]**. But the antibacterial activity of the CuO Nps was examined against only the two gram negative bacterias.

3. Materials, Chemicals and Methods

3.1 Materials, Chemicals and Instruments

Copper(II)sulphate (98.5%),Ferric chloride (99%), Hydrochloric acid (35.4%), Sulphuric acid (98%)(All are products of Loba Chemie Pvt.Ltd, India), Ethanol (99.5%, UNI-CHEM Chemical Reagents), Methanol, Gentamicine (Abcek adtek(P) Ltd), KBr (Uvasol, Germany) Agar Hilten Muller (Oxoid CM, UK), Chloroform(99.9%, Fisher Scientific UK Limited, UK), Sodium Hydroxide (98%), Ammonia solution (25%) (Both of them are products of Blulux Laboratories (P) Ltd), Benedict’s solution, Magnesuim Chloride (98%), Iodine solution (80%), Potassium Iodide (99%) (All of them are produced by Abron Chemicals, India). UV-Vis spectrometer (Aglient technologies, Cary 60UV-Vis) and FT-IR (Perkin Elmer) were alos used.

3.2 Experimental Procedures

Typically, a plant extract mediated bio-reduction involves mixing the aqueous extract with an aqueous solution of the appropriate metal salt. The synthesis of nanoparticle was completed with in few minutes. But results after hours of reactions have also been used to analyze the effect of reaction time on the formation and stability of the CuO Nps. All the glass wares were washed with distilled water and dried in oven. De-ionized and distilled water were used in all of the experimental works.

3.2.1 Preparation of Khat (Catha edulis) Leaf Extract

Fresh green plants of Khat were collected from one of the Khat shops at Bahir Dar city, Ethiopia where the name ’’Abay Dar Khat’’ was given for it. The Khat leaf was collected and used at the months March and May. The leaves were thoroughly washed and rinsed with distilled water, and then finely cuted. The leaves were then homogenized using mortar and pestle. The extraction was carried out by mixing 2 g of the grinded Khat with 100 ml of de-ionized water and was immediately stirred using magnetic stirrer for two-three minutes. It was then heated for about two minutes at a temperature of 50 - 60 ^{0}C. After wards, it was allowed to cool down to room temperature. The mixture was, firstly, filtered using *Abron Qualitative* filter paper and then using the *Quantitative* filter paper, after which the collected filtrate was kept at 4 °C for the nano CuO synthesis **[2]**.

3.2.2 Phytochemical screening-Qualitative analysis

3.2.2.1. Test for Alkaloids: (Wagner's test: Iodine- Potassiumiodide solution)

1.2 gm of Iodine and 2 gm of $H_2SO_4$was mixed and was diluted to 100 ml. 10 ml of the alcoholic extract was acidified by adding 1.5% (v/v) of HCl and a few drops of Wagner's reagent was added. Formation of yellow or brown precipitate was assessed to confirm the presence of alkaloids.

3.2.2.2. Test for Glycosides

A small amount of alcoholic extract was dissolved in 1 ml of water and aqueous NaOH solution was dissolved in 1 ml of water and it was added to the extract. Formation of reddish brown color was taken as an indicator for the presence of glycosides.

3.2.2.3. Test for Tannins: (Ferric chloride test)

1.0 ml extract was stirred with 1.0 ml ferric chloride; the occurrence of a greenish black precipitate for the presence of tannins was checked.

3.2.2.4. Test for Flavonoids

0.2 ml extract was added to 2 ml 10% (m/v) $FeCl_3$ solution and the mixture was shaken. A wooly brownish precipitate for the presence flavonoid was checked.

3.2.2.5. Test for Saponins

0.2 ml extract was mixed with 5.0 ml distilled water, shaken for 20 minutes and the persistence of foams for the presence of saponins was checked.

3.2.2.6 Test for Steroids (Salkowski test)

2 ml of chloroform extract and 1 ml of concentrated H_2SO_4 acid were added carefully along sides of the test tubes. The mixture was then examined to reveal a red color in the chloroform layer which confirms the presence of steroids.

3.2.2.7. Test for Phenols

The Khat extract was treated with 3 - 4 drops of ferric chloride solution. It was then left to form bluish black color that indicates the presence of phenols.

3.2.2.8. Test for Carbohydrates: (Benedict test and Iodine test)

Few drops of Benedict solution was added in to the plant extract and it was checked for its formation of brick read color which is used to confirm the presence of glucose and few drops of Iodine was added in other extract where a dark blue color was expected to confirm the presence of starch **[83]**.

3.2.3 Synthesis of Copper Oxide Nanoparticles

In a typical reaction mixture, 45 ml of aqueous 1 mM $CuSO_4.5H_2O$ was treated with 2 ml extract of 2% (m/v) Khat and stirred magnetically at room temperature; untill the light blue color was changed to light green. Then the mixture was then heated at 50 - 60 °C for 6 minutes. It was allowed to cool down. Afterwards, the mixture was treated with 1 MSodium hydroxide drop by drop for a specific pH of 11. Finally the nanoparticle formed was characterized using the visual observation, the UV-Vis and FT-IR spectras **[8]**. At last, it was examined for its antibacterial activity. The whole procedure has been summerized at fig. 3.1.

3.2.4 Characterization techniques of the synthesized CuO Nps

The synthesized CuO Nps was characterized byvisual observation, UV–Visible absorption spectroscopy and Fourier Transform Infrared spectroscopy analysises.

During synthesis of the nanoparticles, every color change for every step was noticed carefully. The colors changes before and after Khat extract and the precursor were mixed analyzed.

The next characterization method was using the UV-Vis spectroscopy. To do this, Samples of the mixture were collected periodically to monitor the completion of bio-reduction of $Cu^{+\,2}$ ions in aqueous solution and subsequent scanned in UV-visible (UV-Vis) spectra, between wave lengths of 200 to 700 nm in a spectrophotometer, with a resolution of 1 nm. UV-Vis spectras were recorded and analyzed **[20]**.

To Use FT-IR spectroscopy, adrop of the Khat extract was mixed with the KBr powder so as to form a paste. Afterwards the paste was taken in to the FT-IR and got scanned at a resolution of 8 nm in a wave number range of 0 to 4500 cm^{-1}. The same was done for the CuO Nps formed.

