# SYNTHESIS OF ZINC OXIDE NANOPARTICLE USING LEAF EXTRACT OF *LIPPIA ADOENSIS* (KOSERET) AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITY

**By:**

**MERON GIRMA**

**A THESIS SUBMITTED TO DEPARTMENT OF APPLIED CHEMISTRY**

**SCHOOL OF APPLIED NATURAL SCIENCE**

**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMISTRY (PHYSICAL CHEMISTRY STREAM)**

**OFFICE OF GRADUATE STUDIES**

**ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY**

**August, 2018**

**Adama, Ethiopia**

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## APPROVAL OF BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Meron Girma have read and evaluated her thesis entitled "**SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING LEAF EXTRACT OF *LIPPIA ADOENSIS* (KOSERET) AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITY"** and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the Degree of Master of Science in Chemistry.

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# DECLARATION

I do hereby declare that the project work entitled **"SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING LEAF EXTRACT OF *LIPPIA ADOENSIS* (KOSERET) AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITY"** thesis submitted to Department of Applied Chemistry, School of Applied Natural Science, is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Bedasa Abdisa (major advisor) and Dr. Gemechu Deressa (Co-Advisor), Asst. Professors, Department of Applied Chemistry, Adama Science and Technology University, Adama, Ethiopia.

Name: Meron Girma

Signature: _____

This MSc thesis has been submitted for examination with our approval as thesis advisors.

Advisor Name: Bedasa Abdisa (PhD) Co-advisor Name: Gemechu Deressa (PhD)

Signature: _____ Signature: _____

Date of submission: _____

*Dedicated to my family and my beloved one…*

***Family is like branches on a tree, we all grow in different directions but our roots remain as one!!***

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First of all I would like to thank the Almighty God and his beloved mother Saint Virgin Mary for the unreserved and unconditional love, kindness and for keeping me in health, strength and courage in the entire period of this work. If words are considerable as symbols of approval and taken as acknowledgement then let the words play a heralding role in expressing my gratitude.

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# LISTS OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **CFU** | Colony Forming Unit |
| **DTA** | Differential Thermal Analysis |
| ***E. Coli*** | *Escherichia Coli* |
| **EDX** | Energy dispersive X-ray |
| **FDA** | Food and Drug Administration |
| **FTIR** | Fourier Transform Infrared Spectroscopy |
| **FWHM** | Full Width at Half Maximum |
| **IZ** | Inhibition zone |
| **MIC** | Minimum inhibitory concentration |
| **MH** | Mueller-Hinton |
| **$N_C$** | Negative control |
| **NPs** | Nanoparticles |
| **$P_C$** | Positive control |
| **r.p.m** | Revolution per minute |
| **ROS** | Reactive Oxygen Species |
| **SEM** | Scanning Electron Microscope |
| ***S. aureus*** | *Staphylococcus aureus* |
| **TGA** | Thermogravimetric analysis |
| **UV-Vis** | Ultraviolet Visible Spectrophotometer |
| **VOC** | volatile organic solvent |
| **XRD** | X-ray Diffraction |

# *ABSTRACT*

*In this study synthesis of zinc oxide nanoparticle was done using biological/green method from indigenous 'Koseret' Lippia adoensis leaf extract which is endemic medicinal plant and cultivated in home gardens of different regions of Ethiopia. The synthesized ZnO Nanoparticles were analyzed and confirmed, using Thermogravimetric analysis (TGA), X-ray diffraction (XRD), Scanning Electron Microscope (SEM) with Energy dispersive X-ray analysis (EDX), UV-Visible and Fourier Transform Infrared spectroscopy (FTIR). Furthermore, this study also evaluated the antibacterial activity of the synthesized ZnO nanoparticles against clinical and standard strains of Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* (*S. aureus*), *and Enterococcus feacalis by disc diffusion method.*

*According to this study, ZnO nanoparticles synthesized using Lippia adoensis leaf are wurtzite hexagonal structure with an average crystallite size of 18.5 nm at 1:1 reactant ratio is obtained from XRD results. The SEM reveals spherical, nanorod and flake type morphology of nanoparticles and Energy Dispersive X-ray analysis confirms the formation of highly pure zinc oxide nanoparticles. From UV-Visible spectroscopy, band gap energy of 3.42eV is obtained in the near visible region at the wavelength of 362 nm for 3:2 ratio ZnO nanoparticles. ZnO nanoparticles synthesized using Lippia adoensis leaf extract showed promising result against both gram-positive and gram-negative bacterial strains with maximum inhibition zone of 14 nm and 12 nm, respectively in uncalcinated form.*

***Keywords:*** *Zinc Oxide Nanoparticles, Green method, Lippia adoensis, antibacterial activity.*

# 1. INTRODUCTION

Nanotechnology is being widely applied in the field of material science in the past decades. This concept was first explained in an American physicist, Richard Feynman's lecture in 1959 (Kavitha *et al*., 2013). Nanotechnology is dealing with the production of materials in a nanometer scale level, normally from 1 to 100 nm such as carbon nanotube (Martel *et al*., 1998), metal nanowire and nanoparticles (Penner *et al*., 2003) with unique electrical and mechanical properties. In nanotechnology, nanoparticles (NPs) are very small sized particles with enhanced catalytic reactivity, thermal conductivity, non-linear optical performance and chemical steadiness owing to its large surface area to volume ratio (Tabrez *et al*., 2016).

Nanotechnology is emerging as a rapidly growing field with its application in science and technology for the purpose of manufacturing new material at the nanoscale. It emerges from the physical, chemical, biological and engineering sciences where new techniques are being developed to search and plan single atoms and molecules for multiple applications in different field of scientific world. Nanoparticles are of great interest due to their small size and large surface area to volume ratio (Poinern *et al*., 2015) that give rise to some of the superior properties than their bulk phase such as antimicrobial, catalytic, electronic, magnetic and optical properties (Shah *et al*., 2015). The science and engineering technology of nanosystems is one of the most exigent and fastest growing sectors of nanotechnology (Prathna *et al*., 2010). Nanotechnology involves synthesis, characterization and application of nanoparticles by controlling shape and size.

In the field of nanotechnology, metal and metal oxide nanoparticles have attained a great importance due to their unique optical, catalytic, electronic, magnetic and antimicrobial properties owing to their small size (Okuda *et al*., 2005). Nanoparticles synthesis is critical to understand the particulate formation process, modify the physicochemical properties of the nanoparticles and to enable specific functionalities and applications. Recently biological methods for synthesis of zinc oxide nanoparticle using microorganisms, enzymes, and plant extracts have been suggested as possible eco-friendly alternatives to chemical and physical methods (Awwad *et al*., 2014). Green synthesis of nanoparticles and their applications in food processing, production of antimicrobials, cosmetics, and health care products have been

acknowledged by number of researchers (Begum *et al*., 2009). Plant extract as bio-templates can be an economic and efficient alternative for the large- scale synthesis of nanoparticles.

Among, metal oxide nanoparticles, zinc oxide is interesting because of its vast applications in various areas such as optical, piezoelectric, magnetic, solar cells and gas sensing. Besides these properties, ZnO nanostructure exhibits high catalytic efficiency, strong adsorption ability and used most frequently in the manufacture of sunscreens (Seshadri *et al*., 2004), ceramics and rubber processing, wastewater treatment, and as a fungicide. ZnO nanoparticles are also reported by several studies as non-toxic to human cells (Bhumkar *et al*., 2007). This feature necessitated their usage as antibacterial agents, noxious to microorganisms and hold good biocompatibility to human cells (Yin *et al*., 2003). Synthesis of nanoparticles using plant extract is one of the biological/green synthesis methods used because of their environment friendly, cost effectiveness, and uses safe reagents during the production of nanoparticles. In this project work, ZnO NPs were synthesized using leaf extract of *Lippia adoensis* for evaluation of its antibacterial activity against selected gram-positive and gram-negative bacterial strains.

*Lippia adoensis* is one of the five indigenous *Lippia* species in Ethiopia where it occurs as rigid woody shrub up to 1 to 3 m tall. It is endemic medicinal plant and cultivated in home gardens in different regions of Ethiopia with altitudinal range of 1600−2200 m (Abebe and Ayehu., 1993). Two varieties are recognized in Ethiopia, the wild variety (var. adoensis) and the cultivated variety (var. koseret sebsebe). *Lippia adoensis*, locally known as koseret, is widely grown in the central and southern highlands of the country. Traditionally, the dried leaves are used as one of the ingredients in the preparation of spiced butter (Nigist and Sebsebe ., 2009) and also for food flavoring agent and preservative (Riot *et al*., 2005).

The leaves of *Lippia adoensis* are used in Ethiopian traditional medicine for the treatment of various skin diseases including eczema and superficial fungal infections (Hailu *et al*., 2005). The dried leaves powdered together with barely are eaten to get relief from stomach complaints (Megersa *et al*., 2013). Its free radical oxidative stress is implicated in the inhibition of pathogenesis of a variety of inflammatory diseases (Berhanu *et al*., 2001). The chemical compositions of *L. adoensis* var. koseret, investigated so far are essential oils. Lonalool is the major component, and appreciable amount of sesquiterpene hydrocarbons (germacrene, α-copaene, β-cadinrne, and, β- caryophyllene) and uncommon monoterpene ketone, 2-methyl-6-

methylene-2, 7-octadien-4-one (ipsdienone), were also found in the essential oil (Berhanu *et al*., 2001).

