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GREEN SYNTHESIS AND CHARACTERIZATION OF ZINC OXIDE (ZnO) NANOPARTICLES USING NEEM (Azadirachta indica) LEAF EXTRACT AND INVESTIGATION OF ITS ANTIBACTERIAL ACTIVITIES

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DEPARTMENT OF CHEMISTRY

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JULY, 2019

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A Thesis submitted to the school of Graduate Studies of Bahir Dar University and presented in partial fulfillment of the requirements for the Degree of Masters of Science in Chemistry (Physical).

By: Elmineh Tsegahun

Advisor: Muluken Akililu (PhD)

JULY, 2019

Declaration

I hereby declare that, this thesis entitled "**Green synthesis and characterization of Zinc oxide Nanoparticles using Neem(*Azadirachta indica*) leaf extract and investigation of its antibacterial activities"** and the work presented in it are my original work and has not been presented for a degree in any other university and that all sources of materials have been duly acknowledged.

Student's Name: Elmineh Tsegahun

Signature: _____

Date: _____

This thesis has been submitted for examination with my approval as University advisor.

Advisor's Name: Muluken Akililu (PhD)

Signature: _____

Date: _____

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Department of Chemistry, a thesis entitled "**Green synthesis and characterization of zinc oxide Nanoparticles using Neem (*Azadirachta indica*) leaf extract and investigation of its antibacterial activities**" as part of the work recommended in fulfillment of the requirements for a master of science in Chemistry (Physical) at Bahir Dar University.

Board of Examiners

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_____ (Internal examiner I)	_____	_____
_____ (Internal Examiner II)	_____	_____

Dedication

This thesis is dedicated to my beloved Father Tsegahun Gedif whom I lost when I was a First year student at Bahir Dar University, on June 17th, 2008**.**

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Above all I praise God for giving me his helping hands in all of my moves from the beginning up to the end.

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Abstract

In this study, green synthesis, characterization and antibacterial activity of zinc oxide nanoparticles was studied. The zinc oxide nanoparticles were successfully synthesized via a fast, convenient, cost effective, and environmentally friendly method by biologically reducing Zn $(NO_3)_2$ solution with Neem (Azadirachta indica) leaf extract under optimum condition. The presence of active flavonoids, phenolic groups, alkaloids, terpenoids and tannins, which were in the biomass of the Neem leaf extract before and after reduction was identified using qualitative screening methods (observing the colour changes) and FT-IR Spectroscopy. The formation of ZnO NPs was visually indicated by the color changes from colorless to light yellow color. Biosynthesized nanoparticles were also characterized by UV-Visible, FT-IR, and XRD spectroscopies. The reduction process was simple and convenient to handle and was monitored by UV-Visible spectroscopy that showed surface plasmon resonance (SPR) of the ZnO NPs at 321 nm. This result clearly revealed the formation of ZnO NPs. X-ray diffraction was used to investigate the crystal structure. The average particle size of ZnO powder and around 20 nm using the line width of the plane, refraction peak using the Scherrer's equation. Green synthesized zinc oxide nanoparticles were evaluated for antimicrobial activities against Gram positive and Gram negative bacteria. Zinc nanoparticles exhibited maximum zone of inhibition against Escherichia coli (15 mm) while least activity was seen against Staphylococcus aureus.

Keywords: *ZnO NPs, Green synthesis, Neem leaf, UV-Vis, FT-IR, XRD, Antibacterial activity*

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LIST OF ABBREVIATIONS

NPs	Nanoparticles
NMs	Nanomaterials
SPR	Surface Plasmon resonance
ZnO NPs	Zinc oxide nanoparticles
E. coil	Escherichia coli
S. aureus	Staphylococcus aureus
A. indica	Azadirachta indica

1. INTRODUCTION

1.1. Background of the study

Nanotechnology is a science and engineering branch of recent well established technology referring at the nanoscale, i.e. 1 to 100 nm. The field of nanotechnology is one of the most active research areas in modern material science. Over last decades, nanotechnology has established as the great innovation of science and technology. In nanotechnology, a nanoparticle is defined as a small object that behaves as a whole unit in terms of its transport and properties [1]. It is the use and manipulation of matter at a tiny scale. At this size, atoms and molecules work differently, and provide a variety of surprising and interesting uses. Nanotechnology should not be viewed as a single technique that only affects specific areas. Nanotechnology represents the design, production and application of materials at atomic, molecular and macromolecular scales, in order to produce new Nanosized materials [2].

The focus of nanoscience is now mainly shifting towards the assembly of individual Nanoparticles of high order structures and nanomaterial's [3]. Nanoparticles of different size and shapes can be synthesized in different material compositions and surface modifications [4]. The synthesized nanoparticles are found to exhibit size or shape dependent properties [4, 5]. It has prospects in sensors, super capacitors, drug carriers, diodes, photonic and photovoltaic cells, or data storage media application [6, 8]. While the analogies between nanoparticulate building blocks at the nanoscale and the atomic building blocks at the molecular scale appears quite appealing for high order structure [9], yet it must be remembered that nanoparticles are rarely monodispersity and no two particles are ever identical unlike atoms. This intrinsic polydispersity of nanoparticles make self-assembly more complex which affects the overall characteristics derived from the size dependent properties of individual nanoparticles (e.g. magnetic susceptibility, surface Plasmon resonance, (SPR) [10]. In order to synthesize highly ordered structures of nanoparticles with well defined properties and functions, it is highly desirable to lower the polydispersity of their nanoparticulate components to achieve maximum stability [8, 10]. The ability to modify low polydispersity particles is important for different applications, like catalysis where the catalytic activity of the nanoparticles is considered important [11]. Metal oxide nanoparticles have various significant application possibilities, such as anti-microbial, cell line studies and dye degradation properties [12].

The most attractive factor in using ZnO NPs is that it has versatile application where some of them are shown in the figure below.

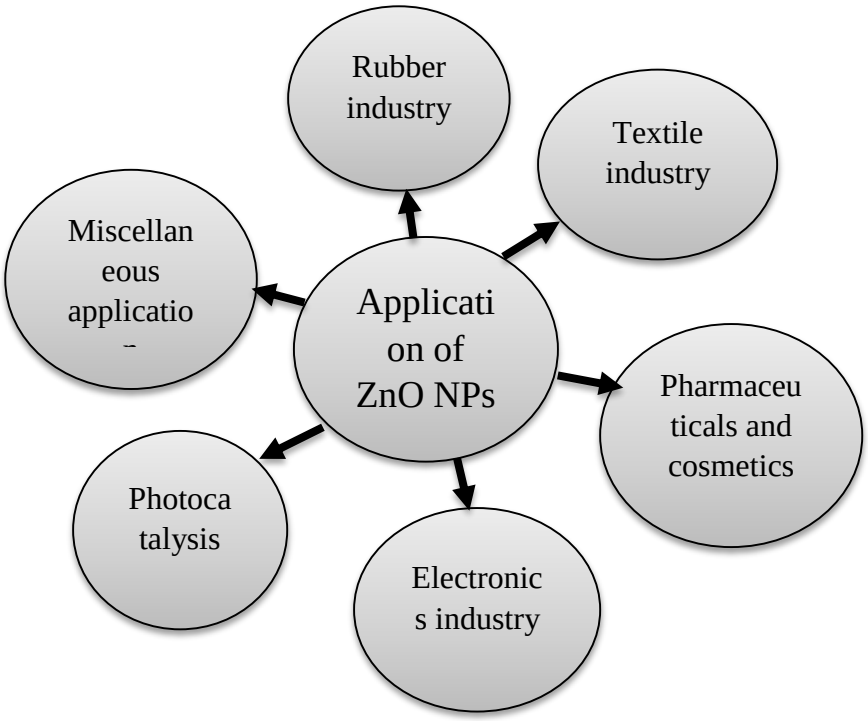


Figure 1.1: Application of ZnO NPs [15]

The most common methods to synthesize ZnO NPs are chemical, physical and biological methods. Several physical and chemical methods are employed for synthesizing and stabilizing ZnO NPs [15]. But most of the chemical methods used for the synthesis of NPs involve the use of toxic like chloroform, pyridine, and hazardous chemicals that create biological risks and these chemical processes are not eco-friendly. This enhances the growing need to develop environmentally friendly processes through green synthesis and other biological approaches [16]. Of these methodologies, biological synthesis plays a major role when compared with the two other methodologies. Biologically-mediated synthesis is further classified into eco-friendly synthesis, which is comprised of plants and plant sources with the corresponding advantages of simplification and lower cost. Therefore, we decided to mainly focus on the green synthesis of NPs [17]. Green routes are used for the synthesis of ZnO because of the least possible number of chemicals utilized that produces least amount of pollutants and are energy efficient as well as cost-effective. A

number of natural moieties such as plants, fungi, algae, bacteria, and viruses are used to synthesize the ZnO [18].

In recent years, Biosynthesis of NPs is an important approach in nanotechnology. Nanotechnology has appealed many researchers from several fields like biotechnology, physics, chemistry, material science, engineering and medicine.

Statement of the problem

These days' different researches on synthesis of metallic and metallic oxide NPs have been reported in many areas of applications. The NPs 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 by-products and need longer time. Hence there arises a growing need to develop green synthesis routes for the NPs. Still, using microorganisms as a green method of synthesis requires lots of maintenance and involves very complex procedures of maintaining microbial cultures [9, 16]. It is also slow which is not very suitable for industrial feasibility because of its requirements of highly aseptic conditions. Different researchers have been used plant extracts to synthesis metallic oxide nanoparticles. The results of their work showed us plant extracts are good sources for the synthesis of metal oxide nanoparticles. These results motivated us to carry out further researches on the use of plant extracts for the preparation of nanoparticles. We know that there are many plants that are grown only in Ethiopia. Researches related with Nanomaterials synthesis using plant mediated are new in Ethiopia. Therefore this study carried out synthesizing zinc oxide nanoparticles by using neem leaf extract. 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. The other driving force is the number of works or literatures on the ZnO NPs are relatively few as compared to other inorganic or metallic oxide nanoparticles. The most studied metallic NPs, Ag and Au, are very much expensive as compared to ZnO NPs. 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, fungi) is becoming a more focused area of study**.** Currently, investigations on the 'green' synthesis method of ZnO NPs are being studied as an alternative to the chemical and physical methods which

are non-compatible, toxic and costly. Unlike these methods, the ‘green’ synthesis method is a compatible, eco-friendlier and cheaper approach.