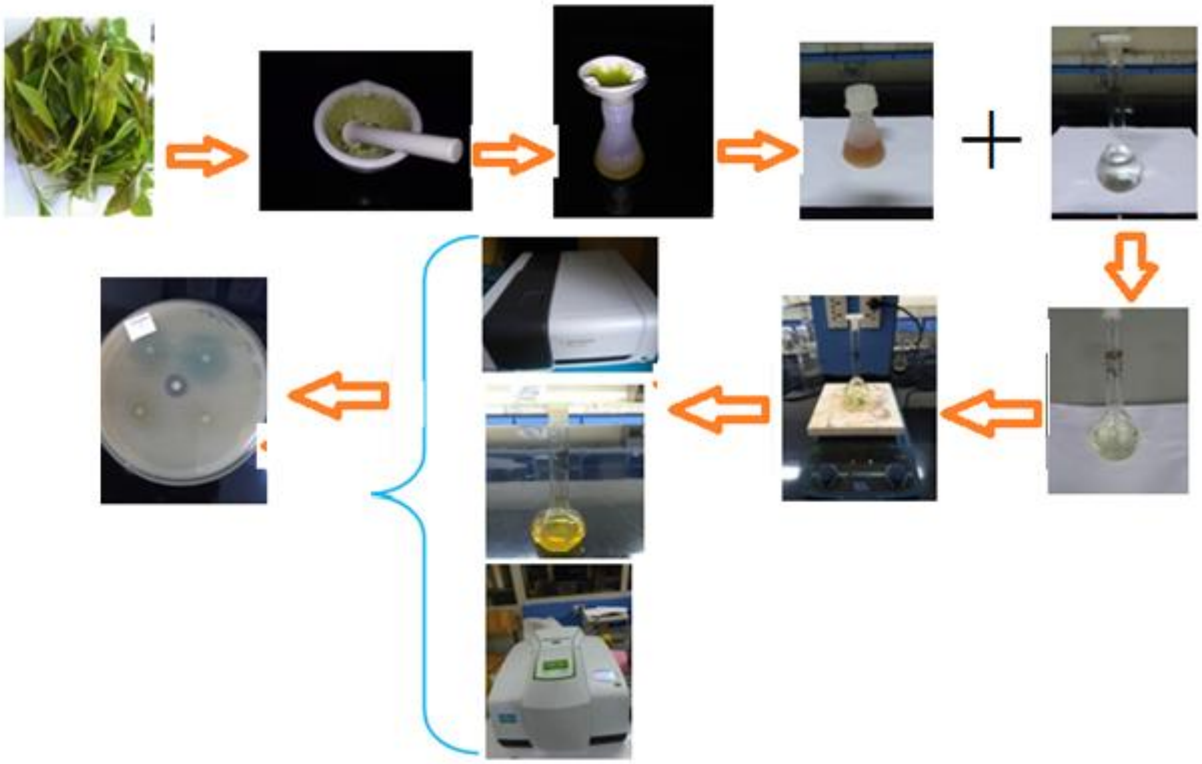


Fig. 3.1 The general scheme of the CuO Nps synthesis, characterization and application

3.2.5 Antibacterial Activity of the CuO Nps

Disc diffusion method

The discs were soaked with double distilled water, 2 % (m/v) Khat extract, copper sulphate penta hydrated solution and solution containing CuO Nps of each type separately. Gentamicine was placed at the center of the plates, used as a positive controller. Then the discs were air dried in sterile condition. The plates containing nutrient agar media were prepared by swabbing them with the microbial cultures (*Salmonella typhimurium* and *Escherichia coli)*. Previously prepared discs were placed on each part of the plate. The discs were placed in the following order: disc soaked with double distilled water as negative control, disc soaked with solution containing plant leaves mediated synthesized CuO Nps, disc soaked with plant leaves extract, and disc soaked with 1 mM copper sulphate solution. The plates were incubated at 37 °C for 24 hr. Then, the maximum zone of inhibition were observed and measured for analysis against each type of test microorganism **[20]**.

3.3 Instrumentation of the spectroscopies

3.3.1 UV-Visible spectroscopy Instrumentation

Fig. 3.2 The UV-Vis spectrometer

UV- Vis spectroscopy is type of absorption spectroscopy in which light of ultra-violet and vissible region (200 - 800 nm) is absorbed by the molecule (Fig. 3.2). Absorption of the radiations results in the excitation of the electrons from the ground state to higher energy state. The energy of the radiation that are absorbed is equal to the energy difference between the ground state and higher energy states, $\Delta E = h\nu$. Generally, the most favored transition is from the highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO). For most of the molecules, the lowest energy occupied molecular orbitals are s-orbital, which correspond to sigma bonds. The p-orbitals are at somewhat higher energy levels, the orbitals (nonbonding orbitals) with unshared paired of electrons lie at higher energy levels. The unoccupied or antibonding orbitals ($\pi^*$and δ^*) are the highest energy occupied orbitals. In all the compounds (other than alkanes), the electrons undergo various transitions. Some of the important transitions with increasing energies are: nonbonding to π^*, nonbonding to δ^*, π to π^*, δ to $\pi^*$and δ to δ^*as illustrated in fig. 3.3 **[84, 85]**.

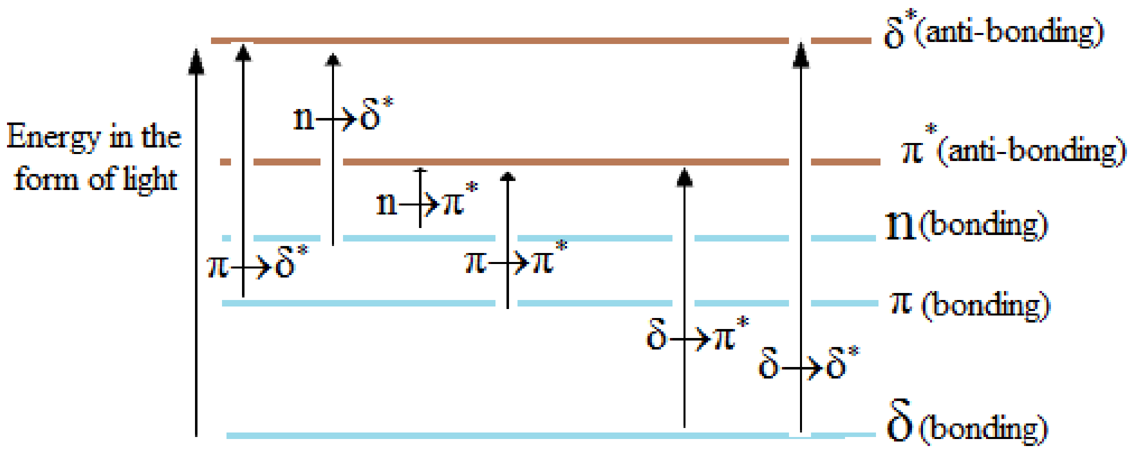


Fig. 3.3 Different transitions between the bonding and anti-bonding electronic states when light energy is absorbed in UV-Visible Spectroscopy

When a sample is exposed to light energy that matches the energy difference between possible electronic transitions within the molecule, a fraction of the light energy would be absorbed by the molecule and the electrons would be promoted to the higher energy state orbital. A spectrometer records the degree of absorption by a sample at different wavelengths and the resulting plot of absorbance (A) versus wavelength (λ) is known as a spectrum. The wavelength at which the sample absorbs the maximum amount of light is known as λ_{max} **[86]**. For example, the spectrum at fig. 3.4 shows the λ_{max} value of pure *Ficus microcarpa* extract at 280 nm **[87]**.

Certain chemical groups or entitities are susceptible to absorb light due to the electronic configuration of the electrons in the functional group. These groups are known as chromophores **[86]**. Chromophore is also defined as any isolated covalently bonded group that shows a characteristic absorption in the ultraviolet or visible region (200 - 800 nm).

Instruments for measuring the absorption of UV or visible radiation are made up of the following components;

1. Sources (UV and visible)
2. Wavelength selector (monochromator)
3. Sample containers
4. Detector
5. Signal processor and readout as can be observed at fig. 3.5.