Yared *et al*., (2014) stated that antibacterial activity profile of L. adoensis against the bacterial strains indicated that gram-positive bacteria (*S. aureus*) is more susceptible than gram-negative bacteria (*E. coli, salmonella typhi* and *P. aeruginosa*). The reason for the difference in sensitivity between gram-positive and gram-negative bacteria might be attributed to the differences in morphological constitutions between these microorganisms. Gram-negative bacteria have an outer lipopolysacharide membrane and this makes the cell wall impermeable to antibacterial chemical substances. The gram-positive bacteria on the other hand are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of gram-negative organisms are more complex in lay out than the gram-positive ones acting as a diffusion barrier and making them less susceptible to the antibacterial agents (Alonso *et al*., 2000). The activity that *Lippia adoensis* showed against *Staphylococcus aureus* **(***S. aureus*) might justify the extensive use of these agents for the treatment of skin disorder. Figure 1 shows uncut (fresh) and dried leaves of *Lippia adoensis* plant.

**Figure 1.** *Lippia adoensis* 'Koseret' Leaves before (left) and after (right) drying, from Adama town. [Photo by Meron Girma].

Due to these evidences about the medicinal values and phyto-chemistry of this plant, the present study applied biomimetic approach for green synthesis of eco-friendly ZnO nanoparticles from *Lippia adoensis* for the study of antibacterial activity against gram-negative, (*Escherichia coli* and *Klebsiella pneumonia)* and gram-positive bacteria (*Staphylococcus aureus* (*S.aureus*), and *Enterococcus feacalis*).

## 1.1. Statement of the Problem

Bacterial infectious diseases are serious health problem that has drawn the public attention in worldwide as a human health threat, which extends to economic and social complications. Increased outbreaks infections of pathogenic strains, bacterial antibiotic-resistance, lack of suitable vaccine in under developed countries, and hospital-associated infections, are global health hazard to human, particularly in children. Thus, developing novel antibacterial agents against bacteria strains, mostly major food pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus feacalis* and *Klebsiella pneumonia* has become greatest demand. These mentioned bacterial strains are selected as they are highly transmittable (Beyth *et al*., 2015); hence this study evaluate the potential antibacterial activity of ZnO nanoparticles against these pathogenic bacteria.

Nanoparticles are being considered as an alternative to antibiotics that can effectively prevent microbial drug resistance in certain cases. The search for new, effective bactericidal materials is significant for combatting drug resistance, and NPs have been established as a promising approach to solve this problem (Linlin *et al*., 2017). This work is explored these problems to induce further investigations in these areas by addressing techniques, benefiting from the unique features of ZnO nanoparticles synthesized from 'Koseret' *Lippia adoensis* leaf extract which are cost effective, green approach and from most common cheap precursors.

## 1.2. Objectives of the Study

### 1.2.1. General objective

The general objective of this study is to synthesize ZnO nanoparticles using leaf extract of 'Koseret' *Lippia adoensis* plant extract and evaluate their antibacterial activity against *Staphylococcus aureus (S.aureus), Enterococcus feacalis*, *Klebsiella pneumonia* and *Escherichia coli*.

### 1.2.2. Specific objectives

The specific objectives are:

- To synthesize ZnO nanoparticles using aqueous leaf extract of 'Koseret' *Lippia adoensis* plant and $Zn(CH_3COO)_2.2H_2O$

- To characterize the synthesized ZnO nanoparticle using techniques such as UV-Visible, FT-IR, XRD and SEM-EDX
- To evaluate potential of ZnO nanoparticle synthesized from 'Koseret' *Lippia adoensis* leaf extract for antibacterial activity against *Staphylococcus aureus* (*S. aureus*), *Enterococcus feacalis*, *Klebsiella pneumonia* and *Escherichia coli*.

## 1.3. Significance of the Study

Metal oxide nanoparticles such as tin oxide, iron oxide, zinc oxide and titanium oxide are very attractive (Mbonyiryivuze *et al*., 2015) and widely used in medicinal fields, electronics, sensors, optics, catalysis, photoelectrochemical devices, drug delivery, biosensors, bio-imaging, antimicrobial activities, food preservation and photonics. In contrast to traditional antibiotics, NPs have characteristic dimensions less than 100 nm. Their uniquely small size results in novel properties, such as greater interaction with cells due to a larger surface area-to-mass ratio and versatile and controllable application (Linlin *et al*., 2017). The benefit of using inorganic oxides for biomedical applications is that they contain mineral elements essential to humans and exhibit strong activity even when administered in small amounts (Arunachalam and Kannappan., 2016).

Among metal oxide NPs, zinc oxide nanoparticles (ZnO NPs) with unique optical and electrical properties are suitable for many applications such as coatings for the solar cells, electronics and chemical sensors. Recent studies demonstrate that ZnO NPs hold considerable promise for biomedical applications and therapeutic intervention (Rasmussen *et al*., 2010). The advantage of using ZnO nanoparticles is that they strongly inhibit the action of pathogenic microbes when used in small concentration (Applerot and Holleman., 2009). Therefore, this study provided important information that contribute to the development of green synthesis approach of ZnO and its antibacterial activity.

## 1.4. Scope of the Study

The scope of the study is limited to synthesis of ZnO NPs using leaf extract of 'Koseret' *Lippia adoensis* plant; characterization of the synthesized ZnO NPs using UV-Vis, FT-IR, XRD, SEM-EDX; and evaluation of antibacterial activity of the synthesized ZnO NPs against selected bacterial strains: *Escherichia coli, Staphylococcus aureus* (*S. aureus*), *Enterococcus feacalis* and *Klebsiella pneumonia.*

# 2. LITERATURE REVIEW

Nanotechnology refers to the production and usage of material with nanoscale dimension. It is a multidisciplinary scientific field undergoing explosive development (Anitha *et al*., 2011) and one of the most active research areas in modern material science. Nanoparticles (NPs) are particles that have at least one dimension in the range of 1-100 nm. Green chemistry utilization of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture, and application of chemical products is current hot issue of the world. Green Synthesis of nanoparticles involves use of biological routes such as those involving microorganisms, plants etc. for the synthesis of nanoparticles. Green chemistry for chemical synthesis addresses our future challenges in working with chemical processes and products by inventing novel reactions that can maximize the desired products and minimize byproducts, designing new synthetic schemes and apparatus that can simplify operations in chemical productions, and seeking greener solvents that are inherently environmentally friendly (Shabnam and Zahra., 2016).

## 2.1. ZnO Nanoparticle

ZnO is an n-type semiconducting metal oxide. Zinc oxide NPs has drawn interest in more than three years due to its wide range of applicability in the field of electronics, optics, and biomedical systems (Oskam., 2006). Several types of inorganic metal oxides have been synthesized and remained in recent studies like $TiO_2$, CuO, and ZnO. Of all these metal oxides, ZnO NPs is of maximum interest because they are inexpensive to produce, safe and can be prepared easily. U.S. Food and Drug Administration (U.S.FDA) have recognized ZnO as safe (Premanathan *et al*., 2011). It possesses a high boiling and melting points which are 2360 $^{o}$C and 1975 $^{o}$C, respectively.

ZnO NPs have very strong antibacterial effect at a very low concentration of gram-negative and gram-positive bacteria as confirmed by studies, they have shown strong antibacterial effect than the ZnO NPs synthesized chemically (Hazra *et al*., 2013). ZnO NPs exhibit tremendous semiconducting properties because of its large band gap (3.37 eV) and high exciton binding energy (60 meV) like high catalytic activity, UV filtering properties, anti-inflammatory, wound healing (Mirzaei and Darroudi., 2017). Crystalline ZnO has a wurtzite (B4) crystal structure at

ambient conditions. The ZnO wurtzite structure has a hexagonal unit cell with two lattice parameters, a and c, and belongs to the space group of $C^4_{6V}$ or $P6_3mc$. ZnO nanoparticles have gathered the increasing interest of the scientific and industrial community due to diverse application in solar energy conversion, sensors, rubber manufacturing, for removing sulfur and arsenic from water, cosmetics, paints, fibers, drug-delivery, antibacterial activity and luminescence properties. Recently, great effort has been made to the synthesis of size controlled ZnO nanoparticles in order to explore their potentials.

## 2.2. Synthesis of ZnO Nanoparticles

Broadly, nanoparticle synthesis methods can be categorized into top-down and bottom-up. The top–down (breakdown) approach involves grinding down of large macroscopic particle into smaller particles. It involves synthesizing large-scale patterns initially and then reducing it into nanoscale level through plastic deformation. The main disadvantage of the top-down approach is the imperfection of the surface structure. The second is the build-up (bottom-up) method that produces nanoparticles starting from atoms of gas or liquids based on atomic transformations or molecular condensations. In the bottom-up approach, atoms and molecules are used as the building blocks in synthesizing complex nanostructures. Bottom-up approaches are more favourable and popular in the synthesis of nanoparticles than top-down method (Jaison *et al*., 2016).

Both approaches are used in the synthesis of metal oxide nanoparticles and relevant for determining the size, shape, and properties of nanoparticles for specific application. The synthesis approaches are also classified into physical, chemical and biological synthesis depending on the processes or materials involved in the production process (Cao., 2004).