1.3. Significance of the study

This work is expected to have its own contribution on the green synthesis of the ZnO NPs using locally available green Neem leaf extracts. These researches will bring a technology transfer and also initiate researchers to explore Ethiopian indigenous and growing plants and use for the nanoparticle preparation. It is also hoped that, it will be used as one possible means of plant-mediated biological synthesis of ZnO NPs of desired quality with low cost, eco-friendliness and convenient methods of preparation so that the toxicity or hazardous nature of the chemical methods of synthesizing ZnO nanoparticle can be avoided. It is believed that this work will have a positive impact on increasing the number of available research works dealing with ZnO NPs synthesis using the indigenous plant and effective antibacterial activity. Having easily available ZnO NPs is also expected to make the protection and /or removal of bacterial microbes easy and effective. And this will definitely be one of the ways of keeping people healthy.

ZnO NPs application is a very promising, efficient and cost effective method for remediating this environmental health concern. There is a need therefore; to develop environment friendly procedures for synthesizing ZnO NPs that avoid toxic by-products, high pressure, and energy and temperature inputs. Neem extracts have shown prospects in ZnO NPs synthesis which is a green “chemistry synthetic” approach.

1.4. Objectives

1.4.1. General Objective

To synthesize, characterizing ZnO NPs using Neem leaf extract and investigation of its antibacterial activity

1.4.2. Specific Objectives

The specific objectives of this research work are:

- Testing the existence of different phytochemicals in the neem leaf extract using different standard solutions as well as FT-IR spectroscopy.
- Synthesizing the ZnO Nps from the Neem leaf extract
- Characterizing the synthesized ZnO Nps using visual observation, UV-Vis, FT-IR, and XRD
- Examining the antibacterial activity of the ZnO Nps by measuring its zone of inhibition over different bacterial species

2. LITERATURE REVIEW

2.1. Historical aspects and growth of Nanoscience and nanotechnology

The term “Nano” comes from the Greek word “dwarf” which generally elaborates the particle of size roughly in the range of 1 to 100 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 [25].

The term "Nanotechnology" was first defined by Norio Taniguchi of the Tokyo Science University in 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 NPs or are manufactured with the help of nanotechnologies [25].

Nanotechnology is a science and engineering branch of recent well established technology referring at the nanoscale, i.e. 1 to 100 nm. The field of nanotechnology is one of the most active research areas in modern material science. Over last decades, nanotechnology has established as the great innovation of science and technology [26]. In nanotechnology, a nanoparticle is defined as a small object that behaves as a whole unit in terms of its transport and properties [1].In modern research era of any branch of science, nanotechnology has find enormous interest. NPs play an essential role as building blocks of nanotechnology. Nowadays, nanoscience as well as nanotechnology is widely applied in different fields mainly in sensor, electronic, antibacterial, water purification, cosmetic, biomedical, pharmaceutical, environmental, catalytic and material applications. The main advantages of NPs synthesis at room temperature and from plant extracts are partly fulfill the green synthesis [15].

Biological systems such as the use of plant materials provide an innovative; ecofriendly alternative for the production of NPs. Biosynthesis using plant sources offers several advantages such as cost effectiveness, eco-friendliness, and the elimination of high pressure, energy, temperature, and toxic chemicals necessary in the traditional synthesis methods. Plants provide a better platform for nanoparticle synthesis as they are free from toxic chemicals as well as provide natural capping agents. The plant extracts contain various organic compounds such as terpenoids that aid nanoparticle synthesis. Terpenoids are surface active molecules that stabilize the NPs [26].

2.2. Nanoparticles/Nanomaterial's

Nanomaterial's (NMs) are defined as materials with at least one external dimension lies in the size range of approximately 1-100 nanometers. While Nanoparticles (NPs) are solid particles with all three external dimensions at the nanoscale that can drastically modify physic-chemical properties compared to the bulk material [15]. Bulk materials possess relatively constant physical properties regardless of their size, but at the nanoscale this is often not the case. The NPs possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired application [27].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, 28]. 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 [29]. 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 [30].

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 [31]. 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, 28].

2.3. Noble metal nanoparticles

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 [32]. 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 nanoparticles are also important because of their superior electronic, electrochemical, paint/ink materials, catalytic and magnetic properties [5, 33]. Metal NPs 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 NPs have found uses in many applications in different fields, such as catalysis, photonics, and electronics [5, 9]. Many kinds of NPs, including metal NPs, oxide NPs, semiconductor NPs, and even composite NPs, have been widely used in electrochemical sensors and biosensors. Metallic NPs are very interesting, because of the fact that these particles show size-dependent characteristics of the material. Especially in the nanometer scale, these size-dependent properties are observable. Metal NPs with at least one dimension approximately 1-100 nm have received considerable attention in both scientific and technological areas due to their unique and unusual physico-chemical properties compared with that of bulk materials. This means the NPs have change in color when their size differs, whereas bulk materials do not have change in color in even if their size varies [34].

Metal oxides have also been serves as sorbents for various environmental pollutants. But the biological methods of synthesis are more favorable than the chemical and physical methods of synthesis since these methods are eco-friendly. The biological methods for synthesis of nanoparticles by using various microorganisms, enzymes, plants, and their extracts have been

suggested as the probable and promising ecofriendly alternatives to the chemical and physical methods of synthesis. Among those ZnO NPs are used in the elimination of toxic chemicals like Arsenic, sulfur from water sources owing to their large surface area by volume ratio than the bulk materials [35].

2.4. ZnO nanoparticles: its application and properties

ZnO NPs are extensively studied material due to their low toxicity, high electron mobility, wide band gap, strong room-temperature luminescence, good transparency and photochemical stability. The attractive properties of ZnO NPs at room temperature and pressure make them enormous use in electronics, optoelectronics and laser technology. At present, ZnO NPs are used in new light-emitting devices, solar cells, biosensors, and Photocatalysis [36]. ZnO is a bio-friendly oxide semiconductor and an inexpensive luminescent material. It has attracted intensive research efforts for its unique properties and versatile applications in antireflection coatings, transparent electrodes in solar cells, ultraviolet (UV) light emitters, diode lasers, piezoelectric devices, spin-electronics, surface acoustic wave propagator, antibacterial agent, photonic material and for gas sensing. Among all the inorganic semiconducting NPs, ZnO NPs has attracted increasing attention because ZnO NPs can be easily synthesized and ZnO is a “green” material that is biocompatible, biodegradable, and nontoxic for medical applications and environmental science. Due to their excellent optical and electrical properties, ZnO NPs have become predominant semiconductor materials for nanoscale devices, such as nano-generators, gas sensors, highly efficient solar cells, field-emission transistors, ultra violet photo detectors, and biomedical systems ZnO is attracting considerable attention for its possible application to UV light emitters, spin functional devices, gas sensors, transparent electronics and surface acoustic wave devices [14].

Zinc oxide is frequently used in several areas of technology. It is worthy to investigate high-quality self-textured ZnO films synthesized on different kinds of substrate [37].Zinc oxide NPs are used in biomedicine like biomedical imaging, drug delivery, gene delivery, and bio sensing [38]. The ZnO powder is widely used as an additive into numerous materials and products including plastics, ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, etc. ZnO NPs are useful as antibacterial and antifungal agents when incorporated into materials, such as surface coatings (paints), textiles,

and plastics. Due to bacteriostatic and fungistatic behavior of Zinc Oxide, it was well studied and utilized in personal care products.

Due to its non-toxicity and compatibility with it skin, makes it a suitable additive for textiles and surfaces that come in contact with human body. Zinc Oxide's UV attenuation properties also make it an effective additive for packaging plastics to prevent UV mediated damage. Zinc Oxide is also used as a catalyst for methanol synthesis. The increase in surface area of nanoscale Zinc Oxide compared to larger powders has the potential to improve the efficiency of both aqueous and organic solvents, allowing for incorporation into most material processes [39].

2.5. Synthesis methods of ZnO nanoparticles

The synthesis of NMs is of current interest due to their wide variety of applications in fields such as electronics, photonics, catalysis and medicine. The synthesis approach significantly impacts the properties of such NPs and these properties in turn have a significant impact on their biomedical applications. Basically there are two main approaches for NP synthesis i.e. Top down and Bottom up approaches as shown in figure 1.1 [40]. In top down approaches, NPs are produced by size reduction from suitable starting material. Size reduction is achieved by various physical and chemical treatments. Top down production methods introduces imperfections in surface structure of the product and other physical properties of NPs are highly dependent on the surface structure.

In bottom up synthesis, the NPs are built from smaller entities, example, by joining atoms, molecules, and smaller particles. The nanostructured building blocks of NPs are formed first and then assembled to produce the final particles. The bottom up synthesis mostly relies on chemical and physical methods of production [41]. A variety of synthetic techniques are used for the synthesis of ZnO. These techniques broadly can be divided into three types, that is, chemical, biological, and physical methods [18].

2.5.1. Physical and chemical synthesis methods

Arrays of conventional methods have been employed in synthesis of nanoparticles. They can be categorized mainly under physical and chemical methods. Some of the physical methods known for the synthesis of nanoparticles include radiolysis, microwave, ultrasonication, laser ablation and electrochemical methods [15]. 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 NPs 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.

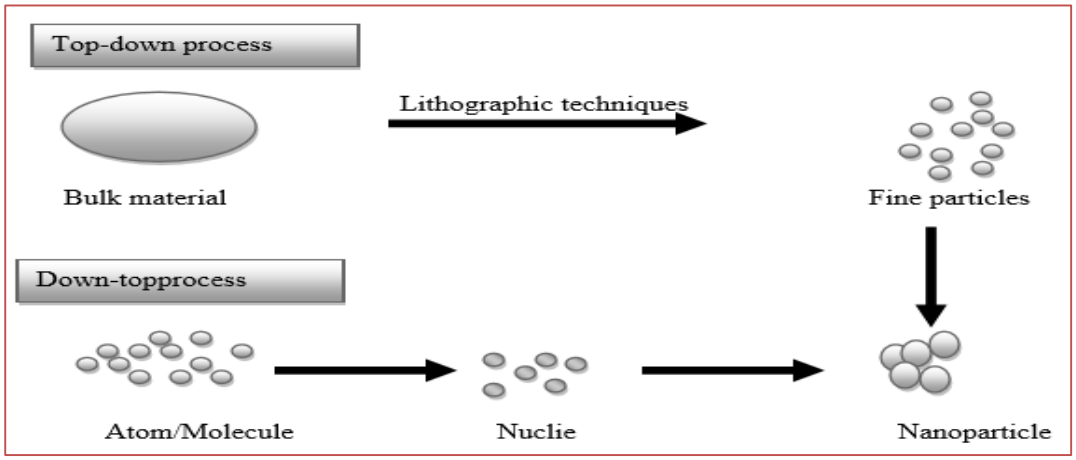


Figure 2.1: methods employed for synthesis of NPs [7-10]

The physio-chemical methods for synthesis of metal nanomaterials are costly; require grand labour and large time. In addition, large quantities of secondary waste are generated resulting from the addition of chemical agents for precipitation and reduction in the processes[42].Chemical and physical methods may successfully produce pure, well- defined nanoparticles. But these techniques are more expensive, energy consuming, and potentially toxic to the environment [43].