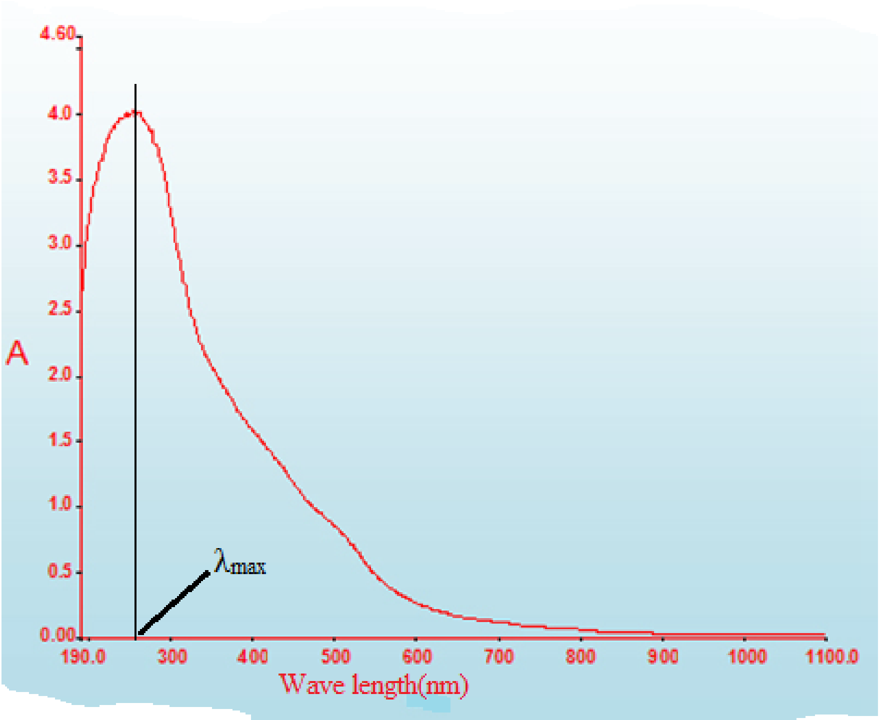


Fig. 3.4 The UV-Vis spectrum of pure *Ficus microcarpa* leaf extract

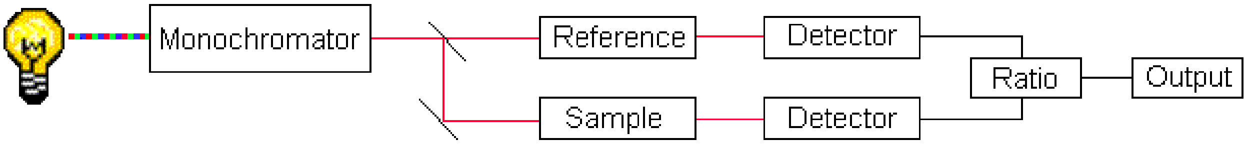


Fig. 3.5 Scheme of the UV-Visible Spectroscopy

The tungsten filament lamp is commonly employed as a source of visible light. This type of lamp is used in the wavelength range of 350 - 2500 nm. The containers for the sample and reference solution must be transparent to the radiation which will pass through them. Quartz or fused silica cuvettes are required for spectroscopy in the UV-Vissible region. These cells are also transparent in the visible region. Quartz cuvettes must be used for those substances or chromophers which do absorb radiation of wavelengths less than 350 nm. Silicate glasses can be used for the manufacture of cuvettes for use between 350 and 2000 nm. The photomultiplier tube is a commonly used detector in UV-Vis spectroscopy. It consists of a photoemissive cathode (a cathode which emits electrons when struck by photons of radiation), several dynodes (which emit several electrons for each electron striking them) and an anode. The alternating current generated in the photocells is transferred to the amplifier. The amplifier is coupled to a small servometer. Most of the time amplifier is coupled to a pen recorder which is connected to the computer. Computer stores all the data generated and produces the spectrum of the desired compound.

There are four types of shifts observed in the UV spectroscopy;

a) Bathochromic effect- This type of shift is also known as red shift. It is an effect by virtue of which the absorption maximum is shifted towards the longer wavelength due to the presence of an auxochrome or change in solvents. The nonbonding to π^* transition of carbonyl compounds observes bathochromic or red shift.

b) Hypsochromic shift- This effect is also known as blue shift. It is an effect by virtue of which absorption maximum is shifted towards the shorter wavelength. Generally it is caused due to the removal of conjugation or by changing the polarity of the solvents.

c) Hyperchromic effect- it is an effect by virtue of which absorption maximum increases. The introduction of an auxochrome in the compound generally results in the hyperchromic effect.

d) Hypochromic effect- It is defined as the effect by virtue of intensity of absorption maximum decreases. Hyperchromic effect occurs due to the distortion of the geometry of the molecule with an introduction of new group.

UV spectroscopy obeys the Beer-Lambert law, which states that: when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution. The expression of Beer-Lambert law is;

$$A = \log\left({}^{I_0}/_{I}\right) = \varepsilon c l$$

Where, A=absorbance,

I_0= intensity of light incident upon sample cell,

I = intensity of light leaving sample cell,

c = molar concentration of solute

l = length of sample cell (cm)

ε = molar absorptivity

From the Beer-Lambert law it is clear that greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of light absorption. This is the basic principle of UV-Vis spectroscopy. Because, in spectrochemical methods only the absorbed radiation is measured **[86]**.

Different research results have shown the formation of various nanoparticles from different salt solutions that give characteristic peaks at 24 hrs time interval at different absorptions using UV-Visible spectroscopy. CuO Nps show characteristic absorption peaks at the range of 200 - 800 nm. UV-Vis absorption spectrum shows peaks characteristics of the surface Plasmon resonance of nanosized particles **[88]**.

3.3.2 Fourier Transform Infrared [FTIR] spectroscopy

Fig. 3.6 Fourier Transform Infrared [FTIR] spectroscopy

The total internal energy of a molecule in a first approximation can be resolved into the sum of rotational, vibrational and electronic energy levels. Infrared spectroscopy is the study of interactions between matter and electromagnetic fields in the IR region. In this spectral region, the EMR waves mainly couple with the molecular vibrations. In other words, a molecule can be excited to a higher vibrational state by absorbing IR radiation. The probability of a particular IR frequency being absorbed depends on the actual interaction between this frequency and the molecule. In general, a frequency will be strongly absorbed if its photon energy coincides with the vibrational energy levels of the molecule. IR spectroscopy is therefore a very powerful technique which provides fingerprint information on the chemical composition of the sample.

Hence infrared spectroscopy is an analytical technique applied to the characterization of molecules. It is based in the fact that molecules absorb specific frequencies that are characteristic of their structure. These absorptions are related to the strength of the bond. In order for a vibrational mode in a molecule to be active in the infrared region, it must be associated with changes in the permanent dipole. Because the interaction between electromagnetic light and a molecule produce a change in its permanent dipole, and when the frequency of the infrared light is the same as the vibrational frequency of a bond, absorption occursas can be observed in fig. 3.7 **[89]**.

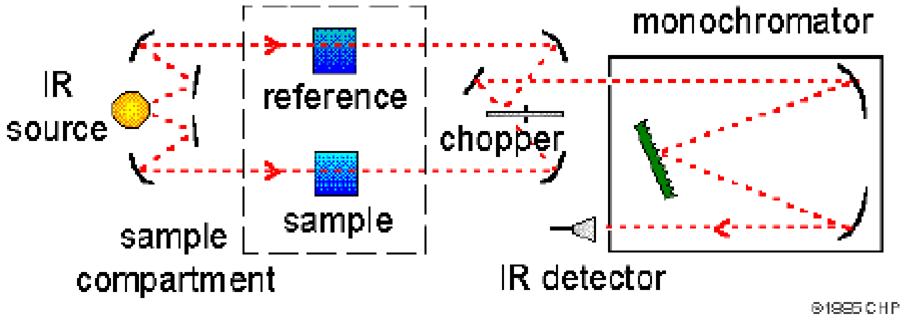


Fig. 3.7 Infrared spectrometer design

Although this technique has been used for many years, conventional dispersive infrared spectrometers have been replaced by Fourier Transform infrared equipment, which incorporates a Michelson interferometer and presents an improvement of energy and the simultaneous measurement of the whole spectral range.