### 2.2.1. Physical method

In the physical method, physical forces are involved in the attraction of nanoscale particles and formation of large, stable, well-defined nanostructures. The physical method often called top-down approach. It uses mechanical forces to break bulk particles or beams of atoms for the formation of nanoparticles (Houdy *et al*., 2007). Physical method includes nanoparticle synthesis through colloidal dispersion, vapor condensation, amorphous crystallization, physical fragmentation and evaporation, pulse laser deposition, sputtering. Physical synthesis methods

are highly useful in producing ultra-thin and highly pure nanostructures and nanoalloys (Yadav *et al*., (2012). The main shortcoming of physical method is the use of costly equipment, high temperature and pressure, large space area for setting up of machines.

### 2.2.2. Chemical method

Moderate reaction conditions and convenient synthetic manipulation have make it an attractive option for accessing ZnO nanoparticles. The method also provides better control of chemical and morphological characteristics (Bhattacharjee *et al*., 2011). There are different chemical methods such as: direct precipitation, homogeneous precipitation, solvothermal method, sonochemical method, pyrolysis, chemical vapours deposition, reverse micelles, sol gel method, and hydrothermal (Kolekar *et al*., 2013).

Chemical method involves the use of toxic chemicals which can prove to be hazardous for the environment and the person handling it. These methods may result in toxic and harsh products which may pose biological risks to the environment; due to high surface charge and high surface area of nanoparticles, harsh chemicals may remain adsorbed onto 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 (Rana and Kalaichelvan ., 2013). These drawbacks have led to the emergence of green synthesis methods of nanoparticles based on the use of environmentally friendly and biocompatible materials such as plants and microorganisms. Therefore, it is essential to optimize green methods for nanoparticle synthesis.

### 2.2.3 Green (biological) method of ZnO nanoparticles synthesis

Green/biosynthesis of nanoparticles is an approach of synthesizing nanoparticles using micro-organisms and plants having biomedical applications. Green synthesis provides more advantages over physical and chemical methods because it is very cost effective, non-toxic, uses safe reagents during the process (Anbuvannan *et al*., 2015), and eco-friendly technique due to use of extracts of plant (leaves, flower, seed and peels), bacteria, fungi and enzymes, as the reducing and stabilizing agents for synthesis of nanoparticles instead of large quantity of chemicals (Vijaylaxmee *et al*., 2014**)**. This method does not require high temperature, high pressure, costly equipment and hazardous chemicals. The three main aspects in the preparation

of nanoparticles that should be evaluated from a green chemistry perspective are the choice of the solvent medium used for the synthesis, the choice of an environmentally favourable reducing agent and the choice of a non-toxic material for the stabilization of the nanoparticles.

The progress of efficient green synthesis utilizing natural reducing, capping and stabilizing agents without the use of toxic, expensive chemicals and high energy consumption have attracted researchers towards biological methods (Mukherjee *et al*., 2001). The biological method of synthesis of ZnO nanoparticles is gaining importance due to its simplicity, eco-friendliness and extensive antimicrobial activity.

Researchers nowadays are more preferred to synthesize nanoparticles using plant, agricultural waste and fruit extract to minimize the uses of toxic chemicals and harsh reaction conditions that consequently contribute significant risk to the environment. In this literature review, ZnO NPs synthesized using green methods are listed into two categories which are fruit extracts (*Artocarpus gomezianus*, trifoliate orange and *Rosa canina* fruit) and agricultural waste and plant extracts (*Duranta erecta L., Ocium tenuiflorum, Camellia sinesis, Limonia acidissima* L., *Trifolium pretense, Aloe vera, Hibiscus subdariffa, Murraya koenigii*) that has been studied by other researchers.

#### 2.2.3.1. Synthesis using agricultural waste or fruit extracts

Nagajyothi *et al*., (2013) Synthesized ZnO NPs using member from Rutaceae family, *Poncirus trifoliate* fruits extract, trifoliate orange and their catalytic activity in Claisen Schmidt condensation reaction between acetophenone and 3, 4-dimethylbenzaldehyde was evaluated. According to Nogata *et al*., (2006), *Poncirus trifoliate* fruits extract was found to be contains large amount of flavonoids, including flavanone, flavone and polymethoxylated flavones that participating in the reduction of zinc ion during the synthesis of ZnO NPs due to their excellent antioxidant ability. Characterization results were shown the spherical particle sizes obtained were 21.12 nm in average according to SEM and TEM images, UV-Vis spectrum, XRD and EDX analysis confirmed the identity of the synthesized ZnO NPs and also FTIR analysis verified the presence of bio-components coated on the surface of NPs by observing certain functional group vibrational modes in the spectrum such as alcohol, phenol, aromatic and $1^{o}$ amine.

*Artocarpus gomezianus* extract assisted synthesis of ZnO NPs and their photocatalytic ability evaluation on Methylene Blue (MB) organic dye degradation was done by (Suresh *et al*., 2015). *Artocarpus gomezianus* belongs to *Artocarpus* family that normally found in Central Western Ghats which are rich in flavonoids, stilbenoids, arylbenzofurons and Jacalin (Jagtap *et al*., 2010). Suresh *et al*., (2015) reported there is 17.16% polyphenol and 22% of flavonoids in *Artocarpus gomezianus* fruit extract. These secondary metabolites were useful in the reduction of zinc ion that leads to the formation of ZnO NPs. The authors characterized the synthesized nanoparticles using XRD and UV-Vis spectrophotometry and studied their surface morphology using SEM and the dye was effectively removed by this synthesized ZnO NPs where > 90% MB degraded under the natural sunlight irradiation.

Demir *et al*., (2014) reported that a member from *Rosaceae* family, *Rosa canina* fruit, rosehip extract possesses high antioxidant activity due to the presence of phenolic acids such as gallic acid, flavonoids such as catechin, proanthocyanidin such as procyanid in $B_2$, sugars and organic acids such as ascorbic acid (AA orvitamin C). Spherical ZnO nanoparticles with crystalline and particle sizes synthesized from microwave irradiation were 11.3 nm and < 50 nm respectively. EDX data confirmed the high purity of synthesized ZnO nanoparticles where the weight percent of Zn gives 65.19% and oxygen gives 34.81%. FTIR spectrum reveals the presence of AA on the surface of ZnO nanoparticles from the detected bond vibrations such as C=O, C-O stretch of ester, C-H, O-H stretch as well as Zn-O stretches.

#### 2.2.3.2. Synthesis Using Plant Extracts

Raut *et al*., (2013) used a member of Lamiacece family, *Ocium tenuiflorum* plant leaves extract to synthesize ZnO NPs. The authors reported that mixture of *Ocium tenuiflorum* plant extract and zinc nitrate solution was stirred together forms a light yellow color ZnO nanoparticles with an average crystalline size of 13.86 nm and 11-25 nm hexagonal particle diameters from XRD analysis. The formation of ZnO NPs was also confirmed by observing the Zn-O bond vibration at 668.29 $cm^{-1}$ in fingerprint region of the IR spectrum.

Shekhawat *et al*., (2016) reported the uses of fruits, leaves, flowers, stems and roots of a Verbenaceae family member often found in tropical and subtropical country around the world which normally used for decoration, *Duranta erecta* plant to synthesize ZnO nanoparticles and

characterized using UV-Vis spectroscopy. Previous study was done by other researchers verified that it contains some phyto constituents in methanolic extract such as polyphenols (tannins and flavonoids), saponins, alkaloids and glycosides (Khanal *et al.*,2014; Sharma *et al.*, 2012). It possesses the antioxidant properties due to the presence of flavonoids, alkaloids, tannins and etc. By referring to the UV-Vis spectra obtained, each part of the plant extract given the different values of $\lambda_{max}$ under the Ultraviolet (UV) region which are 293 nm for roots, 299 nm for flowers and stems, 302 nm for leaves and 317 nm for fruits. These values of $\lambda_{max}$ in the range of 290 nm – 320 nm are the characteristic absorption peaks from ZnO nanoparticles.

Dobrucka *et al*., (2015) was synthesized ZnO nanoparticles in high purity using red clover, *Trifolium pratense* flower extract, a member of Leguminosae family in which they found 60 to 70 nm crystalline size. Synthesized ZnO nanoparticles in this method was characterized by authors using SEM-EDX, XRD, FTIR, UV-Vis spectrophotometry and studied its antimicrobial ability towards five selected microorganisms. The reported bio-components in *Trifolium pretense* were major in estrogenic isoflavones and flavonoids such as biochanin A-G-M, formononetin-G-M and quercetin-G-M (Saviranta *et al*., 2010) that are responsible for reducing and stabilizing purpose.

A very common antioxidant, green tea, *Camellia sinesis* leaves extract was also used to synthesize ZnO nanoparticles and studied its antimicrobial activity on certain selected microbes (Senthilkumar *et al*., 2014). The reported average crystalline size of synthesized ZnO nanoparticles were 16 nm, from signal generated in XRD analysis. Besides, the presence of the λmax in spectrum of UV region at 325 nm to 330 nm corresponds to the absorption from ZnO nanoparticles. FTIR spectrum showed some significant bands such as hydroxyl group, amine, carbonyl, and aromatic stretching produced by the polyphenols, amino acids, proteins and other bio-components. According to the authors, these *Camellia sinesis* leaves extract induced synthesis of ZnO nanoparticles possesses very good antimicrobial activity towards the bacteria (Gram-positive and Gram-negative) and fungal strains as compared with a positive control.