2.5.2. Green chemistry (biological) methods

Green chemistry is the fabrication of products and methods that reduce or eliminate the use and ultimately the disposal of hazardous substances out of the environment. Passed in 1990 as a Pollution Prevention Act, green chemistry is a new approach for dealing with pollution by preventing environmental problems before they happen. Recently, synthesis of NPs using plants and microorganisms including bacteria, viruses, fungi and yeast has been reported to be an eco-friendly alternative method of synthesizing a wide variety of NPs. Of these biological entities, synthesis of NPs using plant parts and plant extracts has been demonstrated to be cost-effective and much simpler because it doesn’t require multi-step procedures that involve complex culturing and isolation techniques. The ability of plants to hyper-accumulate and biologically reduce metal

ions has been known since the early 1900s and has been used for extracting precious metals from land unjustifiable to mine. However, the use of whole plants in the synthesis of NPs has certain limitations where the heterogeneity of the size and morphology of NPs produced hinders their use. For instance, in clinical applications whereby the size and shape play a critical parameter effecting cell uptake, rate and site specific for drug delivery from the system. In this regard, using plant extracts have been favored as a simpler alternative for synthesizing NPs.

Plant extract containing various biomolecules with functional groups such as C=C (alkenyl), C=N (Amide), -O-H (phenolic and alcohol), N-H (amine), C-H and COO- (carboxylic group) are mainly responsible for the reduction and stabilization of metallic ions into metal oxide NPs. The extraction method involves boiling the plant part. The obtained extract contains the reducing and capping agents needed to reduce metallic ions. Some of the plants extracts used in the bioreduction of ZnO NPs are reported in table 2.1.

Table 2.1: List of different plants used for synthesis of ZnO NPs [44].

Scientific name	Common name	Source of extract	Precursor	Size(nm)	Shape
Abrusprecatorius	Rosary pea	Seed	Zn(Ac)	90-500	Spherical
Aloe barbadenis miller	Aloe Vera	Leaves	$Zn(NO_3)_2$	25-40	Spherical
Ocimum tenuiflorum	Holy basil	Leaves	$Zn(NO_3)_2$	11-25	Hexagonal
Ruta graveolens	Rue	Stem	$Zn(NO_3)_2$	~28	Hexagonal
Agathosma betulina	Buchu	leaves	$Zn(NO_3)_2$	~19.4	Spherical
Azadirachta indica	Neem	Leaves	Zn(Ac)	9.6-25.5	Spherical

2.5.3. Limitation of conventional methods

Chemical methods have drawbacks of contamination by toxic solvents and production of hazardous byproducts. The drawback of the physical technique is that; resultant NPs 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. This method also requires strong and weak chemical reducing agents as well as capping agents like sodium borohydride, sodium citrate and alcohols. These agents are generally highly toxic, flammable, cannot be easily disposed of. 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 using of toxic chemicals and it's dangerous by products, concentrated reducing agents, high level of radiation, and contamination from precursor chemicals. Hence it is a fact that reproducibility and stability of the NPs 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 NPs which is alarming threat in every aspect of flora, fauna and human health [16].

Biological methods using microorganisms are clean, nonhazardous, eco-friendly and energy efficient. These reduce metal ions at faster rate and are carried out at ambient conditions. Microorganisms can survive in difficult conditions such as high concentration of metals by adopting survival mechanisms such as efflux system, extracellular combination and precipitation and chemical detoxification [40].

2.5.4. Green synthesis of metal oxide nanoparticles

In order to overcome the negative impacts of the chemical methods, a new science for the research and commitment has 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 NPs, such as; ZnO Nps [12].

The following figure shows the comparison of conventional method and green synthesis methods.

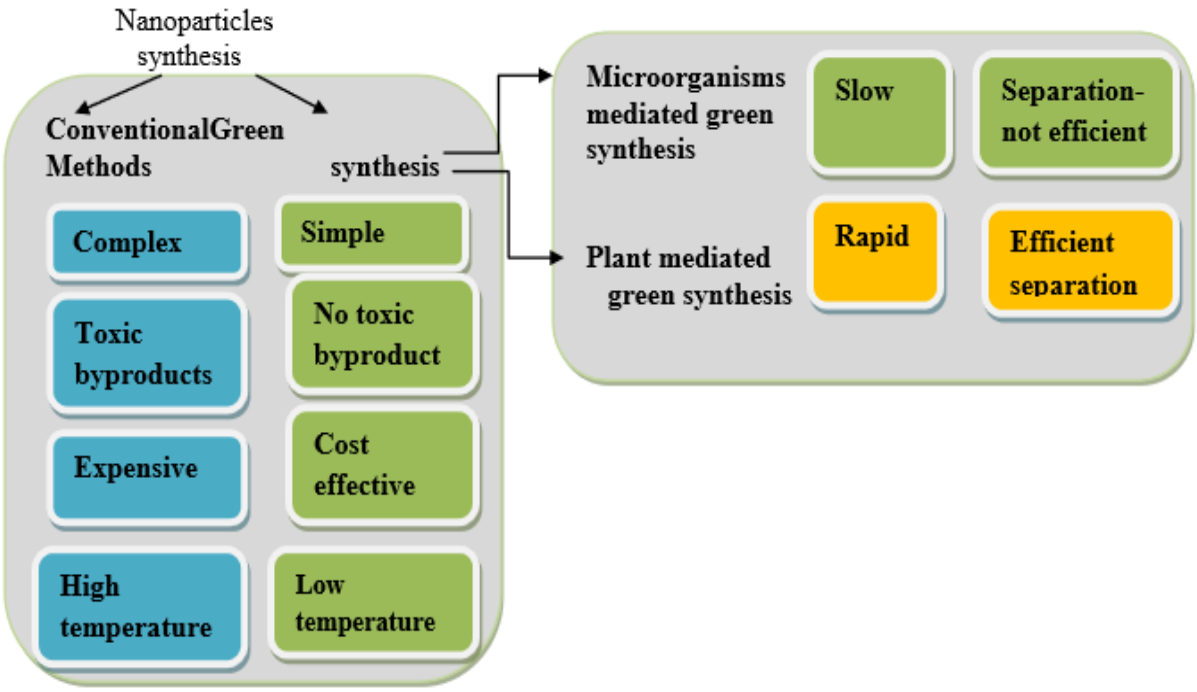


Figure 2.2: Comparison of conventional methods and Green synthesis methods of nanoparticles

Biosynthesis of Metal or metal oxide nanoparticles using environmentally friendly methods without the use of harsh, toxic reducing agents (e.g. 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, NPs 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 NPs that may have adverse effect when used for medicinal purpose.

Green chemistry is the design, development and implementation of chemical products and processes to reduce or eliminate the use and generation of substances hazardous to human health and the environment. In synthesis of metal oxide NPs 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 [45].

2.5.4.1. Microorganisms mediated green synthesis of ZnO NPs

Microbes are considered as eco-friendly factories of NPs synthesis. Interactions between metals and microbes have been exploited for various biological applications in the fields of bioremediation, biomineralization, bioleaching, and biocorrosion. It is one of the most sustainable, eco-friendly techniques of are in spite of its few limitations. Both prokaryotes and eukaryotes are used for the synthesis of metal/metal oxide especially ZnO. Further, the synthesis may be intracellular or extracellular. Fungi are decomposers as well as parasite in nature. In intracellular synthesis, fungal biomass is incubated for a particular time period in dark along with a zinc salt solution, while in extracellular synthesis fungal filtrates are treated with the precursor solution and synthesis is assessed. A number of studies are available on using fungi as ZnO synthesizer. However, a complete understanding of synthesis mechanism occurring in microorganisms is yet to be fully developed. Bacteria are known to synthesize metallic NPs by either intracellular or extracellular mechanisms this is because each type of microorganism tends to behave and interact differently with particular metallic ions. The interaction and biochemical processing activities of a specific microorganism and the influence of environmental factors such as pH and temperature ultimately determines the formation of NPs with a particular size and morphology [46].

In nature, bacteria are frequently exposed to diverse and sometimes extreme environmental situations. Survival in these harsh conditions ultimately depends on their ability to resist the effects of environmental stresses. Natural defense mechanisms exist in bacteria to deal with a variety of stresses such as toxicity arising from high concentrations of metallic ions in the environment. Biological strategies for dealing with high concentrations of metallic ions include changes in metal ion concentration via red-ox state changes, efflux systems, intracellular precipitation, and accumulation of metals, and extracellular formation of complexes. Size, shape, and composition of a nanoparticle can be significantly influenced by pH and temperature [47]. For example, particle size is an important factor since novel and unique physicochemical properties are more pronounced

at smaller sizes. Therefore, there is a need to optimize synthesis parameters during nanoparticle formation to enhance the overall particle properties [47].

2.5.4.2. Plant mediated green synthesis of ZnO nanoparticles

Plants and plant extracts as machinery for metal NPs synthesis are fascinating as they eliminate the need of using hazardous materials as well as the tedious process of culturing and downstream processing. However, plant extracts are more attractive because the methodology is much simpler and cost-effective. The mechanism of synthesis of such NPs by plants is their capability to uptake metals from the soil and water, hyper accumulation, and further reduction to recoverable NPs. Such techniques are extensively used in phytoremediation and phytomining. However, the focus is majorly on silver and gold NPs on using plants. Literature is limited in the synthesis of ZnO by plant and plant extract based routes.

In vitro approaches make use of plant extracts to bioreduce a particular zinc salt (zinc nitrate, sulphate, chloride, and many other) and provide a control over size and shape of the nanoparticles. Basically, plants contain a number of primary and secondary metabolites, for example, tannins, terpenoids, saponins, starches, polypeptides, flavonoids, and phenolic, that act as an excellent reducing as well as capping agents. Mild solvents like water, ethanol, or methanol are used for the extraction of the plant metabolites, which are allowed to react with zinc salt solution under different conditions to obtain a maximum yield [43]. The phytochemicals present in plant leaf extracts have uncanny potential to reduce metal ions in a much shorter time as compared to fungi and bacteria, which demands the longer incubation time. Therefore, plant leaf extracts are considered to be an excellent and benign source for metal as well as metal oxide nanoparticle synthesis. Additionally, plant leaf extract play a dual role by acting as both reducing and stabilizing agents in nanoparticles synthesis process to facilitate nanoparticles synthesis. The composition of the plant leaf extract is also an important factor in nanoparticle synthesis, for example different plants comprise varying concentration levels of phytochemicals.