The Michelson interferometer consists of a fixed mirror, a moving mirror and a beam splitter. The beam splitter is made of a material that transmits and reflects light equally. The two beams reflect back to the beam splitter from the mirrors and interfere constructively and destructively according to the optical path difference produced by the moving mirror **[90]**.

The Michelson Interferometer;

- ✓ a broad-band light source which emits light covering the mid-IR range,
- ✓ a beamsplitter made of KBr or CsI,
- ✓ two front surface coated mirrors – one moving and one fixed, and
- ✓ a detector

Interferogram is determined experimentally in FTIR spectroscopy, and the corresponding spectrum–frequency against intensity plot, is computed using Fourier transform. This transformation is carried out automatically and the spectrum is displayed. The detector sees all the frequencies simultaneously, whereas in a dispersive spectrometer, only one frequency can reach the detector at one time.

The FT-IR spectrometer is considered as a useful instrument during the synthesis of nanoparticles (Fig. 3.6). It is one of the instruments used in characterizing a synthesized nanopartcles by detecting functional groups found in both plant extract and the surface of the nanoparticles **[91, 92]**.

4. Results and Discussion

4.1 Phytochemical test

Khat has been extracted and for it to be used as a reducing agent and caping agent, it was necessary to asses whether the phytochemicals, such as flavonoids, polyphenols, carbohydrate etc, do naturally exist in the extract or not. The results of qualitative phytochemical analysis of the Khat extract are shown in fig. 4.1 and table 4.1 which revealed the presence of secondary metabolites such as phenols, flavonoids, carbohydrates, tannis, alkaloids, etc. Therefore Khat is composed of phytochemicals which are capable of reducing the Cu^{2+} ion by donating electrons, capping and stabilizing the formed nanoparticles **[73]**. For instance, the poly phenolic compounds found in plants including khat are very important plant constituents because of the scavenging ability of their –OH groups. The antioxidant property of polyphenolic compounds is mainly due to its redox property which allows them to act as reducing agents. These constituents are the ones which do make the Khat extract to be helpful at reducing the Cu^{2+} ion of the salt solution **[7]**.

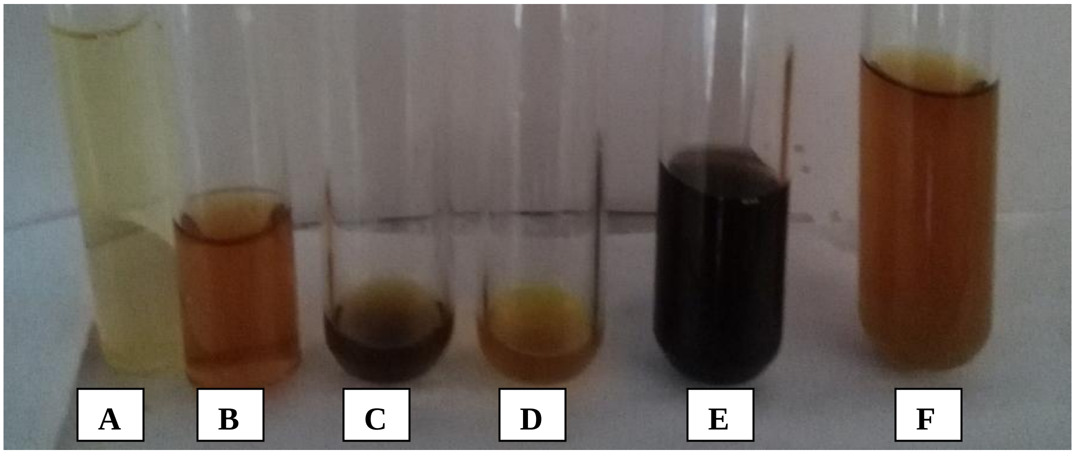


Fig.4.1 The colors observed when the Khat extract is tested for A) Alkaloids B) Glycosides C) Tannis D) Flavonoids E) Poly phenols and F) Carbohydrate

Table 4.1: The qualitative analysis of phytochemicals in the Khat leaf extract

No	Phytochemicals	Chemical test	Result	Colors observed
1	Alkaloid	Wagner's test	+	Yellow
2	Glycoside	Alkaline reagent test	+	Redish brown
3	Tannin	Ferric Chloride test	+	greenish black
4	Flavonoid	Ferric Chloride test	+	Wooly brown
5	Phenols	Ferric Chloride test	+	Bluish Black
6	Carbohydrate (glucose)	Benidict's test	+	Brick red

The color changes are indicators of formation of different complexs as a result of oxidation and reduction reactions. For instance the yellow color for alkaloids indicates that the nitrogen or oxygen atoms of the amide groups of the alkaloids involment in a reaction. In most of the Ferric chloride tests, the iron (III) ion forms complexes having different colors depending on the nature of the complexes. As can be observed from table 4.1 bluish black, wooly brown and the greenish colors are expected to be the results of the iron complexes formed from the iron (III) ion and the benzoate groups**[85]**.

4.2 Characterization of the synthesized CuO Nps

The formation of CuO nanoparticles was confirmed primarily based on change in color of the reaction mixture. Analyzing the UV-Vissible and FT-IR spectrums were also used as main characterizing methods.

4.2.1. Visual observation

Formation of copper oxide nanoparticles was easily discernible due to changes in color of the solution. The color changes arise due to excitation of surface plasmon resonance in the metal nanoparticles indicating the formation of CuO Nps. The colorless 1 mM$CuSO_4.5H_2O$ solution started changing its color to light green at the time when the Khat leave extract was added to it. As soon as the NaOH solution comes in contact with the solution, the light green mixture was spontaneously changed to yellowish green (Fig.4.2), indicating the formation of water soluble CuO Nps. This color of the nanoparticle has persisted for days. But then brownish black precipitate was observed. It is the real and final color of the CuO Nps formed. There after no further color change had been observed. This indicates that copper ion found in the reaction mixture has been reduced completely **[8]**. In contrast, the color of control solutions, the Khat extract and the $CuSO_4.5H_2O$ solution, remained unchanged.