Lakshmi *et al*., (2012) have reported that the antibacterial study of zinc oxide nanoparticles synthesized from Aloe vera hot extract (ZnO-AH), cold extract (ZnO-AC) and chemical method (ZnO-C) on six clinically isolated strains namely, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella*

*pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus*. They conclude that significant activity was seen in zinc oxide nanoparticles synthesized by chemical method and nanoparticles obtained using Aloe vera cold extract ZnO-AH showed lesser activity. There was a significant difference in the antibacterial activities of ZnO-AH and ZnO-AC though both synthesized in a similar manner. This variation was because of the size as the size of ZnO-AH is much more than that of ZnO-AC.

Niranjan *et al*., (2015) prepared ZnO nanoparticles using *Hibiscus subdariffa* leaf extract and found that the growth of the ZnO nanoparticles depend on the annealing temperature. It is confirmed that the increase of temperature increases the size of the particle. Antibacterial activity of the particles is analyzed using gram-positive (+ve) and gram-negative (-ve) bacteria. Sagar *et al*., (2015) investigated various parameters of ZnO nanoparticles such as physical properties; antibacterial property, high piezoelectricity and binding energy. Larger potential of Zinc Oxide nanoparticles in pharmaceutical, textile, paint and rubber industries is highlighted.

Elumalai *et al*., (2015) carried out a study to the antimicrobial activity of the leaf extract of Murraya koenigii using five bacterial strains such as *S. aureus*, *B. subtilis*, *P. aeruginosa E. coli*, *P. mirabilis* and two fungal strains such as *C. albicans* and *C. tropicalis* as per the disc diffusion and dilution technique. From the study concluded that the zone of inhibition increased with increase in zinc oxide nanoparticle concentration and decrease in particle size. The ZnO nanoparticles were found to be effective for both *S. aureus* and *E. coli* and *P. aeruginosa*.

To the best of my knowledge, there are no reports in the scientific literature on green synthesis of ZnO nanoparticle using *Lippia adoensis* leaf extracts. Hence, the current study is aimed to synthesize ZnO nanoparticle using *Lippia adoensis* and evaluate its antibacterial activity.

## 2.3. Properties of ZnO Nanoparticles

As the dimension of the semiconductor materials continuously breaks down to nanometer or even smaller scale, some of their physical properties undergo changes known as the “quantum size effects”. Investigation of the properties of ZnO nanostructures is essential for developing their potential as the building blocks for future nanoscale devices. This section will review the physical properties of ZnO nanostructures, including, electrical, optical and chemical properties.

### 2.3.1. Electrical properties

The fundamental study of the electrical properties of ZnO nanostructures is crucial for developing their future applications in nanoelectronics. ZnO has a relatively large direct band gap of 3.37 eV at room temperature. Advantages associated with a large band gap include higher breakdown voltages, ability to sustain large electric fields and lower electronic noise. ZnO is well-known as n-type semiconductor where its electrical conductivity is due to the excess of zinc interstitial position (Khan *et al*., 2013). The origin of n-type character is typically the non-stoichiometry analysis. Due to the imperfections like the oxygen vacancies, zinc interstitials and ZnO nanowires are reportedly show n-type semiconductor behavior. The major hindrance of ZnO is the broad series of applications in electronics and photonics with the complexity of p-type doping.

### 2.3.2. Optical properties

ZnO is a wide band gap semiconductor having high optical transparency and luminescence in visible and near ultraviolet range of spectrum. Therefore, it is usually used in light emitting diodes and solar cells. Optical properties and processes in ZnO as well as its refractive index were extensively studied many decades ago. The renewed interest in ZnO is fuelled and fanned by its prospects in optoelectronics applications owing to its direct wide band gap of 3.37 eV at room temperature with large exciton energy of 60 meV and efficient radiative recombination. The strong exciton binding energy, which is much larger than that of GaN (25 meV), and the thermal energy at room temperature (25 meV) can ensure an efficient exciton emission at room temperature under low excitation energy (Lili *et al.*, 2008). As a consequence, ZnO is recognized as a promising photonic material in the blue UV region.

### 2.3.3. Chemical properties

ZnO occurs as the mineral zincite or as white powder known as zinc white. It is usually orange or red in color due to manganese impurity. Crystalline zinc oxide is thermochromic, which changes from white to yellow colour when heated and reverting to white colour on cooling. This change in colour is caused by a very small loss of oxygen at high temperatures. ZnO dissolves in both acidic and basic solutions and the range where it is stable is very limited. With acid it reacts to form compound such as zinc sulfate. Also, when it reacts with alkali it forms zincates.

Zinc oxide exposed to air absorbs both water vapour and carbon dioxide. This results in the formation of basic zinc carbonate (Omkar., 2011).

## 2.4. Applications of ZnO Nanoparticles

Because of its diverse properties, both chemical and physical, zinc oxide is widely used in many areas. It plays an important role in a very wide range of applications, ranging from tyres to ceramics, from pharmaceuticals to agriculture, and from paints to chemicals. However, the most fundamental application of ZnO nanomaterial is its technological applications such as sensor, energy generator, optoelectronics, bio-medicine and drug delivery design (Kennedy *et al*., 2014). Because of its hardness and rigidity, it is an important material in the ceramics industry, while its low toxicity, biocompatibility and biodegradability make it a material of interest for biomedicine and in pro-ecological systems such as cosmetics (Ozgur *et al*., 2005). Presently, ZnO NPs have found application in sun screens, paints and coatings as they are transparent to visible light and offer high UV absorption and are also being used as an ingredient in antibacterial creams, ointments and lotions, self cleaning glass, ceramics and deodorants .

### 2.4.1. Antibacterial activity of ZnO nanoparticles

Zinc oxide nanoparticles has been seen as a possible solution to stop infectious diseases due to its antimicrobial properties. Antibacterial activity is related to compounds that locally kill bacteria or slow down their growth, without being in general toxic to surrounding tissue. It is also described as a function of the surface area in contact with the microorganisms (Wahab *et al*., 2010).

Nanomaterials as antibacterial complementary to antibiotics are highly promising and are gaining large interest as they might fill the gaps where antibiotics frequently fail. This includes combating, multidrug-resistant, mutants and biofilm (Pelgrift and Friedman., 2013). The most important application of ZnO nanoparticles would be as antibacterial agents. Antibacterial activity of ZnO nanoparticles depends on the surface area and concentration; if the concentration is higher and the surface area is larger ZnO nanoparticles possess satisfactory antibacterial activity (Rezende *et al*., 2009). The increase in surface area and smaller size of these particles make them an ideal antibacterial agent. ZnO nanoparticles have been lately tested for their antimicrobial potential and seem to possess both antibacterial and antifungal potential. They are

active against both gram-positive and gram-negative bacteria and also show considerable activity against more resistant bacterial spores (Azam *et al*., 2011).

### 2.4.2. Antibacterial activity investigation methods

An antibacterial agent is considered as bactericidal if it kills bacteria or as bacteriostatic if it inhibits their growth. Different methods have been adopted for the assessment and investigation of antibacterial activity invitro. These methods include disc diffusion, broth dilution, and agar dilution method (Premanathan *et al*., 2011).

**Broth dilution method:** Broth dilution is a technique in which a suspension of bacterium of a predetermined optimal or appropriate concentration is tested against varying concentrations of an antimicrobial agent (usually serial two fold dilutions) in a liquid medium of predetermined, documented formulation. Because of the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories.

**Agar dilution method:** Agar dilution involves the incorporation of varying concentrations of antimicrobial agent into an agar medium, usually using serial two fold dilutions, followed by the application of a defined bacterial inoculum to the agar surface of the plate. These results are often considered as the most reliable for the determination of minimum inhibitory concentration (MIC) for the test bacterium/antimicrobial combination. The advantages of agar dilution methods include: i) the ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time, ii) the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range.

Agar dilution methods also have certain disadvantages, such as: if not automated, they are very difficult and require substantial economic and technical resources, once the plates have been prepared, they normally should be used within a week (or less, depending on the antimicrobials tested), the endpoints are not always easy to read nor is the purity of the inoculum easy to verify.

**Disc diffusion method**: refers to the diffusion of an antimicrobial agent of a specified concentration from disks, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disc. The

diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. Generally, the larger the zone of inhibition, the higher the concentration of antimicrobial required to inhibit the growth of the organisms (Clinical and Laboratory Standards Institute., 2008). Disk diffusion is easy to perform, reproducible, results easily interpreted and does not require expensive equipment. Due to such mentioned advantages this methods used for antibacterial tests in this study.

### 2.4.3. Mechanism of antibacterial activity of ZnO-NPs

Antibacterial activity of ZnO-NPs has been referred to a number of issues, but the exact toxicity mechanism is not completely illuminated and still controversial, as there are some questions within the spectrum of antibacterial activity requiring deep explanations. Distinctive mechanisms that have been put forward in the literature are listed as following: direct contact of ZnO-Nanoparticles with cell walls, resulting in destructing bacterial cell integrity (Zhang *et al*., 2007), liberation of antimicrobial ions mainly $Zn^{2+}$ ions, and reactive oxygen species (ROS) formation. However, the toxicity mechanism varies in various media as the species of dissolved Zn may change according to the medium components besides the physicochemical properties of ZnO-Nanoparticles (Li *et al*., 2011).