In recent years, plant-mediated biosynthesis of zinc oxide nanoparticles has been achieved in *Parthenium hysterophorus*, *Sapindus rarak*, *Acalypha indica*, *Passiflora foetida*, *Ficus benghalensis*, *Zingiber officinale* etc [43].

2.6. Neem (Azadirachta Indica)

Scientific classification

Table 2.2: Classification of Neem

Kingdom:	Plantae
Division:	Magnoliophyta
Order:	Sapindales
Family:	Meliaceae
Genus:	Azadirachta
Species:	Indica

Figure 2.3: Description of Neem plant (photograph)

Neem (*Azadirachta Indica* A. Juss) is a plant of the Meliaceae family belonging to the Indian subcontinent. It was later introduced into many tropical countries of America and Africa. Neem is a fast-growing tree that can reach a height of 15–20 m, rarely to 35–40 m. It is evergreen, but in severe drought it may shed most or nearly all of its leaves. *Azadirachta Indica* is variously known as Sacred Tree. The Neem tree is noted for its drought resistance [48].

The *A. Indica* is a very useful traditional medicinal plant in the African sub-continent and each part of the tree has some medicinal property. Neem trees (leaf, stem, bark and seed) are known to antibacterial, antifungal activities against different pathogenic microorganisms and antiviral activity against Vaccinia, Chikungunya, and measles. Neem also contain biologically active principles isolated from different parts of the plant include: azadirachtin, Meliacin; gedunin, salanin, nimbin, valassin, and many other derivatives of these principles. Meliacin forms the bitter principles of Neem seed oil; the seed also contain tignic acid (5-methyl-2-butanicacid) responsible for the distinctive odour of the oil. These compounds belong to natural products called triterpenoids (limonoids). The active principles are slightly hydrophilic, but freely lipophilic and highly soluble in organic solvent like hydrocarbon, alcohols, ketones and esters. Also, Neem twigs are used as tooth brushes in some tropics [49].

2.6.1. Studies Related to Phytochemical Constituents of Neem leaf

The Medicinal plants are rich in secondary metabolites which include alkaloids, flavonoids, saponins and related active metabolites which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry. These secondary metabolites are reported to have many biological and therapeutic properties. Recently number of studies had been reported on the Phytochemistry of medicinal plants, particularly on the vegetative parts like leaves and stems etc. Alkaloids, flavonoids, glycosides have been reported to exert multiple biological effects like anti-inflammatory, anti-allergic, antioxidant, anti-diabetic, anti-viral and anti-cancer activities, anti-leprosy activities, antimicrobial activity etc. The phytochemical constituents are well known for its curative activity against several human problems such as ulcers, swollen liver, malaria, dysentery, diarrhea etc. A variety of herbs and herbal extracts contain different phytochemicals with biological action that can be of valuable therapeutic index. Much of the protective effect of herbal plants has been attributed by phytochemicals, which are the non-nutrient compounds [51]. The most commonly active compounds found in neem are azadirachtin, nimbin and nimbidine. The most important of these bioactive constituents of plant are alkaloids, tannins, flavonoids and phenolic compounds. The most active chemical compounds are slightly hydrophilic in nature; however, they are freely lipophilic and more soluble in organic solvents, such as water, alcohol, ketones and esters which could be used as reducing, stabilizing, chelating and capping agents to react with zinc ions and as scaffolds to direct the formation of ZnO NPs in solution [49].

2.6.2. Mechanism of Zinc oxide nanoparticles formation

Various plant constituents, including terpenoids, polyphenols, alkaloids, phenolic acids play an important role in the biosynthesis of zinc oxide nanoparticles Flavonoids are Polyphenolic compounds and contain various functional groups that are capable of donating electron for the reduction of Zn^{2+} ions to ZnO NPs formation. The active ingredient responsible for reduction of Zn^{2+} ions varies depending up on the plant extract used or bioactive molecules.

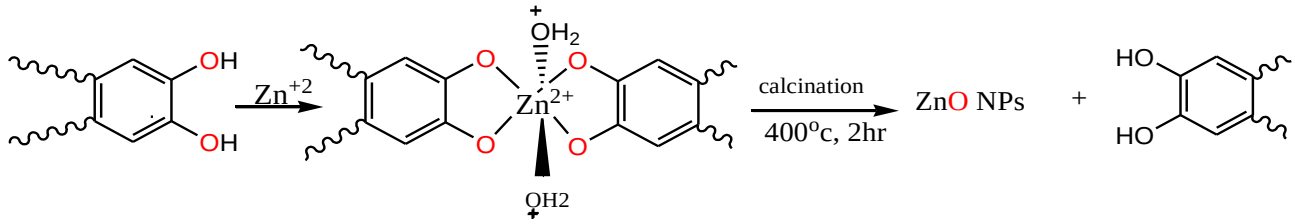
In this study, zinc oxide nanoparticles was prepared using extracted neem leaf with zinc nitrate solution in Green synthesis method. The neem leaves possess biomolecules such as Phenolic acids, flavonoids, proteins, terpenoids, and alkaloids which could be used as reducing, stabilizing, chelating and capping agents to react with zinc ions and as scaffolds to direct the formation of ZnO NPs in solution. Although the exact mechanism for the synthesis of nanoparticles using extracts

has not been devised yet, it was suggested that different polyol components are responsible for the synthesis of the nanoparticles. The FT-IR spectrum again confirmed the formation of ZnO NPs in the presence of plant extracts. The peaks of the spectrum have indicated the secretion of some water-soluble organic components (phytochemicals) from the plants, which might have contributed in the formation of ZnO NPs via similar mechanism. This gives strong evidence for the involvement of polyphenols in the rapid biosynthesis and for the stability of metallic oxide nanoparticles in the aqueous medium**.** This definitely reveals that flavonoids, poly phenols and other phytochemicals found in plant extracts are the ones responsible for the synthesis of nanoparticles like ZnO NPs by firstly donating electrons to the metallic cation and stabilizing the newly formed nanoparticles. Since neem leaves which is composed of phytochemicals like Phenolic acids, flavonoids, and alkaloids, it was expected to be helpful in synthesizing the ZnO NPs by donating electrons to the zinc (II) cation of the zinc nitrate solution [52].

The overall observation proved the existence of some phenolic compounds, terpenoids or proteins that were bound to the surface of ZnO nanoparticles. The stability of ZnO nanoparticle could be due to the free amino and carboxylic groups interacted with the zinc surface. The bonds of functional groups such as –CO–C–, –C–O– and –C=C– were derived from heterocyclic compounds and the amide bands derived from the proteins were present in the leaf extract and were the capping ligands of the nanoparticle. Moreover, the proteins presented in the medium prevented agglomeration and aided the stabilization by forming a coat, covering the metallic nanoparticles.

In green synthesis of ZnO NPs, biomolecules found in the Neem leaf extract induce the reduction of Zn^{2+} ions from $Zn(NO_3)_2.6H_2O$ to ZnO NPs or in another synthesis, the plant extract of neem acts as ligation, and the aromatic hydroxyl group present in Polyphenolic ellagic acid ligate with zinc ions to form zinc-ellagate complex. Calcination of this complex at 400 °C for 2 hrs. In static air leads to the formation of ZnO NPs [53].

$Zn(NO_3)_2 \rightarrow Zn^{2+} + 2NO_3^-$



Schceme.2: Possible mechanisms of formation of ZnO NPs by using the plant leaf extracts [53]

2.7. Antimicrobial activity of ZnO 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 NMs have also increased because microorganisms are developing more resistance towards existing antibiotics [55]. Current advances in the field of Nanobiotechnology, particularly the ability to prepare metal oxide NMs 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 (microbicidal or growth-inhibiting) activity have been synthesized [56]. 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 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. 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, zinc oxide can be an excellent candidate for production of antimicrobial textiles.

Zinc oxide has received increasing attention as antibacterial agent in recent years among the various metal oxides studied for their antibacterial activity, as zinc oxide nanoparticles are highly toxic to prokaryotic cells. More over their stability under harsh processing conditions and relatively low toxicity combined with the potent antibacterial properties favors their application as antibacterial. Little is known regarding interaction of nanoparticles with other bacteria and importantly less is known about the mechanism underlying the antibacterial [57].The antibacterial activity of chemically synthesized zinc oxide nanoparticles against two strains of pathogenic bacteria such as Escherichia coli, and Staphylococcus aureus was done by using well diffusion method.. Literature revealed that the electrostatic interactions between ZnO NPs and cell walls resulting in destroying bacterial cell integrity, the liberation of antimicrobial Zn^{2+} ions which is related to an accumulation of ZnO NPs into the bacterial cells shown in Figure 2.4 [61].

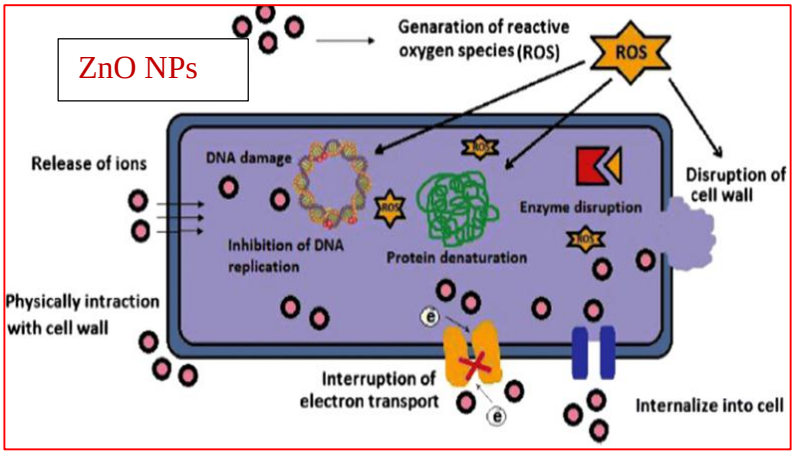


Figure 2.4: Schematic illustration of antibacterial activity of ZnO NPs

Therefore it was clear that green synthesized ZnO NPs has been used as an antibacterial agent. And the neem leaves synthesized ZnO NPs was expected to be effective in killing bacteria. In this work antibacterial activity of ZnO NPs was examined by disc diffusion method for two bacterial strains. This was quantitatively done by measuring the clear zone of inhibition. Generally, strains are thought to present a major public health problem. The antibacterial activity of the ZnO NPs was examined against the one Gram-positive and Gram-negative bacteria.