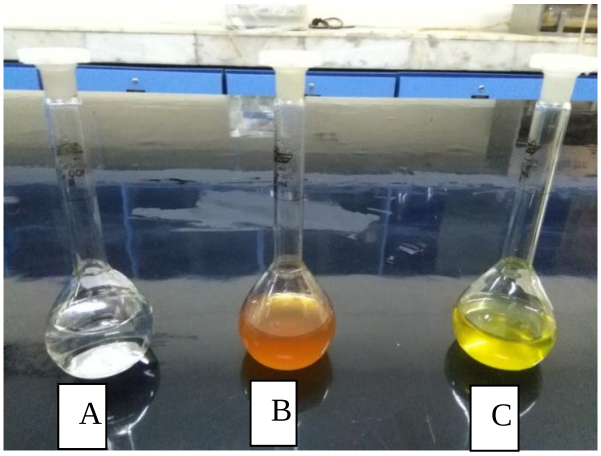


Fig. 4.2 The color changes observed during the formation of CuO Nps A) precursor B) the Khat extract and C) the nanoparticle

4.2.2 UV-Vissible spectra analysis

Metals can be treated as free-electron systems. Such metal called plasma contains equal numbers of positive ions which are fixed in position and conduction electrons which are free and highly mobile. Under the irradiation of an electromagnetic wave, the free electrons are driven by the electric field to oscillate coherently. These collective oscillations of the free electrons are called Plasmon. These plasmons can interact, under certain conditions, with visible light in a phenomenon called surface plasmon resonance (SPR). The position, the shape and intensity of the surface plasmon resonance strongly depend on various factors including the size, shape and monodispersity of the Nps, as well as the composition of the surrounding media and interactions between stabilizing ligands and the Nps. Therefore the effect of different factors on the size, shape and monodispersity of the nanoparticles should be observed to design a suitable formulation for production of nanoparticles. Metal nanoparticles exhibit the absorption of visible electromagnetic waves by the collective oscillation of conduction electrons at the surface. This is known as the surface plasmon resonance effect. It is the resonant oscillation of conduction electrons at the interface between a negative and positive material stimulated by incident light. The resonance condition is established when the frequency of incident photons matches the natural frequency of surface electrons oscillating against the restoring force of positive nuclei. The interest in this effect is the possibility of using it as a tracer for the presence of metal nanoparticles with a simple UV-Visible spectrometer **[84]**.

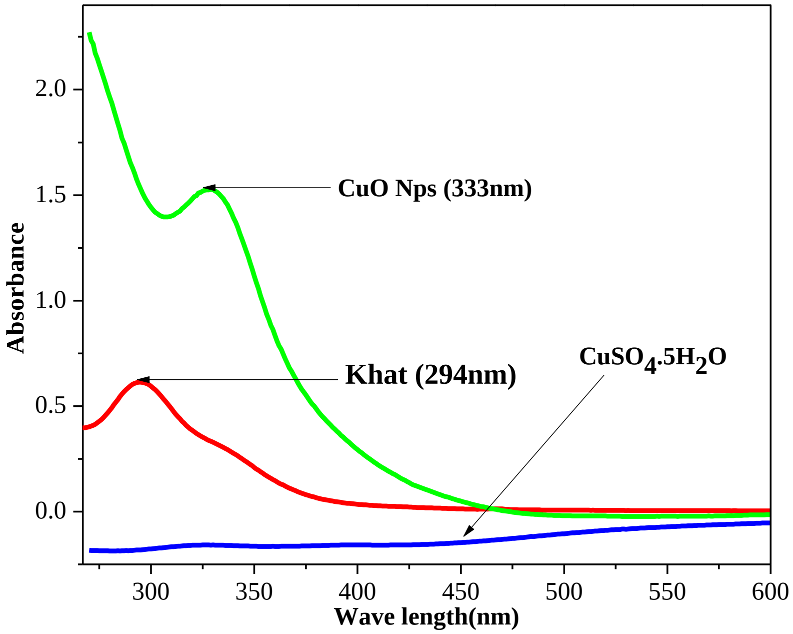


Fig. 4.3 UV–Visible spectra of the precursor, Khat extract and CuO nanoparticles

The UV–Vis spectra result revealed a strong absorbance at 333 nm suggesting the formation of copper oxide nanoparticles (Fig. 4.3). While the pure Khat extract has a strong absorbance at 294 nm. This result definitely agrees with the range of λ_{max} values of the CuO Nps, 250 nm - 395 nm, at different previous works using plants other than Khat **[4 - 6, 8, 19, 83]**. This shows, during the formation of the CuO Nps, there is a red shift in wavelength which can be considered as an indicator of a newly formed shape and size of particles with different Surface Plasmon Resonance, SPR. The peak obtained in this work is even sharper and more intense than some other works on the green synthesis of CuO Nps **[4]**. This is expected to be the effect of the smaller size of the nanoparticles synthesized which enable the CuO Nps to be more applicable. Therefore the color changes and the above UV-Vis values confirm the formation of the CuO Nps **[84]**.

Here parameters were optimized so as to select the best conditions used for effective synthesis of the CuO Nps. These were the concentration of both Khat extract and the precursor, as well as pH of the mixture.

4.2.2.1 Optimization of precursor concentration

To optimize the concentration of the precursor two stages were taken. The first stage was at the time when pH was not adjusted. Here the peaks for the CuO Nps were all closer to the peak of the pure Khat extract (294 nm) as can be seen at fig. 4.4. But they also show small red shift in wave length where the maximum λ_{max} was obtained at 296.5 nm while 1 - 3 mM $CuSO_4.5H_2O$ solutions were used. On the other hand, 4 mM and 5 mM have shown peaks very much closer to the pure Khat extrat (295.4 nm). It was then easy to conclude that the nanoparticle was not fully formed yet. According to different previously done studies on the synthesis of CuO Nps, there was a need to change the media to a basic one. And this was done by adding NaOH solution so as to adjust the pH, based on some prevous works **[8]**.

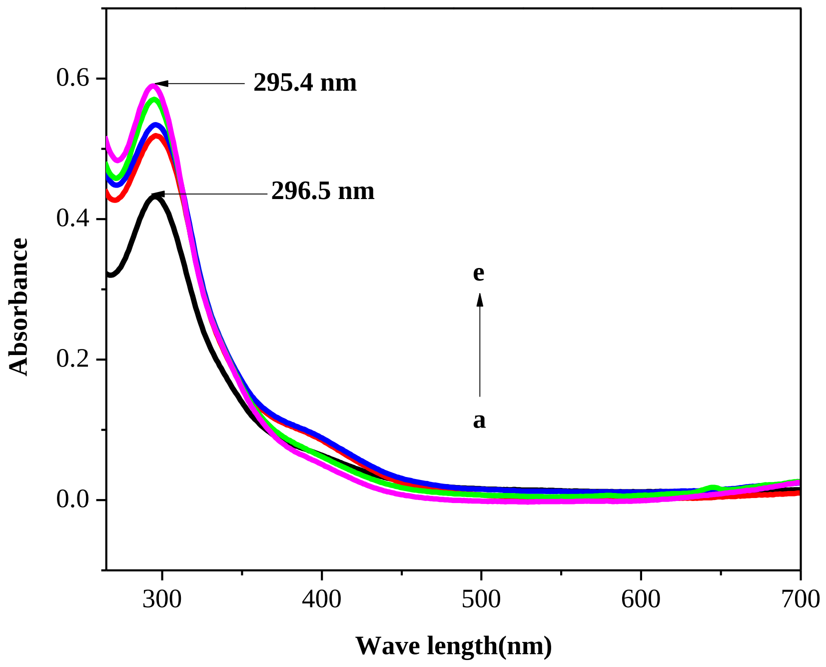


Fig. 4.4 The UV-Vis spectra of CuO Nps formed from 2 ml extract of 2 % (m/v) Khat and 45 ml of (a, b, c, d, e), (1, 2, 3, 4, 5 mM) respectively before pH was adjusted

Hence, adding NaOH solution with the recommended pH (10, before optimization) value was taken as the next step. As soon as it was added, remarkable change in the position of the peaks was observed. All of the peaks have shown more shift in position. Comparing the peaks obtained, it was founded that 1 mM$CuSO_4.5H_2O$ was the best concentration of the precursor to be used for the synthesis of the CuO Nps with an intense peak and more red shift as compared to the 2 mM, 3 mM, 4 mM and 5 mM, as can be seen at fig. 4.5. This result definitely agrees with the concentration of $CuSO_4$ used for the synthesis of copper oxide nanoparticles using aqueous extract of flowers of *Cassia alata*. For the 2 mM and 3 mM, the peaks became broader due to the increase in number of the copper ions to be reduced and form particles of lager size **[5]**. But for the 4 mM and 5 mM pale blue colour was observed. The color was different from greenish yellow colors of the CuO Nps formed from 1 - 3 mM precursor. This shows that when the concentration of the copper ions in the solution was increased, it has exceeded the amount of reducing agent phytochemicals of the Khat extract. It can even form a respective hydroxide solution. This is expected to happen when the added NaOH solution does react directly with the copper ions and form $Cu(OH)_2$ with a pale blue colored water insoluble precipitate **[7]**. This might be the reason why the peaks have shown different trends from the CuO Nps synthesized using 1, 2, and 3 mM$CuSO_4.5H_2O$.