ZnO is found to possess high photocatalytic efficiency among all inorganic photocatalytic materials, and is more biocompatible than $TiO_2$ (Zhang *et al*., 2011). ZnO can highly absorb UV light (Nirmala *et al*., 2010), and it has a better response to UV light, thus its conductivity dramatically enhances, and this feature significantly activates the interaction of ZnO with bacteria. UV illumination rapidly causes desorption of loosely bound oxygen ($O^-_2$, $O_2^{2-}$), from the surface. This results in reducing the surface electron depletion region and causing improved photoconductivity (Bao *et al*., 2011). The photocatalysis is described as a photo-induced oxidation process that can damage and inactivate organisms (Baruah *et al*., 2010). ZnO-NPs in aqueous solution under UV radiation have phototoxic effect that can produce ROS such as hydrogen peroxide ($H_2O_2$) and superoxide ions ($O^{2-}$). The generated active species are, capable

to penetrate into the cells, thus inhibit or kill microorganisms. Figure 2 shows mechanisms of ZnO NPs antibacterial activity.

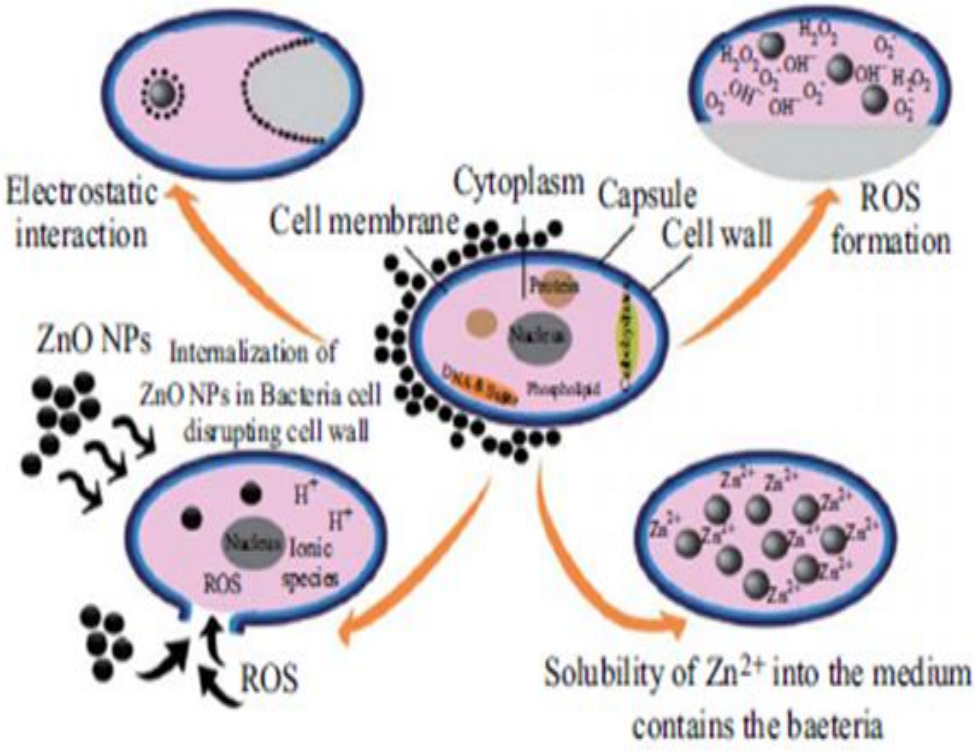



**Figure 2.** Mechanisms of ZnO-NPs antibacterial activity, including: ROS formation, $Zn^{2+}$ release, internalization of ZnO-NPs into bacteria, and electrostatic interactions (Amna *et al*., 2015).

# 3. MATERIALS AND METHODS

## 3.1. Experimental Site

Experiments such as synthesis of ZnO nanoparticles, TGA, and XRD characterization were done in Adama Science and Technology University at Material Science and Engineering Department Laboratory. FT-IR characterization was done at chemistry department, Addis Ababa University. UV-Visible spectroscopy, EDX and SEM were conducted at Department of Material Science and Engineering Laboratory, National Taiwan University of Science and Technology. Antibacterial activities of synthesized ZnO nanoparticles against selected bacterial strains were tested at Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory, Adama.

## 3.2. Equipment and Apparatus

Equipment and apparatuses used in this study were: XRD-7000 (Shimadzu, XRD-7000 South Korea), SEM (Model: VEGA 3, TESCAN, Czech Republic), EDX, FT-IR Spectrophotometer (Perkin Elmer, 65), UV-Visible Spectrophotometer (Perkin Elmer, Lambda 950 UV/VIS/NIR Spectrophotometer), Muffle Furnace (Amb+ ℃ to 1000 ℃, Digital PID Controller type program/p), DTG-60H (Room temperature to 1500 ℃, South Korea), Drying Oven, 150 $\mu m$ size sieves, Analytical Precision Balance (Weighing capacity 252g/0.1mg A&D Inc. Korea), Mortar and pestle, Ceramic crucibles, Volumetric flasks, Conical flask, Funnel, Whatman Filter paper#1, Magnetic stirrer, Measuring cylinder, Glass Beakers, Gloves, and Glass filter.

## 3.3. Chemicals and Reagents

Chemicals used in this project work were all analytical grade and used without further purifications. These chemicals were: Zinc acetate dihydrate ($Zn(CH_3COO)_2$. $2H_2O$, UNI=CHEM, Sodium hydroxide (NaOH pellet, Alpha Chemika), Ethanol 96% ($CH_3CH_2OH$, Ranchem Industry & Trading) and deionized water.

## 3.4. Experimental Procedure

### 3.4.1. Collection and Preparation of Plant Material (Broth Solution)

Fresh leaves of *Lippia adoensis* 'koseret' were collected from Adama town, East Ethiopia. The collected leaves were thoroughly washed three times using distilled water to remove the dust particles and then sun dried to remove the residual moisture. The dried leaves were cut and ground into powder. Then, the ground powder was sieved by using 150 $\mu m$ size sieves. The aqueous extract of sample were prepared by boiling 5 g of fine powdered leaves in 250 ml glass beaker along with 100 ml of double distilled water, at 80 ℃ for 60 minutes while stirring using magnetic stirrer at 800 r.p.m until the color of the aqueous solution changed from green to brown (Figure 3). The extract was cooled to room temperature and filtered using Whatman filter paper. The extract was stored in a refrigerator at 4 ℃ in order to be used for further experiments. The filtrate aqueous extract were used as reducing and capping agent for synthesis of ZnO nanoparticles (Sharmila and Gayathri., 2014).

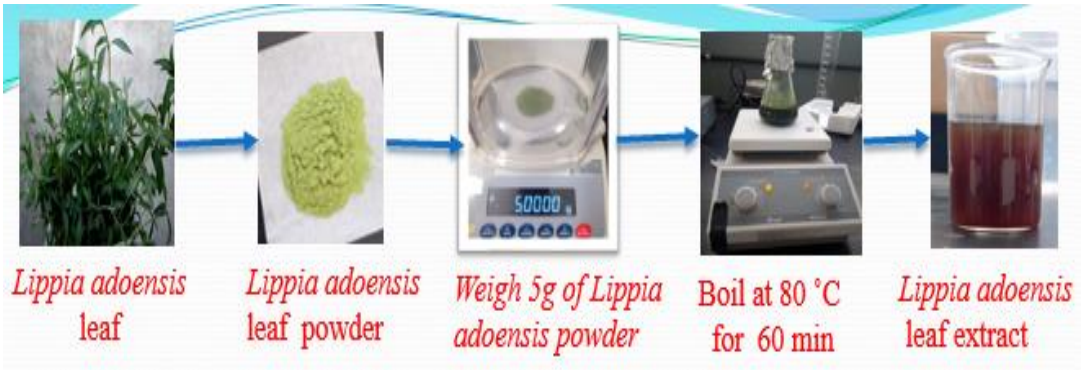



**Figure 3.** Schematic representation of extraction preparation of *Lippia adoensis* leaf.

### 3.4.2 Preparation of Zinc Acetate Dihydrate and synthesize ZnO nanoparticles

Zinc acetate dihydrate [$Zn(CH_3COO)_2.2H_2O$] was used as precursor to synthesize ZnO nanoparticles using aqueous extract of *Lippia adoensis* leaf. 0.45 M Zinc acetate solution was prepared by dissolving 24.6937 grams of Zinc acetate dihydrate in 250 mL double distilled water using 250 mL volumetric flask and stored for further use (Sharmila and Gayathri., 2014) and 0.45 M NaOH was prepared using double distilled water by dissolving 4.5 gram Sodium hydroxide in 250 mL volumetric flask to be used as precipitating agent. Figure 4 shows

schematic representation of green synthesis of ZnO nanoparticles using aqueous leaf extract of *Lippia adoensis* plant.