3. MATERIALS, CHEMICALS, AND METHODS

3.1. Materials and chemicals

Zinc nitrate solution, 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(sigma Aldrich, Israel), Gentamicin(HI media laboratories, India), KBr (Uvasol, Germany) Agar Hilten Muller (Oxoid CM, UK), Chloroform (99.9%, Fisher Scientific UK Limited, UK), Sodium Hydroxide(Blulux, India), Ammonia solution (25%) (Both of them are products of Blulux Laboratories (P) Ltd), Benedict's solution, Magnesium Chloride (98%), Iodine solution (80%), Potassium Iodide (99%) (All of them are produced by Abron Chemicals, India).

3.2. Instruments and Apparatus

The necessary apparatus and instruments used for this study were UV-Vis spectrometer (Agilent technologies, Cary 60 UV-Vis), FT-IR spectroscopy (Perkin Elmer), electronic beam balance, Furnace, store chamber (Lec refrigerator plc. England), and others were used for different purposes.

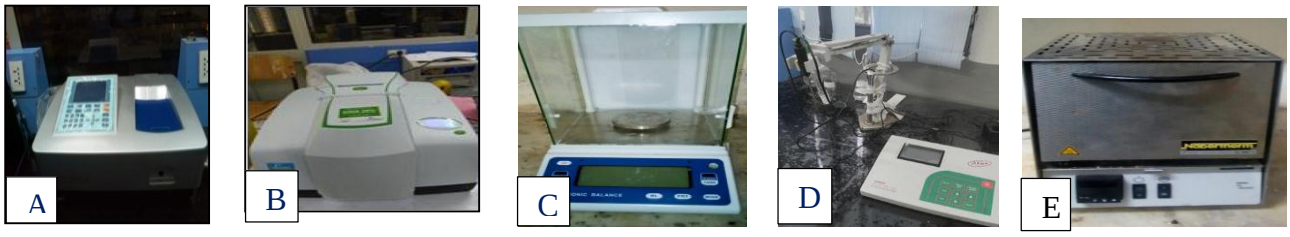


Figure 3.1: Some of the instruments used during the experiment. (A) UV-Vis spectrometer (B) FTIR spectrometer (C) Electronic beam balance (D) digital PH meter (E) Furnace

3.3. Experimental Procedures

Typically, a plant extract mediated bio-reduction involves mixing the aqueous extract with an aqueous solution of the appropriate metal salt. A ZnO NPs has been prepared by using aqueous Neem leaves mixed with zinc nitrate solution. The synthesis of nanoparticles was carried out at room as well as other 60-80°c and was completed within 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 ZnO NPs. All the glassware is washed with distilled water and dried in air. De-ionized and distilled water were used in all of the experimental works.

3.3.1. Preparation of Neem (Azadirachta Indica) leaf extract

Ethiopia medicinal plant typically, Green neem tree was selected from Peda campus, Bahir Dar University, on the basis of cost-effectiveness and ease of availability. Fresh neem leaves were collected locally and rinsed thoroughly first with tap water followed by distilled water to remove all the dust and unwanted visible particles, cut into small pieces and the leaves were then homogenized using mortar and pestle. The aqueous leaves extract was prepared by placing 5 g of washed and dried fine cut leaves in 250 ml glass beaker along with 50 ml of distilled water. The mixture is then heated at 60°c for 20 minutes. Then, the extract is cooled to room temperature and filtered through Whatman No.1 filter paper to remove particulate matter and to get clear solution. The filtrate was collected and then stored in refrigerator at 4°C in 250 ml conical flask in order to be used for further experiments [60].

3.3.2. Phytochemical screening- Qualitative analysis

Chemical tests were carried out for the screening and identification of bioactive chemical constituents like alkaloids, carbohydrates, glycosides, saponins, terpenoids, Phenolic compounds, proteins, amino acids, flavonoids, and tannins, in the neem plants were used as reducing and capping agent, to cheek whether these components are found in neem leaves by using standard procedure [58, 52].

3.3.2.1. Test for Alkaloids: (Wagner's test: Iodine- Potassium iodide solution)

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

3.3.2.2. Test for Glycosides

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

3.3.2.3. 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.

3.3.2.4. Test for Flavonoids

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

3.3.2.5. 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 has been checked.

3.3.2.6. Test for Steroids (Salkowski test)

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

3.3.2.7. Test for Phenols

The extract Neem 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.

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

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

3.3.3. Synthesis of ZnO nanoparticles

For synthesizing of Zinc oxide nanoparticles, 2 mM of zinc nitrate was taken in a beaker and dissolved in 100 ml of distilled water under mild stirring for 10 minutes using a magnetic stirrer. After being stirred, 2 ml of the reducing agent and the capping agent of the leaf extract were added into the precursor solution and heated at 60 ^{0}C- to 80 ^{0}C. The solution turned from clear colorless to a deep yellow colored paste confirming the formation of ZnO NPs. The paste was transferred into a ceramic crucible and kept in a furnace heated at 400 ^{0}C for 2 h. Finally the resultant powder was used for further characterization using the visual observation, UV-Vis spectra, FT-IR, and XRD. At last, it was examined for its antibacterial activity.

3.4. Characterization techniques of the synthesized ZnO Nps

The synthesized ZnO Nps was characterized by visual observation, UV–Visible absorption spectroscopy, X-ray diffractomtere spectroscopy, and Fourier Transform Infrared spectroscopy analyses. During synthesis of the nanoparticles, every color change for every step was noticed carefully. The colors changes before and after Neem 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 Zn^{+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 spectra's were recorded and analyzed [28]. To Use FT-IR spectroscopy, a drop of the 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 400 to 4000 cm^{-1} [59]. The same was done for the ZnO Nps formed

3.5. Antimicrobial activity of ZnO NPs

Disc diffusion methods

The discs is soaked with double distilled water, 5 % (m/v) Neem extract, Zinc nitrate hexa hydrated solution and solution containing ZnO Nps of each type separately. Gentamicin 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 (staphylococcus aureus 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 ZnO Nps, disc soaked with plant leaves extract, and disc soaked with 2 mM zinc nitrate 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.

4. RESULT AND DISCUSSIONS

4.1. Phytochemical test Analysis of the Extracted Neem leaf

Phytochemical analysis of plant extracts supported the results obtained from FT-IRspectrum. It was observed that phenolic, flavonoids, ascorbic acids, and alkaloids present in the Neem plant extract can trigger the reduction of zinc nitrate and control the size of synthesized ZnO NPs. The results of qualitative phytochemical analysis of the Neem leaf extract are shown below in Figure.4.1and table 4.1. The Aqueous extract of Azadirachta indica showed the presence of alkaloids, flavonoids, glycosides, reducing sugar, terpenoids, tannins and phenols. The preliminary phytochemical investigations it was observed that alkaloids, tannins, phenols, flavonoids, terpenoids, and glycosides were present in the extract. The presence of these secondary metabolites in plants extract is used as reducing and capping agent, it was necessary to check whether the phytochemicals, such as flavonoids, polyphenols, alkaloids, carbohydrates, tannins, saponins etc., do naturally exist in the extract or not. Therefore; the Neem leaf extract is composed of phytochemicals which are capable of reducing the Zn^{+2} by donating electrons, capping and stabilizing the formed nanoparticles [50, 60].

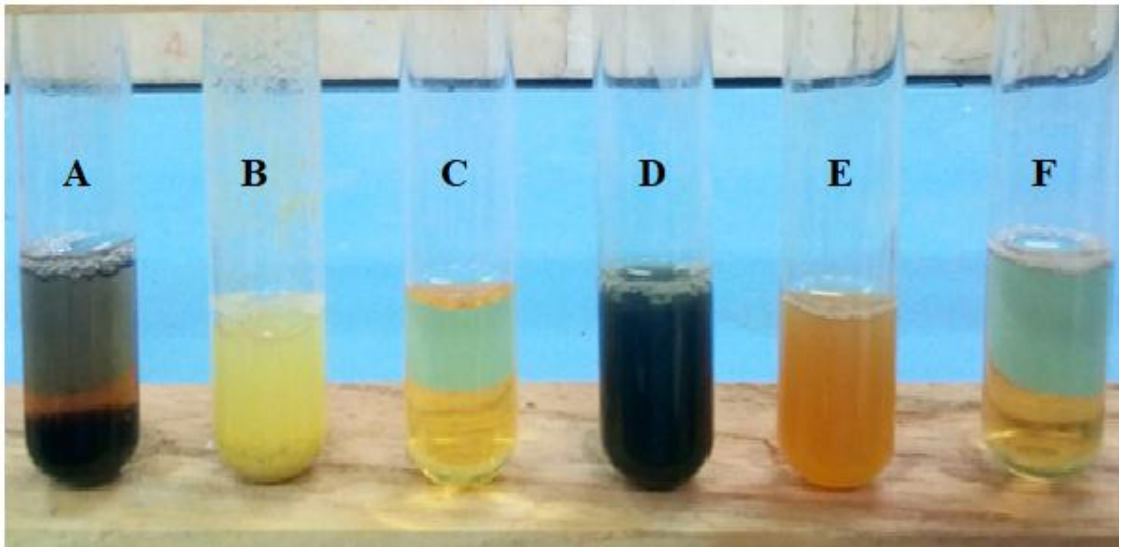


Figure 4.1: The colors observed when the extract is tested for (A) Alkaloids (B) Flavonoids (C) Glycosides (D) Tannins (E) Phenols and (F) Terpenoids

For instance, the Polyphenolic compounds 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. The color changes are the results of formation of different complexes as a result of oxidation and reduction reactions. In most of the Ferric chloride tests, the iron (III) ion forms complexes having different colors [50]. These different colors assures the presence of alkaloids, flavonoids, phenolic compounds and tannins in extracted neem leaf.

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

No	Phytochemicals	Chemical test	Results	Colors observed	
1	Alkaloid	Wagner's test	+	Reddish brown	
2	Flavonoids	Ferric Chloride test	+	Yellow(orange)	
3	Glycoside	Alkaline reagent test	+	Yellow	(+) = presence
4	Tannin	Ferric Chloride test	+	Dark green	
5	Phenols	Ferric Chloride test	+	Intense color	
6	Terpenoids	Lieberman butchered	+	Forms layer	

4.2. Characterization of the synthesized Zinc Oxide Nanoparticles

The formation of ZnO NPs nanoparticles was confirmed primarily based on change in colors of the reaction mixture, and then using UV-Vis, FT-IR, and XRD spectrophotometer which were frequently used to characterize the synthesized metal oxide nanoparticle

4.2.1. Visual observations for ZnO NPs

Figure 4.2: shows the synthesis of zinc oxide nanoparticles from aqueous neem extract was confirmed by visual observation. When colorless zinc nitrate was mixed with neem extract, a pale yellow colour was produced, which confirmed the formation of zinc oxide nanoparticles. The color changes arise due to excitation of surface Plasmon resonance in the metal oxide nanoparticles indicating the formation of ZnO Nps. The colorless 2 mM Zn $(NO_3)_2.6H_2O$ solution started changing its color to light yellow at the time when the Neem leaves extract was added to it.