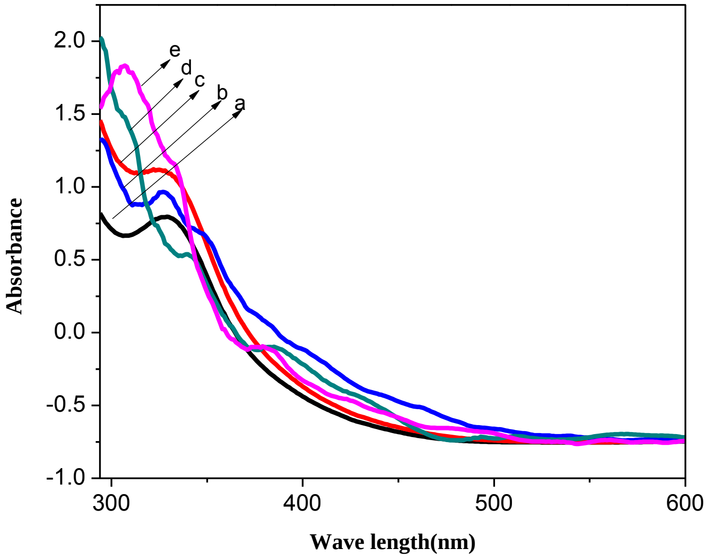


Fig. 4.5 The UV-Vis spectra of CuO Nps formed from 2 ml extract of 2% Khat and 45 ml of (a) 1 mM (b) 2 mM (c) 3 mM (d) 4 mM (e) 5 mM at a pH of 10

4.2.2.2 Optimization of the concentration of Khat

Fig. 4.6 shows that the concentration of Khat which gave intense peaks were 3, 4, 5 and 6 % (m/v) relative to the 1 % and 2 %. However, it was so clear that the peaks are full of noises. This reveals that the Khat extract has dominated the solution and the nanoparticle formed has become more unstable. The increase in sharpness indicates that the size of the nanoparticle decreases more as the concentration of the Khat extract is increased which agrees with the concept that; progressive increase in the characteristic peak with increase in concentration of biological extracts with the same amount of salt ions is a clear indicator of nanoparticle formation **[89]**. Despite of all these facts, extract of 2 % (m/v) Khat was taken as the best concentration of the extract for its smooth peak with good intensity. Because increasing the concentration above 2 % (m/v) was noticed making the peaks noisier. Therefore, increasing the concentration above this value might altimately make the nanoparticle unstable **[5]**.

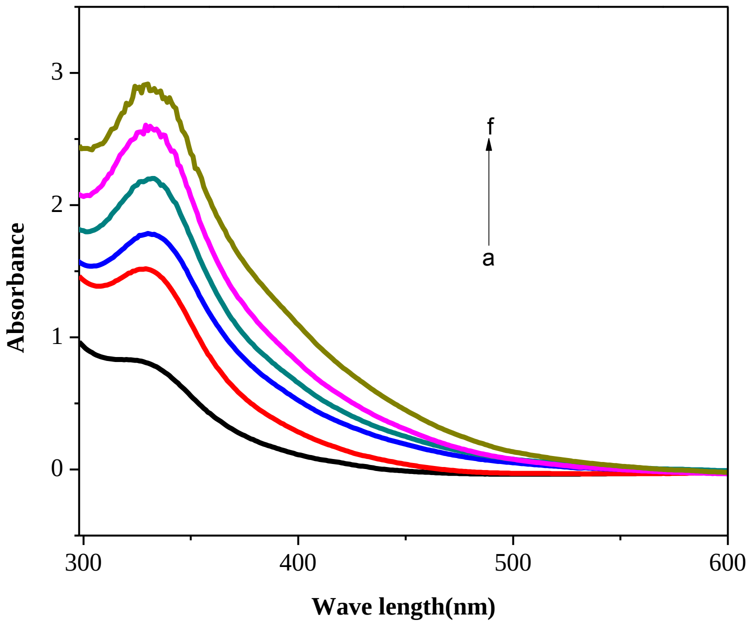


Fig. 4.6 UV-Visible spectra for the CuO Nps formed from 45 ml of 1 mMCuSO$_4$.5H$_2$O and (a, b, c, d, e, f), (1, 2, 3, 4, 5, 6 % (m/v)) Khat respectively at a pH of 10

4.2.2.3 Optimization of pH of the mixture of Khat extract and the precursor

Other parameter to be optimized was the pH of the CuO Nps solutions. The pH of the precursor, the extract, and the nanoparticle before pH was adjusetd were 5.4, 5.5 and 4.15 respectively. The nanoparticle solutions of different pH values (6, 7, 8, 9.5, 10, 11, 12, and 13) were analyzed based on intensity of their peak and red shift of their respective λ_{max} values. The Best result was obtained for a CuO Np solution with a pH value of 11, as it is revealed at fig. 4.7. This shows that a more basic media is vey suitable for the CuO Nps synthesis **[7]**. The plasmon resonance was clearly stronger for pH 9 to 12. At pH 12, the peak is still strong but ofcourse weaker as compared to pH 11.

The maximum blue shift in SPR peak around the maximum value at pH 11 could be attributing to the decrease in the particle size. The added NaOH solution is expected to act as a catalyst for the formation of the nanoparticle by making particles to collide and connect to each other so as to form homodispersed nanoparticles **[11]**. But increasing the pH above 12 might hinder the formation of the CuO Nps. This is expected to happen when the added NaOH solution does react directly with the copper ions and form $Cu(OH)_2$ with a pale blue color **[30]**.