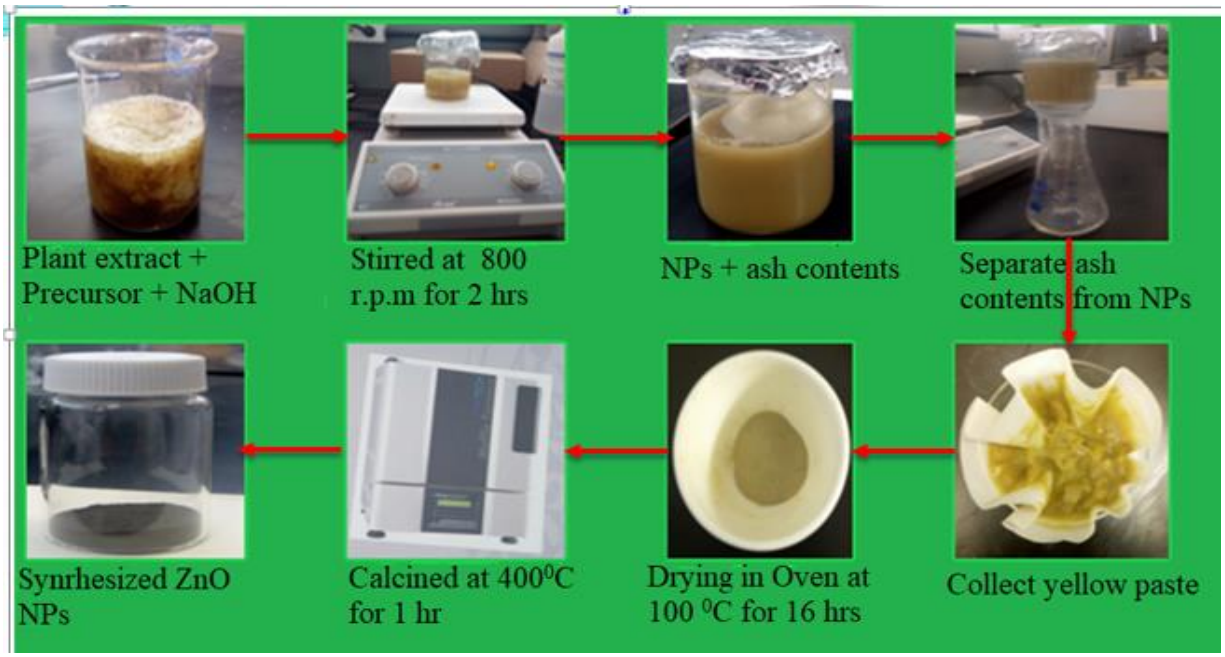



**Figure 4.** Schematic representation of green synthesis of ZnO nanoparticles using aqueous extract from *Lippia adoensis* leaf.

Then three different samples were prepared by taking different ratios by volume (Precursor salt solutions to *Lippia adoensis* leaf extract).

Sample 1: $Zn(CH_3COO)_2$. $2H_2O$: *Lippia adoensis* Extract (9:1) Ratio (90 mL: 10 mL)

Sample 2: $Zn(CH_3COO)_2$. $2H_2O$: *Lippia adoensis* Extract (3:2) Ratio (60 mL: 40 mL)

Sample 3: $Zn(CH_3COO)_2.2H_2O$: *Lippia adoensis* Leaf Extract (1:1) Ratio (50 mL:50mL)

**Synthesis of ZnO nanoparticles:** Required volume of *Lippia adoensis* leaf extract, Precursor salt 0.45 M $Zn(CH_3COO)_2$. $2H_2O$ and 0.45 M NaOH was mixed in beaker. Then, the mixture was stirred continuously for 2 hours on magnetic stirrer at 800 r.p.m resulting in yellow precipitate solution (as indicated in Figure 4). The precipitate was filtered using glass filter and washed repeatedly with distilled water followed by ethanol in order to remove the impurities. The paste was collected in crucible and dried in an Oven at 100 °C for 1 hr. After that, the light yellow color powder was obtained and mashed using mortar and pestle. Finally, yellow powder

obtained was calcined at 400 ℃ for 1 hr and grinded to fine powder and packed for characterization.

## 3.5. Characterization Methods of Zinc Oxide Nanoparticles

### 3.5.1 Thermal Gravimetric Analysis (TGA) of ZnO NPs

Thermal gravimetric analysis (TGA) was carried out using a simultaneous DTA-TG apparatus (DTG-60H, Shimadzu Co., South Korea) to characterize the decomposition and thermal stability of ZnO nanoparticles measured at the heating rate of 10 ˚C/min. About 8.785 mg of the uncalcinated ZnO nanoparticle was placed in a platinum crucible on the pan of the microbalance and heated from room temperature to 1000 °C, using Nitrogen gas as inert material.

### 3.5.2. Characterization of ZnO NPs using XRD

Crystal structure and phase of synthesized zinc oxide nanoparticles were characterized using XRD-7000. Small amount of the powders were taken and ground to fine particle and analyzed by XRD equipped with a Cu target for generating a Cu Kα radiation with λ = 0.154060 nm. XRD spectrums were recorded from $10^0$ to $80^0$ with 2θ angles using CuKα radiation operated at 40 kV and 30 mA. The particle size (D) of the powders was calculated by Scherer's formula;

$$D = \frac{0.94\lambda}{\beta cos\theta} \quad \text{..... (1)}$$

Where, K= 0.94 is the Scherrer's constant, λ is the x-ray wave length, θ is the Bragg's diffraction angle, and β is the peak width of the diffraction line at half of the maximum intensity.

### 3.5.3. Characterization of ZnO NPs using SEM

Morphologies of the samples were investigated by scanning electron Microscope (SEM, Model: VEGA 3, TESCAN, Czech Republic) instrument operating at constant beam energy. To prepare the sample small amount of the synthesized powder was taken and placed in the pellet maker. The SEM images were captured at the same magnification levels and the morphological features of the particles were studied.

### 3.5.4. Characterization of ZnO NPs using EDX

The elemental analysis or chemical composition of synthesized zinc oxide nanoparticles was estimated by energy-dispersive X-ray spectroscopy (EDX). To prepare the sample small amount of the synthesized powder was scooped and placed in the pellet maker. The prepared pellet was then coated with carbon before mounting in the machine. The operation was done at excitation energy of 20 keV with scan or acquisition time of 600s.

### 3.5.5. Characterization of ZnO NPs using FT-IR Spectroscopy

Fourier transformer infrared spectroscopy (FTIR) spectra of synthesized zinc oxide nanoparticles were recorded at room temperature using FTIR spectrophotometer in order to analyze and detect surface functional groups at the scanning range of 4000 – 400 $cm^{-1}$. For the FT-IR characterization, ZnO NP was mixed with KBr and compressed to form a pellet which was used for FT-IR analysis.

### 3.5.6. Characterization of ZnO NPs using UV-Visible Spectroscopy

Solid films (pellets) of the synthesized ZnO NPs were formed and used for UV-Vis analysis. The absorption of all synthesized zinc oxide nanoparticles samples were measured by scanning the wavelength from 200 - 800 nm.

## 3.6. Method for Antibacterial Activity Test

Nutrient broth 1.3g, Nutrient agar 5.6g, Agar-agar 0.5g, petriplates, antibiotic discs, cotton swabs, *Staphylococcus aureus* (ATCC22953), *Enterococcus feacalis (ATCC 229212)*, *Escherichia coli* (ATCC 25922) and *Klebsiella pneumonia* (ATCC 700603) were used for antibacterial activity test of the synthesized ZnO nanoparticles using disc diffusion method.

### 3.6.1 Preparation of inoculum

Nutrient broth (1.3 g in 100 mL deionized water) was prepared in 4 conical flasks and sterilized. Clinically isolated strain of *Staphylococcus aureus*, *Enterococcus feacalis*, *Escherichia coli*, and *Klebsiella pneumonia* were added into the first, second, third, and fourth conical flasks, respectively. These bacterial cultures inoculated in nutrient broth were kept on rotary shaker for 24 hours at 100 r.p.m (Haritha *et al*., 2011).

### 3.6.2 Inoculation of test plate

Nutrient agar was prepared (5.6 g nutrient agar and 0.5 g Agar-Agar in100 mL deionized water) and sterilized. The agar suspension within 15 min was used to inoculate plates by dipping a sterile cotton-wool swab into the suspension and removed the excess by turning the swab against the side of the container (Haritha *et al*., 2011). Then, the inoculum was spread evenly over the entire surface of the plate by swabbing in three directions. The plate was allowed to dry before applying to antibiotic discs.

### 3.6.3 Disc diffusion method for antibacterial activity test

The antibacterial activity of zinc oxide nanoparticles and uncalcinated Zinc oxide on gram-positive (*Staphylococcus aureus* and *Enterococcus feacalis*) and gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) bacteria were tested by disc diffusion method according to the procedures reported (Sawai., 2003). Microbial strains were grown aerobically in nutrient broth for 24h at 37°C until the turbidity of bacterial suspensions was achieved to $1.5 \times 10^8$ CFU/mL (colony forming unit per milliliter) by comparison with the 0.5 Mac Farland Standard. The disc diffusion assay was carried out by swabbing each test strain on Mueller-Hinton (MH) agar plate.

Bacterial cultures were maintained on nutrient Muller-Hinton agar at 37 $^0$C and the cultures were kept in appropriate media slants and stored at 4 $^0$C until used. The antibiotic Vacomycin and Carbenicilin were used as positive control ($P_C$) and dimethyl sulfoxide (DMSO) solvent was used as negative control ($N_C$). Discs were applied to the surface of an agar plate that was previously dried. The plates were then turned upside down and incubated at 37 $^0$C for 24 h in an incubator. The plates were shaken gently to allow evenly mixing of bacteria cells and agar. 60 mm plate accommodated two discs and ZnO nanoparticles without unacceptable overlapping of zones. Agar plate was divided into 3 sections: antibiotic disc, zinc oxide nanoparticles sample, and both antibiotic disc and zinc oxide nanoparticle sample.