Earlier, previous reports observed deep yellow colour while synthesizing zinc oxide nanoparticles from the leaves of C. *procera*, yellow colour while synthesizing zinc oxide nanoparticles from *Punica granatum peel* and pale yellow, while synthesizing zinc oxide nanoparticles from leaf, stem and root extracts of *Hybanthus enneaspermus* [53].

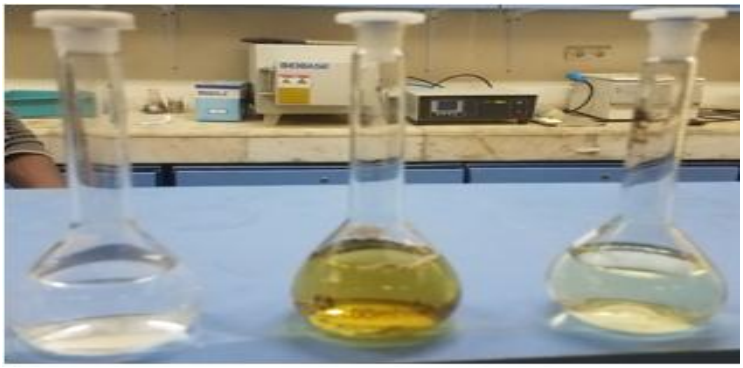


Figure 4.2: colour changes for the formation of ZnO NPs (A) Extract of Neem (B) Zinc nitrate (C) light yellow of ZnO NPs

4.2.2. UV- Visible Spectra results 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 Plasmon's 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 oxide 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. [61].

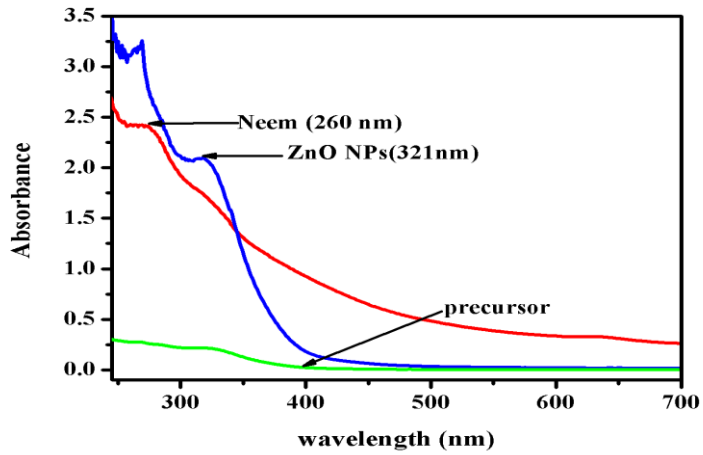


Figure 4.3: UV–Visible spectra of the precursor, Neem extract and ZnO nanoparticles at pH =9

The UV–Vis spectra result revealed a strong absorbance at 321 nm suggesting the formation of zinc oxide nanoparticles (Figure. 4.3) while the pure Neem extract has a strong absorbance at 260 nm. This result definitely agrees with the range of λ_{max} values of the ZnO Nps, 280 nm - 380 nm, at different previous works using plants other than Neem [20, 22, 26, and 42]. This shows, during the formation of the ZnO 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.

4.3. Optimization of Different Parameters of ZnO Nps result analysis

4.3.1. Effect of Zinc nitrate concentration on the synthesis of ZnO NPs

In this optimization, we have taken different concentration of zinc nitrate solution mixed with constant concentration of Neem extract. The result showed that at different concentration different maximum wave length and peak intensity were obtained. In this work, 321 nm and 1.863 at 2 mM, 315 nm and 2.159 at 4 mM, 318 nm and 2.958 at 6 mM, of maximum peak wave length and absorbance intensity were obtained respectively.At the lower concentrations 0.25-1 mM, there was no peaks formed. As shown in Figure 4.4**,** as the concentration of zinc nitrate increases, the intensity of the peak would also increases. Comparing the peaks obtained, it was shown that 2 mM Zn $(NO_3)_2$ was the best concentration of the precursor to be used for the synthesis of ZnO NPs with an intense peak and more smooth as compared to the others.This result definitely agrees with the concentration of Zn $(NO_3)_2$ used for the synthesis of ZnO NPs using aqueous leaf extract of

Catharanthus roseus [62]. Agglomeration was formed on peaks at higher concentration,This shows that when the concentration of the zinc ions in the solution was increased, it has exceeded the amount of reducing agent phytochemicals of the neem extract. This Higher concentration of zinc nitrate suggests the formation of larger ZnO NP. The large size and aggregation of nanoparticles was occurred due to the competition between Zn^{2+} and functional groups of 2 ml extract of Neem leaf. This investigation concludes that the optimum zinc nitrate concentration (2mM) was suitable for ZnO nanoparticles synthesis using neem leaf extract. Because the peak is more intense and sharp.

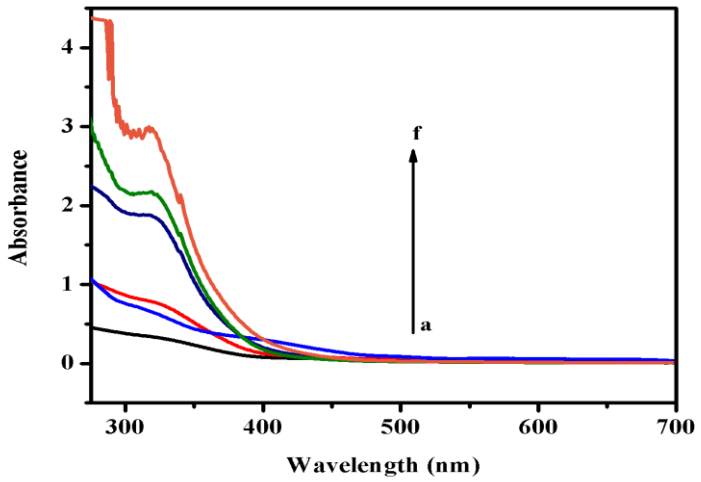


Figure 4.4:The UV-Vis spectra of ZnO NPs from 2 ml extract of 5 % (m/v) Neem leaf and 25 mL of (a, b, c, d, e, f,), (0.25, 0.50, 1, 2, 4, and 6 mM) Zn(NO3)2.6H2O respectively at pH = 9

4.3.2. Effects of concentrations of Neem leaf on synthesis of ZnO NPs

The results were obtained from UV-Visible spectroscopy analysis of the sample. Figure 4.5 shows that the absorption spectra of the Zinc oxide nanoparticles were obtained. Figure 4.5 shows that; the concentration of Neem leaf extracts which gave a broader peak were 1- 4 % (m / v) relative to the 5 %, 6% and 7 %. This reveals that the neem leaf extract is dominating the solution and the nanoparticle formed has become more unstable. Despite of all these facts, extract of 5 % (m/v) neem was taken as the best concentration of the extract for its smooth peak with good intensity. Because increasing the concentration above 5 % (m/v) was noticed making the peaks noisier. Therefore, increasing the concentration above this value might ultimately make the nanoparticle unstable [63].

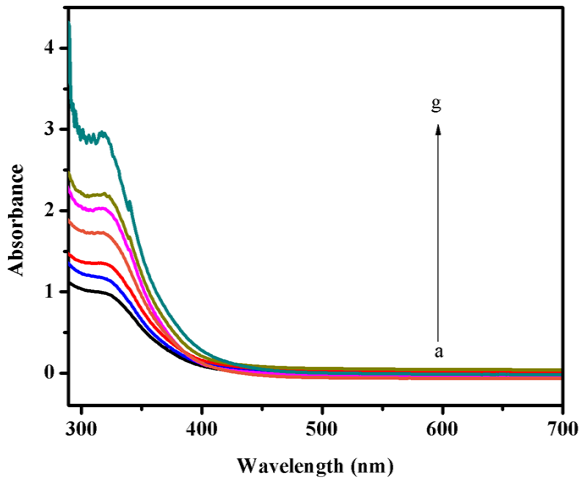


Figure 4.5: UV-Visible spectra for the ZnO NPs formed from 25 ml of 2 mM Zn (NO3)2. 6H2O) and (a, b, c, d, e, f, g), (1, 2, 3, 4, 5, 6 and 7 % (m / v)) neem leaf extract respectively at pH = 9

4.3.3. Effect of pH on synthesis of ZnO NPs

PH is considered as an important parameter in nanoparticle synthesis. In our study, the solution was adjusted to different pH (1, 3, 5, 7, 9, 11, and 13) and using 0.1 M HCl and 0.1 M NaOH. After pH adjustment, absorbance (200 to 700 nm) of the resulting solution was measured spectrophotometrically. To study the eff ect of pH, considered the most important parameters aff ecting the NPs formation, leaf extract (2.0 ml), bulk zinc nitrate (2 mM), and incubation time were kept constant. The pH of the solution before addition of NaOH were 5.3. In this experiment further pointed out, as shown in Figure 4.6, the difference in the intensity of the colour as pH increases (1, 3, 5, 7, 9, 11,and 13) at constant concentration of the reducing agent (*Azadirachta indica* leaf extract) and precursor. This is because as the pH increases, the rate of formation of the nanoparticles increases and intensity of colours also increases. Typically, at pH 1, 3, and 5 these colours were not primarily confirmed with synthesizing ZnO NPs and the result is in good agreement with previous reported work [73], broad SPR bands at low pH, which indicated the formation of large size nanoparticles.

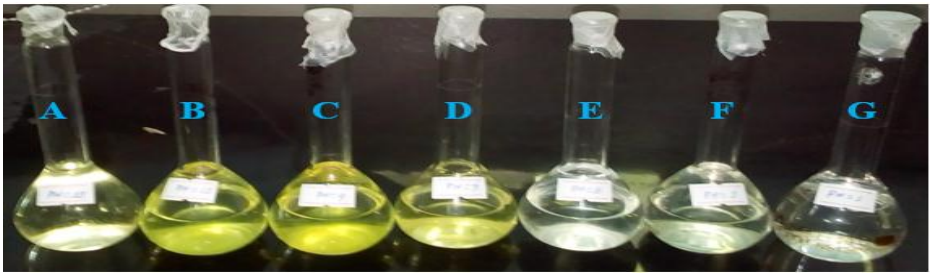


Figure.4. 6: The colour changes observed during the formation of ZnO NPs by changing pH at (A) 13 (B) 11 (C) 9 (D) 7 (E) 5 (F) 3 (G) 1.