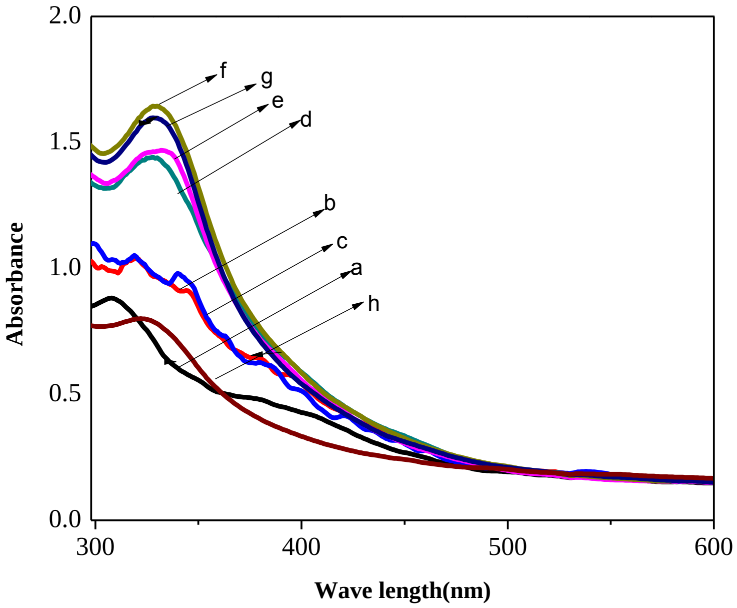


Fig. 4.7 The UV-Vis spectra of CuO Nps formed from 2 ml extract of 2 % (m/v) Khat and 45 ml of 1 mM at a pH of (a) 6 (b) 7 (c) 8 (d) 9 (e) 10 (f) 11 (g) 12 (h) 13

Its stability was assesed by comparing the UV-Vis spectrum of CuO Nps of different age. It was then founded that the UV-Vis spectrum of the nanoparticle after 24 hr was extaly the same in intensity, broadness and in λ_{max} value with the spectrum one hour after it was prepared. Even after six days no visible change was observed in the peak intensity and λ_{max} value of the nanopartcile as can be observed at fig. 4.8. There was, of course, small increase in intensity of the absorbance and broadness. This is expected to be the result of small increase in size of the particles and rate of agregation due to the increase in nucleation and collusion of the nanoparticles formed **[7]**.

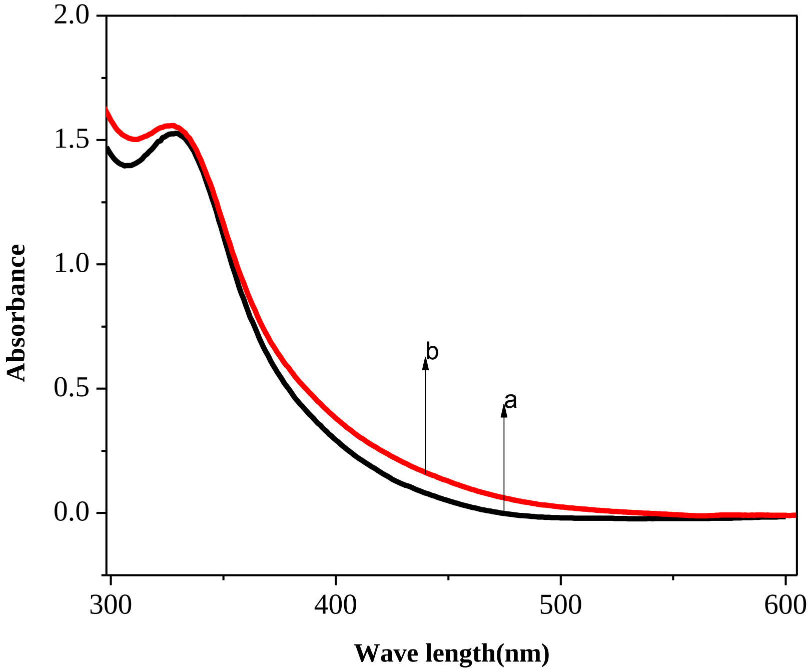


Fig. 4.8 The UV-Vis spectra of CuO Nps (a) after one day (b) after 6days

4.2.3 The FT-IR spectra analysis

The dual role of the plant extract, as a reducing as well as capping agent, and presence of some functional groups in both the Khat extract and CuO Nps were investigated by FT-IR analysis **[91]**. This was done by measuring the FT-IR of the pure extract and the CuO Nps. FT-IR analysis was used to identify and get an approximate idea of the possible biomolecules that are responsible for capping and stabilization of the CuO Nps with the Khat leaf extract **[8]**.

A strong peak at 3471 cm^{-1} can be attributed hydrogen bonded O-H groups of alcohols and phenols and also to the presence of amines N-H of amide, fig. 4.9. This agrees with the conclusion that Khat extract was composed of poly phenols, flavonoids, alkaloids and other similar phytochemicals containing O-H and N-H bonds confirmed using visual observation. This peak shifted to lower field at 3447 cm^{-1} in the synthesized CuO Nps (Fig. 4.10). The shoulder peak at 1643 cm^{-1} in Khat leaf extract could be attributed to C=C stretching, vibrations about C=O amide, and conjugated C=O of the proteins that are responsible for capping and stabilizing of CuO Nps. The peaks observed in the range of 686cm^{-1} has been assigned to phenolic groups, C-N stretching vibrations of aliphatic and aromatic amines.

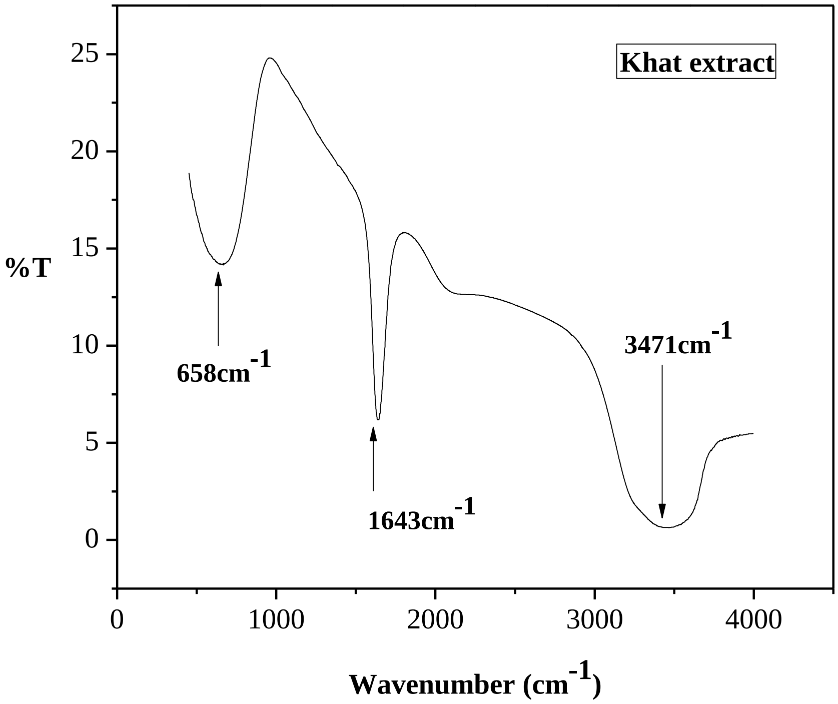


Fig. 4.9 FT-IR of Khat leaf extract

The major peak observed at 717cm^{-1}should be a stretching of Cu-O which is very closer to the results of some prevous works **[33]**. Disappearance of the peak of Khat extract at 658 cm^{-1} shows that some aromatic phytochemicals have been already oxidized while reducing the copper cation. The bands at 1643 cm^{-1} of the leaf extract shifted to lower field at 1631 cm^{-1}, in the product (Fig. 4.10). These bands are assigned to C-O stretching, C=O stretching and N-H bending. The shifting in these bands is clearly indicating that the coordination of carboxylic acids in the protein of Khat leaf extract with CuO Nps play a major role on dispersing, stabilizing and capping of CuO Nps **[2, 8]**.