Then, 100 mg of ZnO nanoparticles were dissolved in 200 µL dimethyl sulfoxide (DMSO). From the prepared sample, 200 µL of each concentration were diluted in 200 and 400 µL dimethyl sulfoxide (DMSO) solvent to obtain 1:4 and 1:8 dilution factors. From each sample, 200 µL of each concentration saturated with discs (6 mm diameter disc) was placed on plate and incubated at 37 $^0$C for 24 hours. Clear inhibition zones formed around the discs indicated the

presence of antibacterial activity. The results were taken by considering the zone of growth and inhibition of the bacteria by the test fractions. Antibacterial activity was evaluated by measuring the diameter (mm) of the inhibition zone (IZ) around the disc against the test organisms by using caliper.

# 4. RESULTS AND DISCUSSION

## 4.1. Synthesis of Zinc Oxide Nanoparticles

The synthesis of ZnO NPs involved a redox process. *Lippia adoensis* leaf extract was first added into salt of zinc acetate dihydrate aqueous solution that reduced Zn (II) to Zn (0) and maintains the size of particles formed in nano scale by capping them from coming into contact with each other. During the mixing cloudy solution was formed that is an indication of the occurrence of reduction reaction. Sodium hydroxide (NaOH) was added as an accelerant to enhance the rate of reduction and as precipitating agent during nucleation process of $Zn^{2+}$ to $Zn(OH)_2$ followed by loss of water to form ZnO NPs (Balavandy *et al*., 2015). The related chemical reactions are shown in the equation below:

$$Zn(CH_3COO)_2.2H_2O + \text{Plant extract} + 2NaOH \rightarrow ZnO\downarrow + 2NaOOCCH_3 + 3H_2O + \text{byproduct} \quad (4.1)$$

In this work, ZnO nanoparticle was synthesized using *Lippia adoensis* leaf extract and zinc acetate dihydrate precursor using different volume ratios. Figure 5 shows photographs of the synthesized ZnO nanoparticles. Sample (a), (b), and (d) which appeared brown in color are ZnO nanoparticles synthesized using different ratios (1:1, 3:2, and 9:1) by volume of precursor solution and leaf extract, respectively and calcinated at 400 $^{o}$C. Whereas, sample (c) which appeared yellow in color is ZnO NP synthesized using precursor solution and leaf extract using 1:1 ratio by volume without calcination. The brown color appearance of the nanoparticles up on calcination (heating) is most probably due to burning of the organic compounds from the plant extract producing soot that might in turn adhere to the nanoparticles.

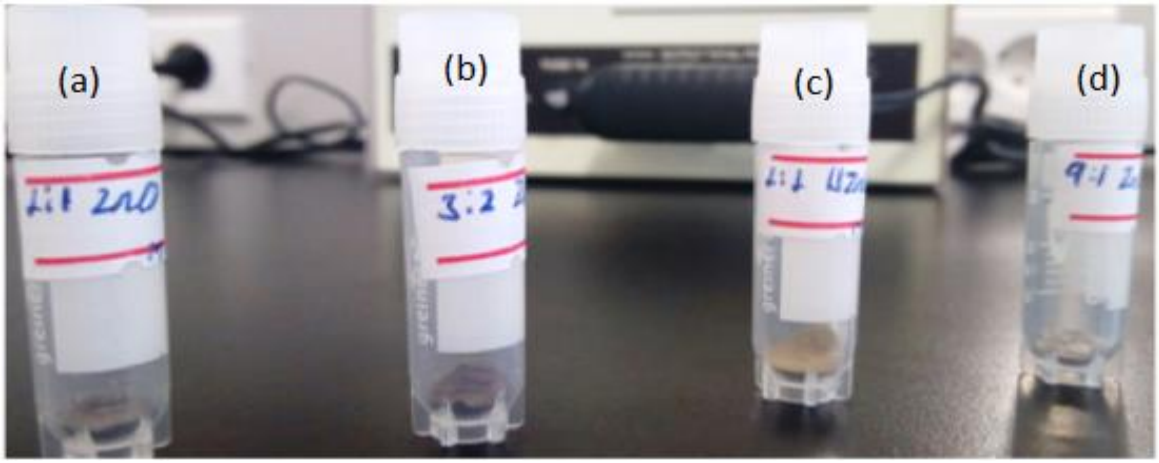



**Figure 5.** Photograph of ZnO NPs synthesized using different reactant ratios by volume of: (a) 1:1, (b) 3:2, (c) 1:1 (uncalcinated) and (d) 9:1.

Generally, this synthesis approach was considered green as it involved the uses of plant extract to replace toxic chemical such as surfactants or polymers (to control the growth of particles), cheap and ecofriendly precursors zinc acetate dihydrate salt, universal green solvent, water ($H_2O$) instead of volatile organic solvent (VOC) and low energy consumption (reaction proceeded in room temperature) that fulfilled the principles of green chemistry.

## 4.2. Thermal Gravimetric Analysis (TGA)

Figure 6, shows the thermogravimetric analysis (TGA) and differential thermal analysis (DTA) of the uncalcined ZnO nanoparticles (9:1 UZnO). TGA curve shows mass loss of the mass whereas DTA curve indicates the energy gain or loss during the process. TGA/DTA transition shows a loss of 36.270 % up to 400 $^0$C and no considerable loss in mass is observed after this temperature up to 1000 $^0$C. TGA-DTA data for $Zn(OH)_2$ precursor registered also strong endothermic peak at 400 $^0$C. This indicates that after 400 $^0$C there is no weight loss for the synthesized ZnO nanoparticles and the nanoparticle is thermally stable. Therefore, 400 $^o$C was selected to be used for calcinations of all other synthesized ZnO nanoparticles.

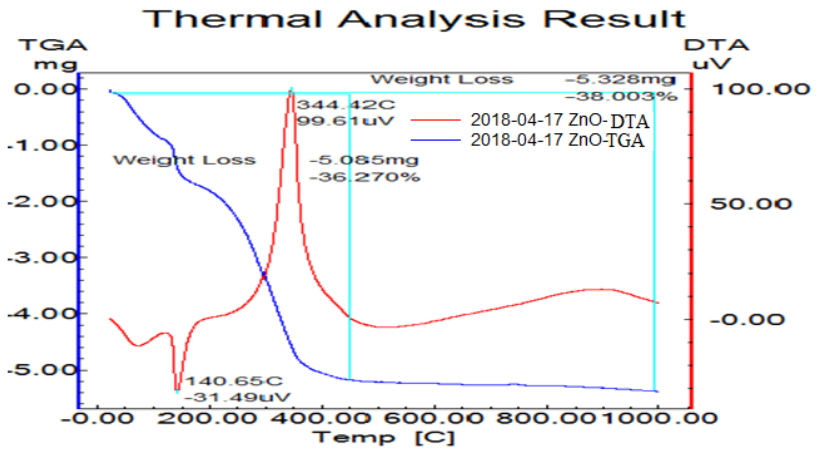



**Figure 6.** Thermal analysis result of synthesized uncalcinated ZnO NPs using zinc acetate dihydrate and *Lippia adoensis* leaf extract (9:1 ratio).

## 4.3. XRD Analysis

Crystal structure and phase of green synthesized zinc oxide nanoparticles were characterized using XRD. Powdered sample was used by a Cu Kα - X Ray Diffractometer for confirming the formation of ZnO and analyze the structure. Figure 7 shows XRD patter of ZnO nanoparticles synthesized using zinc acetate dihydrate and *Lippia adoensis* leaf extract with different ratios by volume. In Figure 7, the peaks appeared at 2θ value of 31.76°, 34.42°, 36.24°, 47.54°, 56.59°, 62.86°, 66.41°, 67.97° and 69.06° corresponds to (100), (002), (101), (102), (110), (103), (200), (112) and (201) crystal planes confirmed the presence of ZnO NPs (Saravanan *et al*., 2011). All the peaks were properly assigned using JCPDS file card No. 0361451. All characteristic peaks for pure ZnO were present in the XRD patterns of sample. The location of the peaks was compared to literature values (Renata D. and Jolanta D., 2016) and the presence/formation of zinc oxide nanoparticles were confirmed. The average grain size of the ZnO nanoparticles was calculated from the three most intense peaks using Debye-Scherrer"s formula and presented in the following Tables.