The Best result was obtained for the ZnO NPs solution with a pH value of 9, as it is revealed at Figure. 4.7 (e). This shows that a more basic media is vey suitable for the ZnO NPs synthesis. The surface plasmon absorbance of ZnO NPs was obtained in the range of pH 9-11. At high pH = 13 and lower pH 1-7, no absorption peaks were observed **(**Figure 4.7). It is suggested that at pH = 9, zinc nitrate hexahydrate was converted to ZnO NPs, which showed the metal oxide reduction and synthesis. Similar results were observed with extract of *Catharanthus roseus*, where increasing concentrations of zinc ions were used to optimize the synthesis of ZnO NPs [62].

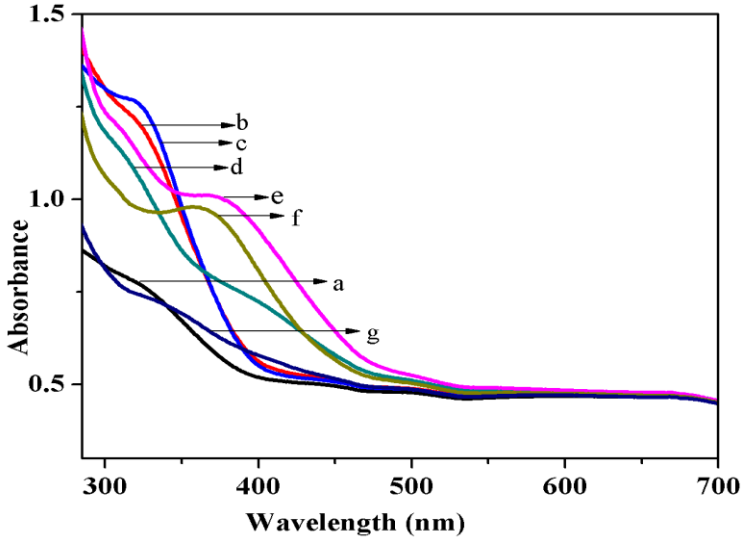


Figure 4.7: The UV-Vis spectra of ZnO NPs formed from 2 ml of 5 % (m/v) Neem leave extract and 25 ml of 2mM zinc nitrate at a pH of (a)1 (b)3 (c)5 (d)7 (e) 9 (f) 11 and (g) 13

4.3.4. Effect of reaction time on synthesis of ZnO nanoparticles

For the synthesis of ZnO NPs, a reaction time of 2, 24, and 48 hrs were tested. It was then founded that the UV-Vis spectrum of the nanoparticle after 24hr. was similar in intensity, broadness and in λ_{max} value with the spectrum after 2 hr. Even after 48 hr. no visible change was observed in the peak intensity and λ_{max} value of the nanoparticle as can be observed at figure. 4.8. The UV-Vis graphs in Figure 4.8 showed that there was small significant difference in the synthesis of ZnO NPs irrespective of the reaction time. 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 aggregation due to the increase in nucleation and collusion of the nanoparticles formed. This results is the same that has been done in the previous report [64].

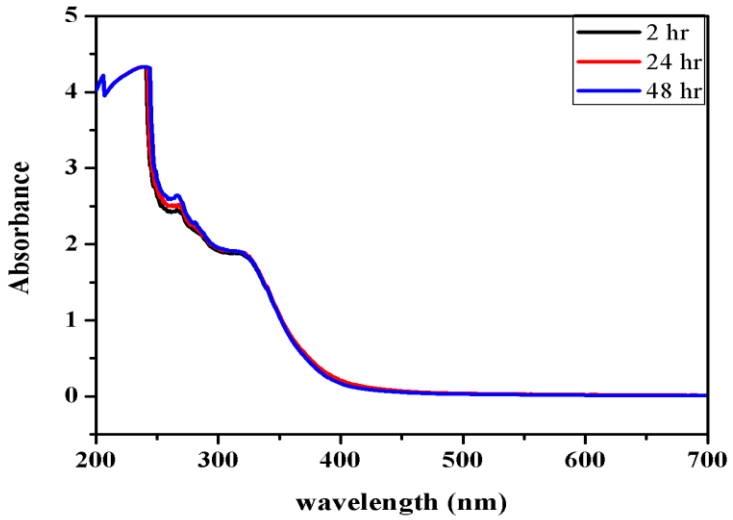


Figure 4.8: The UV-Vis spectra of ZnO Nps from 25 mL of 2mM zinc nitrate with 5% Neem extract after 2 hr., after 24 hr., and 48 hrs

4.4. The FT-IR spectra analysis

FT-IR spectroscopy, was used to evaluate chemical bonds in surface atoms and functional groups on the surface of nanoparticles. It can be used to characterize physical properties of nanomaterial's and their functions. In the biosynthesis of metal oxide NPs using plant extracts, FT-IR spectroscopic measurements were carried out to identify the possible biomolecules in extracts responsible for capping and leading to efficient stabilization of NPs [65]. The dual role of the plant extract, as a reducing as well as capping agent, and presence of some functional groups in both the Neem extract and ZnO Nps were investigated by FT-IR analysis [66].

The FT-IR spectrum was used for the characterization of the pure neem leaf extract and the resulting Zinc oxide nanoparticle (Figure.4.9) The Absorbance bands in the region of 4000 - 400 cm^{-1}, the FT-IR spectrum of neem leaf extract with strong bands at 3465 cm^{-1}, 1638 cm^{-1}, and 690 cm^{-1}, was shown in (Figure. 4.9). A strong, broad band peak at 3465 cm^{-1} in neem leaf can be attributed hydrogen bonded O-H groups of alcohols, phenols and amides. This agrees with the conclusion that the neem leaf extract was composed of poly phenols, flavonoids, alkaloids and other similar phytochemicals containing -OH and -NH bonds. The peak that was observed at 1638 cm^{-1} in neem leaf extract could be attributed to C=C stretching, 1720 cm^{-1}vibrations about C=O amide, that are responsible for capping and stabilizing of ZnO Nps. The peaks observed in the range of 690 cm^{-1} has been assigned to phenolic groups, C-N stretching vibrations of aliphatic and aromatic amines. On the other hand, the FT-IR spectrum of synthesized zinc oxide nanoparticles in Figure 4.9, shows the presence of major peaks at 3435, 2929, 2337, 1632, 1434, 1385, 1122, 873, and 544 cm^{-1} which are associated with –OH stretching vibrations, C-H stretching of alkanes, (NH) C=O group appeared sharp and intense peak at 1632 cm^{-1}, and at 1434 cm^{-1}, C- C stretching was observed. Moderate levels of absorption in the region covering 1430–1385 cm^{-1} imply the presence of an aromatic ring [67]. The major peaks observed should be a M-O (Zn-O) bond stretching which is found in very closer range, 400-600 cm^{-1}, to the results of some previous works [68,69,70]. The region between 400 and 600 cm^{-1} is assigned for metal-oxygen bond. In addition to the absorption bands of the biomolecules used as reduction and stabilization (capping agents), the absorption peak at 544 cm^{-1} indicate the presence of ZnO NPs [71].

From the analysis of FT-IR spectrum in Figure 4.9 the major peaks of neem plant extract were observed completely peak shifted in ZnO NPs. The slight shift in the vibrational bands of ZnO Nps from the corresponding vibrational bands of neem plant extract implies the interaction of bulk ZnO with plant extract. The shifting of the band increases and decrease is due to the various functional groups such as, ascorbic acid, flavonoids, tannins, and polyphenols acted as both stabilizing and reducing agents for the ZnO NPs formation reaction. The FTIR study also confirmed result of preliminary phytochemical investigation discussed that proteins, phenolic compounds, alcoholic compounds present in neem extract acted as a reducing agent and stabilizing agent in the formation of ZnO Nps and further prevented its agglomeration [72]. The presence of some sharp and prominent peaks in neem leaf extract spectrum and absence or weak presence in ZnO NPs spectrum suggested that those functional groups performing the job of capping,

dispersing and stabilizing agents for ZnO NPs [73]. The main role of the capping ligands is to stabilize the nanoparticles to prevent further growth and agglomeration.

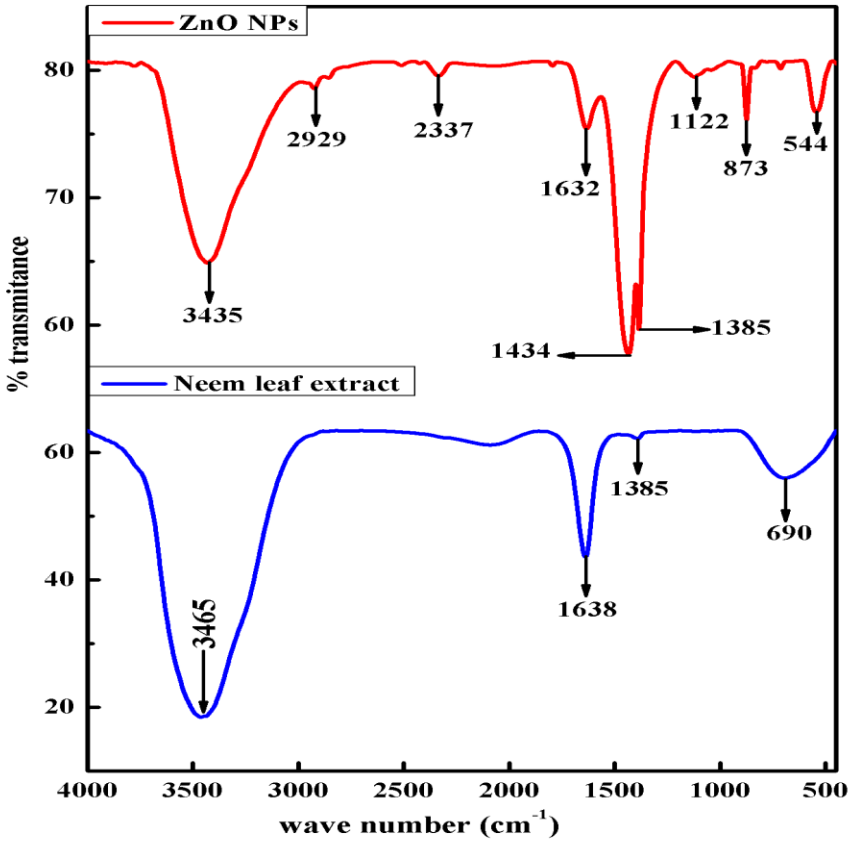


Figure 4.9: FTIR spectra of Neem leaf extract and ZnO NPs

FT-IR analysis concluded that the presence of N–H and O–H bonds in the FTIR spectrum revealed that the proteins or phenolic compounds in the neem leaf extract involved in the bio reduction of Zn^{2+} ions to ZnO NPs. FT-IR analysis confirmed that neem leaves extract can perform dual functions of reduction of Zn^{2+} to ZnO and also stabilization of ZnO NPs. FTIR spectrum of ZnO NPs suggested that ZnO NPs were surrounded by different organic biomolecules such as terpenoids, alcohols, ketones, aldehydes , carboxylic acids, flavones, terpenoids, and amides, which are responsible for bioreduction of nanoparticles. Flavonoids contain various functional groups, which have an enhanced ability to reduce metal ions.