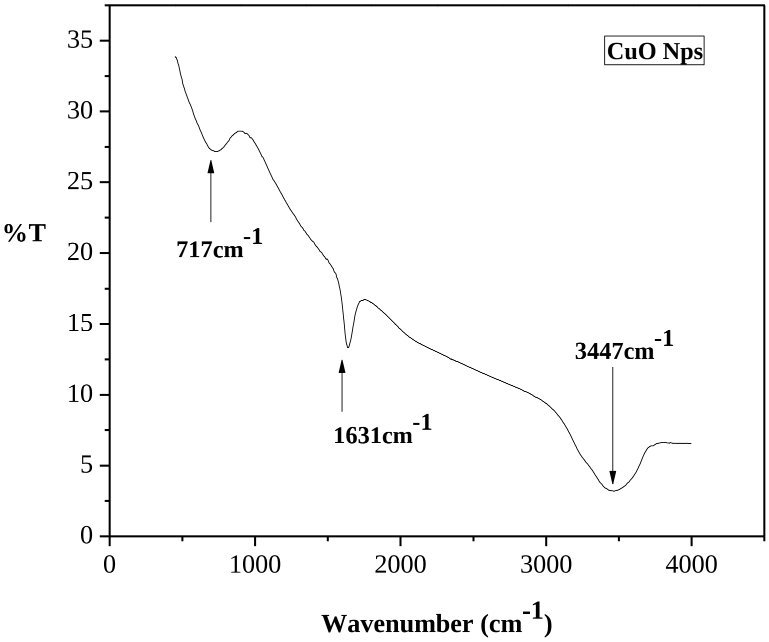


Fig. 4.10 FT-IR of synthesized CuO Nps

4.3 The antibacterial activity of the CuO Nps

The antimicrobial properties of CuO Nps were investigated using *Salmonella typhimurium* and *Escherichia coli* bacterium specieses. The bactericidal effects of CuO Nps were studied based on diameter of inhibition zone in disk diffusion tests of nanoparticles dispersed in batch cultures. As can be observed at table 4.2, the CuO Nps showed good antibacterial activity against *Salmonella typhimurium*, with a maximum inhibition zone of 14 mm. But for the *Escherichia coli,*the nanoparticle have shown relatively lower zone of inhibition, 10 mm, after 24 hour incubation with two trials which agrees with results obtained so far **[2, 8]**. The results revealed that the bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. It was also possible to observe from the fig. 4.11 that the CuO Nps was more sensitive to the bacterial as compared to the Khat extract and the precursor, $CuSO_4.5H_2O$. This is expected to be due the reason that the size of the copper oxide particles is smaller than particles of the precursor and the Khat particles.

Table 4.2 In vitro antimicrobial activity of some human pathogenic bacteria on CuO Nps by disc diffusion assay (* (1- first trial) and (2- second trial))

S.No.	Test organism / Bacterial type	Zone of inhibition (mm)
1	*Salmonella typhimurium* (1)	11
2	*Salmonella typhimurium*(2)	14
3	*Escherichia coli* (1)	10
4	*Escherichia coli* (2)	6

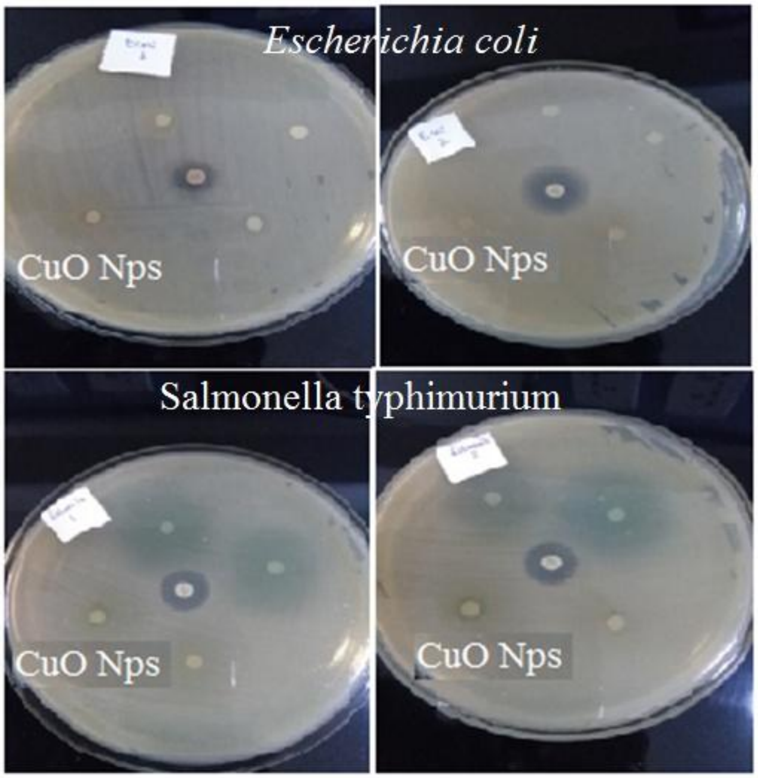


Fig.4.11 Zone of inhibition produced by CuO Nps against; A) *Escherichia coli* (1)

B) *Escherichia coli* (2) C) *Salmonella typhimurium* (1) D) *Salmonella typhimurium* (2)

Based on these results, it can be concluded that these synthesized CuO Nps had significant antibacterial action on both of the bacterias.

5. Conclusion and Recommendation

5.1 Conclusion

The green chemistry approach used in the present work for the synthesis of copper oxide nanoparticles using Khat as reducing, stabilizing and capping agent was simple, cost effective and the resultant nanoparticles are highly stable and reproducible. Khat is one of the plants composed of many phytochemicals used to synthesize nanoparticles. The synthesized copper oxide nanoparticles were characterized using visual obseravation (color change), UV-Vis absorption and FT-IR which reaffirmed the formation of the CuO Nps using Khat extract. The synthesized CuO Nps has been found to have a maximum absorbance at a wave length of 333 nm which is very much different from the 294 nm of the Khat extract. The UV–Visible absorption spectrum indicates red shift with increasing concentration of the plant extract to a certain value (2% (m/v)) and decreasing the precursor concentration (1 mM) in the reaction mixture during the synthesis. The FT-IR also showed that there was newly formed peak that is different from the spectrum of the pure Khat. Mainly the strong peak at 717 cm^{-1}, which was not found at the pure extract, was considered to be the major indicator for the formation of the CuO Nps. Therefore, it is possible to synthesize CuO Nps using $CuSO_4.5H_2O$ as a precursor and Khat as a reducing and stabilizing agent. CuO Nps prepared from above route do have appreciable application in killing bacterias. It was founded that the CuO Nps has an average inhibition zone of 10 mm. This green method of synthesizing CuO Nps is expected to be extended to fabricate other, industrially important metal oxides.

5.2 Recommendations

Visual observation, UV-Visible and FT-IR spectroscopeshad been used as characterization methods for the synthesized CuO Nps. These methods were helpful in confirming the synthesis of the CuO Nps. But further works are recommended for the convenience of the work. Therefore, determining the size and crystalline nature of the CuO Nps using XRD, TEM and SEM are considered as future works to be done after graduation. Studying the composition of the CuO Nps surface using EDS is also another test needed to be performed. Because there is a possibility for the copper metal nanoparticle to be found mixed with the copper oxide nanoparticle. Hence determining the major component of the surface is believed to be very much important.

On the other hand, the antibacterial activity of CuO Nps was investigated against only two human pathogenic bacteria. Hence testing the killing nature of the CuO Nps on other bacterium specieses is also recommended and planned to be done next.

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