4.5. X-ray Diffractometre

X-Ray diffraction is a well-known technique for the structural identification and determination of crystalline size of the synthesized ZnO nanoparticles. The crystalline size and structural properties of the ZnO nanoparticles are revealed by using Powder X-ray diffraction. The XRD is carried out with Cu Kα radiation (k = 0.15406 nm) and 2θ ranges from 10° to 80°. The XRD pattern of bio-synthesized ZnO nanoparticles from leaf extract of Neem is shown in Figure. 4.10. This shows the x-ray diffraction (XRD) spectrum of the synthesized zinc oxide nanoparticles. The main peaks found correspond to Bragg reflections with 2θ values of: 31.74°, 34.38° 36.24°, 47.46°, 56.54°, 62.88°, 66.43°, 67.96°, and 68.99°, Locations of the characteristic Bragg reflections were indexed to (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0), (1 1 2), and (2 0 1), planes of ZnO hexagonal wurtzite structures, respectively, (standard JCPDS card # 36-1451) and this confirms the presence of ZnO NPs. The characteristic peaks we have obtained for ZnO NPs are in good agreement with previous experimental findings [67, 74]. The crystallite size (D) of ZnO NPs was calculated from the full-width at half-maximum from XRD patterns by using Debye Scherer equation:

$$D = \frac{K\lambda}{\beta \cos\theta} \tag{4.1}$$

$$\beta = \frac{FWHM\ in\ 2\theta\ x\ \pi}{180^o} \tag{4.2}$$

Where D is the crystallite size, λ= 0.15406 nm, which is the wavelength of the X-ray for Cu target Kα radiation, β is the peak width at half maximum of an XRD, K = 0.89, which is the Scherer's constant and θ is the Bragg diffraction angle. The strong and narrow diff raction peaks (Figure. 4.10) indicate that the Nps have a good crystallinity and size. Crystallite size of the synthesized ZnO Nps was calculated using Debye-Scherrer formula [67] and the average crystallite size of the sample was estimated to be 20 nm which is derived from the FWHM of the most intense peak corresponding to (101) plane located at 36.24°. The unassigned and assigned peaks by (*) indicates the dissolved metals present in the synthesized nanoparticle.

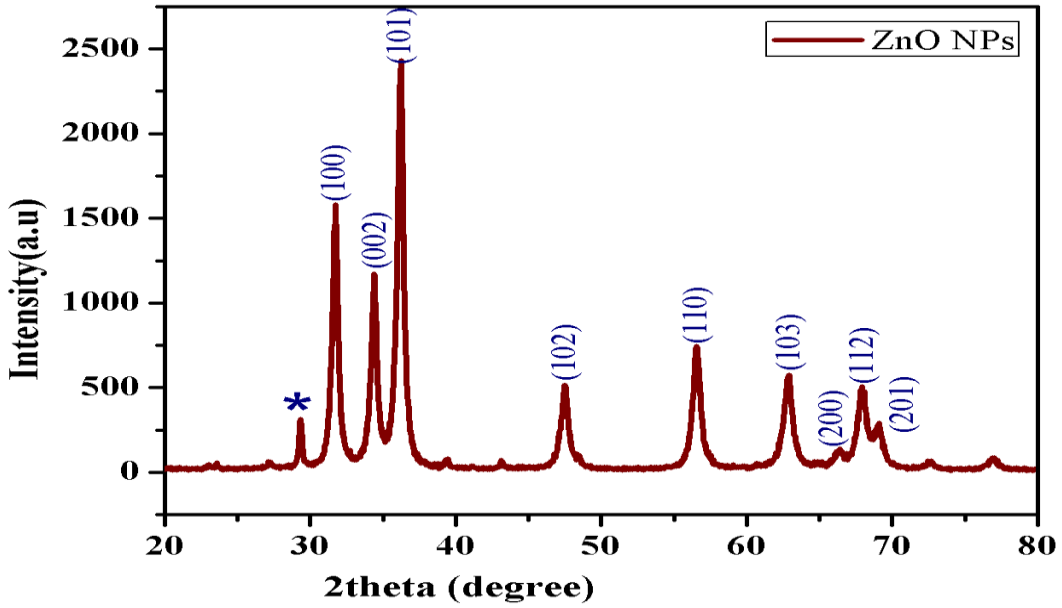


Figure 4.10: X-ray diffraction patterns of ZnO nanoparticles

4.6. The Antibacterial Activity of ZnO NPs

The antibacterial activity of the synthesized zinc oxide nanoparticles was investigated against the one Gram-negative bacterial species (*Escherichia coli*) and one Gram-positive bacterial species (*Staphylococcus aureus*) through disc diffusion method. The positive control (Gentamicin) against all the bacteria and the zone of inhibition values was measured as shown in Figure. 4.11. The results are given in Table 4.2 from the table, indicate that the synthesized zinc oxide nanoparticles (ZnO NPs) have shown a considerable antimicrobial activity for all the two bacteria's studied; As can be observed at table 4.2, the ZnO Nps showed good antibacterial activity against *E. coli*, with a maximum inhibition zone of 15 mm. But for the *S. aureus* ,the nanoparticle have shown relatively lower zone of inhibition, 13 mm, after 24 hour incubation that coincides with results obtained in [75] . The results revealed that the bacterial sensitivity to nanoparticles was found to vary depending on the antibacterial species. It was also possible to observe from the figure 4.11. That the ZnO Nps was more sensitive to the bacterial as compared to the Neem extract and the precursor, Zn $(NO_3)_2$. $6H_2O$.This is expected to be due to the reason that the size of the ZnO NPs is smaller than particles of the precursor and the plant extract particles.

Table 4.2: In vitro antimicrobial activity of some human pathogenic bacteria on ZnO Nps by disc diffusion assay

Name of Bacterial	Zone of inhibition in (mm)		
	Neem	ZnO NPs	Gentamicin
Escherichia coli(E. coli)	9	15	28
Staphylococcus aureus(S. aureus)	7	12	24

The results revealed that gram-negative bacteria are more sensitive to ZnO NPs treatments than gram-positive bacteria, and this could attributed to the presence of a thick layer in the cell walls (peptidoglycan) of the latter group. Previous study shows that gram-negative, is more sensitive than gram- positive bacteria. They concluded that the resistance in gram-positive bacteria is due to the presence of a thick layer of peptidoglycan in their cell wall [69, 76, and 77]. Other reports show that strong antibacterial potential against gram-negative bacteria using ZnO NPs synthesized from cumin seeds [76], and concluded that the slight resistance of gram-positive bacteria is due the presence of a thick layer of peptidoglycan in their cell wall, which is similar to the results achieved in this work.

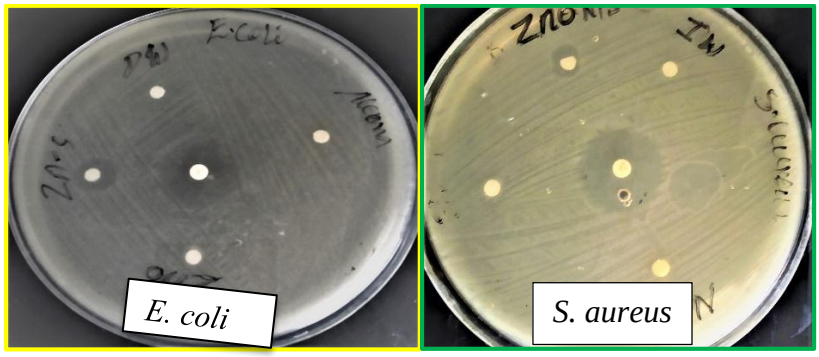


Figure 4.11: Zone of inhibition ZnO NPs against the one, Gramm-negative, (A) E. coli and the one Gramm- positive bacteria (B) S. aureus

5. Conclusions and Recommendations

5.1. Conclusions

In this work, ZnO NPs has been successfully synthesized through a green synthetic pathway with the aid of neem extract as reducing, stabilizing as well as capping agent which is simple, cost effective, and environmentally friendly. The synthesized zinc oxide nanoparticles were characterized using visual observation (color change), UV-Vis absorption, XRD, and FT-IR, which confirmed the formation of the zinc oxide nanoparticles using Neem extract. The synthesized ZnO Nps has been found to have a maximum absorbance at a wave length of 321 nm which is very much different from the 260 nm of the Neem extract.

FTIR spectrum explained that the phytochemicals present in neem extract involves in the bioreduction process for the nanoparticle synthesis. Mainly the strong peak at 544 cm^{-1}, which was not found at the pure extract, was considered to be the major indicator for the formation of ZnO Nps. The crystalline nature and the hexagonal structure of the synthesized ZnO NPs were confirmed by powder X-ray diff raction analysis. The average crystallite size through Debye–Scherrer method was estimated around 20 nm. ZnO NPs synthesized through the above green route do have better antibacterial activity against Gram- negative bacteria than Gram- positive bacteria. It was found that the ZnO Nps has an average inhibition zone of 14 mm.

5.2. Recommendations

In this work the ZnO NPs strong peak has been obtained using fresh Neem leaf. Therefore fresh Neem leaf is recommended to be used. The effect of extracted neem leaf on the size of ZnO NPs formation in green method has been studied. To do so; ZnO NPs was synthesized by differing the concentration of extracted Neem leaf, the concentration of zinc nitrate solution ,the effect on PH and the formation of reaction time. Moreover, the effect of ZnO NPs on bacteria was studied by disc diffusion method. Based on the results of this study it is recommended that:

- ✓ Further studies should be done on the effect of temperature on the synthesis of ZnO NPs
- ✓ The synthesized ZnO NPs should be characterized by other techniques like SEM and TEM
- ✓ Further studies should be done on the Photocatalytic activity of ZnO NPs

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