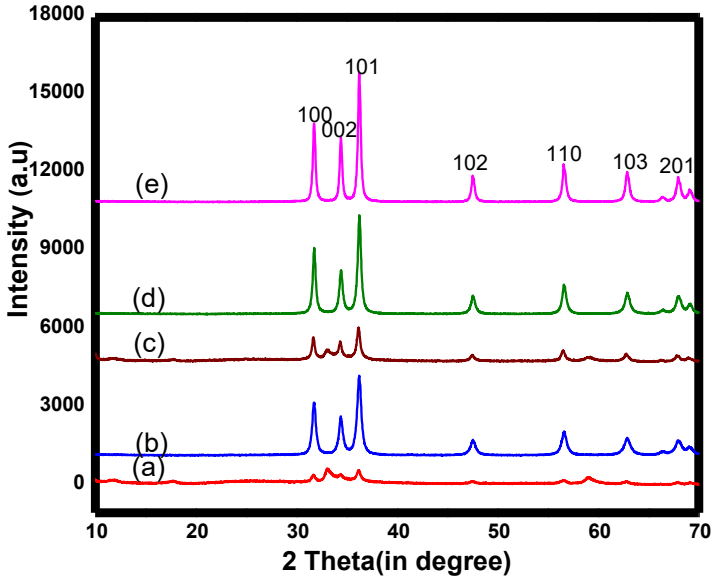



**Figure 7.** XRD diffraction patterns of ZnO Nanoparticles synthesized using zinc acetate dihydrate and *Lippia adoensis* leaf extract at different ratios by volume a) 1:1 (uncalcinated) b) 1:1 c) 3:2 (uncalcinated) d) 3:2 and e) 9:1

XRD spectrum showed sharp Bragg diffraction peaks around $37^0$, which are indexed the (101) of the wurtzite crystal structure of ZnO. Intense Bragg reflections suggest that strong X-ray scattering centers in the crystalline phase and could be due to capping agents. Therefore, XRD results also suggested that the crystallization of the bio-organic phase occurs on the surface of the ZnO nanoparticles or vice versa. As shown in appendix 1 and 3, additional peaks which are present in uncalcinated ZnO nanoparticles but removed after calcinating the nanoparticle at 400 $^oC$ are due to biomolecules from *Lippia adoensis* leaf extract. Moreover, XRD results indicated grain size of ZnO nanoparticles increase from 18.5 nm to 22.6 nm to 26.7 nm with increasing ratio by volume of precursor from 1:1 to 3:2 to 9:1, respectively. Table 1 presents FWHM (Full Width at Half Maximum) values; average crystallite sizes calculated using Scherer's Formula, d–spacing ($d_{hkl}$) and Miller indices of ZnO NPs synthesized using zinc acetate dehydrate and *Lippia adoensis* leaf extract at ratios by volume of 3:2 and 1:1.

**Table 1**. FWHM values, average crystallite sizes calculated using Scherer's Formula, d–spacing ($d_{hkl}$) and Miller indices of calcinated and uncalcinated ZnO NPs synthesized using zinc acetate dihydrate and *Lippia adoensis* leaf extract at ratios by volume of 3:2, 1:1and 9:1.

| | | | 3:2 ZnO NPs | 3:2 UZnO NPs | 1:1 ZnO NPs | 1:1 UZnO NPs | 9:1 ZnO NPs |
|---|---|---|---|---|---|---|---|
| h k l | 2θ(degree) | d –spacing ($d_{hkl}$) | FWHM (degree) | FWHM (degree) | FWHM (degree) | FWHM (degree) | FWHM (degree) |
| 1 0 0 | 31.7773 | 2.81369 | 0.35610 | 0.35010 | 0.44830 | 0.68000 | 0.30140 |
| 0 0 2 | 34.4411 | 2.60192 | 0.38560 | 0.41040 | 0.46960 | 1.18660 | 0.29280 |
| 1 0 1 | 36.2598 | 2.47548 | 0.39210 | 0.39870 | 0.48830 | 0.75380 | 0.30810 |
| 1 0 2 | 47.5471 | 1.91083 | 0.45350 | 0.45000 | 0.57290 | 0.65330 | 0.28000 |
| 1 1 0 | 56.6034 | 1.62471 | 0.43830 | 0.41840 | 0.55160 | 0.72000 | 0.34300 |
| 1 0 3 | 63.3832 | 1.46627 | 0.35500 | 0.45180 | 0.65800 | 0.80000 | 0.37210 |
| 2 0 0 | 66.4138 | 1.40671 | 0.46700 | 0.32400 | 0.66500 | - | 0.24580 |
| 1 1 2 | 67.9799 | 1.37788 | 0.55040 | 0.52670 | 0.70180 | 0.76000 | 0.27340 |
| 2 0 1 | 69.0795 | 1.35861 | 0.51600 | 0.45550 | 0.70660 | 0.78000 | 0.41430 |
| **Average Crystallite size** | | | **22.6 nm** | **22.5 nm** | **18.5 nm** | **9 nm** | **26.7 nm** |

As compared to ZnO nanoparticles synthesized using 3:2 and 1:1 ratio, XRD result showed that the average crystallite size of ZnO nanoparticles in 1:1 ratio has a smaller particle size (18.5 nm) than that of the 3:2 (22.6 nm). This difference may be due to the greater amount of leaf extract (50 mL) used in 1:1 ratio during synthesis process resulting in more capping agents that effectively stabilize the synthesized nanoparticles and hindered aggregation. In addition, in the Table 1, it is clearly seen that the peak width of the diffraction line at half of the maximum intensity (FWHM) of 3:2 ratio ZnO nanoparticles is less than that of 1:1 ratio. The broadening of peaks in the XRD patterns of solids is attributed to smaller particle size. This is due to the reason that, from Scherer's formula, the particle size increases with decreasing of the peak width of the diffraction line at half of the maximum intensity.

Table 1 shows the average crystalline size of 1:1(uncalcinated) ZnO nanoparticle is smaller (9 nm) than 3:2 (uncalcinated) (22.5 nm) ZnO nanoparticle. This difference is most probably due to the greater amount of leaf extract (50 mL) used in 1:1 ratio during synthesis process resulting in more capping agents that effectively stabilize the synthesized nanoparticles in a similar fashion with the calcinated once (Table 1). As indicated in Figure 7, the XRD diffraction patterns for 3:2 (uncalcinated) ZnO nanoparticles is sharper than 1:1 (uncalcinated) which suggest the nucleation and growth of the crystal.

## 4.4. SEM Analysis

The surface morphologies of synthesized ZnO nanoparticles were studied by SEM and the results are shown in Figure 8. Figure 8 (a) shows that, the morphology of uncalcinated ZnO NPs synthesized at 1:1 mixing ratio of reactants has spherical shapes and the particles are also found to be in clinged together. These are due to the presence of more capping agent (uncalcinated bio-molecules from *Lippia adoensis* leaf extract) that stabilize nanoparticle. It was observed on figure 8 c, from the ratio of 3:2 precursors that used for ZnO synthesis, equal size spherical shapes nanoparticles. On the other hand, the morphology of ZnO nanoparticle synthesized at reactant ratio of 1:1 (Figure 8 (b)) formed spherical grains and rods morphologies of ZnO nanoparticles. The nanoparticle synthesized from 9:1 reactant ratio (Figure 8 (d)) contains both nanorod and flake types.

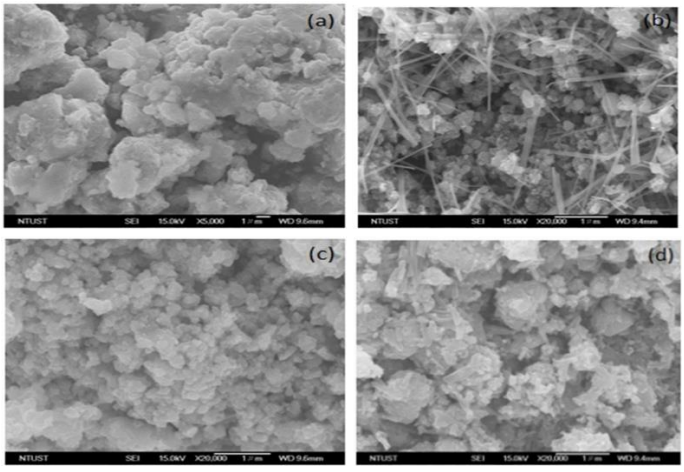



**Figure 8.** SEM images of a) 1:1 UZnO b) 1:1 ZnO c) 3:2 ZnO and d) 9:1 ZnO.

## 4.5. EDX Analysis

EDX was carried out to analyze the chemical composition and formation of ZnO nanoparticle. The energy dispersive X-ray analyses of all ZnO nanoparticles at different mixing ratios are shown in Figures 9 to 12. It is obvious from the X-ray patterns of all the samples in which Zn and O are found in the respective spectrum. The EDX studies of Figures 9 to 12 present three peaks between 1 kV and 10 kV. Those peaks are directly related to Zinc and Oxygen in the tested material. The results indicated that the reaction product was composed of high purity ZnO NPs as there is no peak that represent any other foreign material from the spectrum.

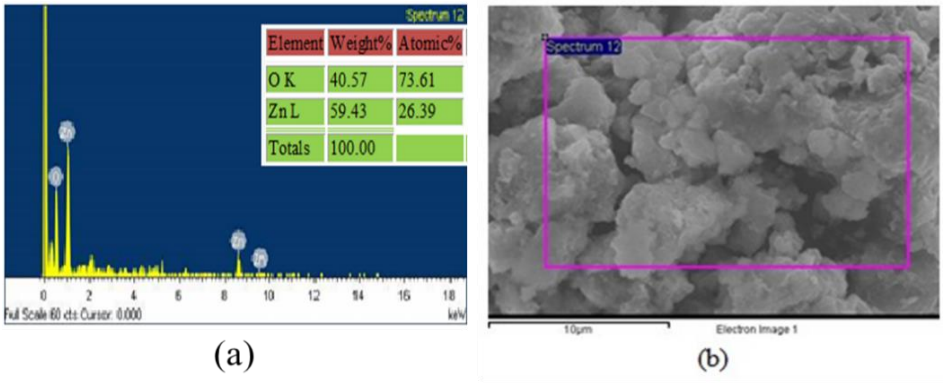



**Figure 9.** (a) EDX Spectrum and quantitative analysis of 1:1 UZnO (b) SEM images of selected area for EDX spectra of 1:1 UZnO.

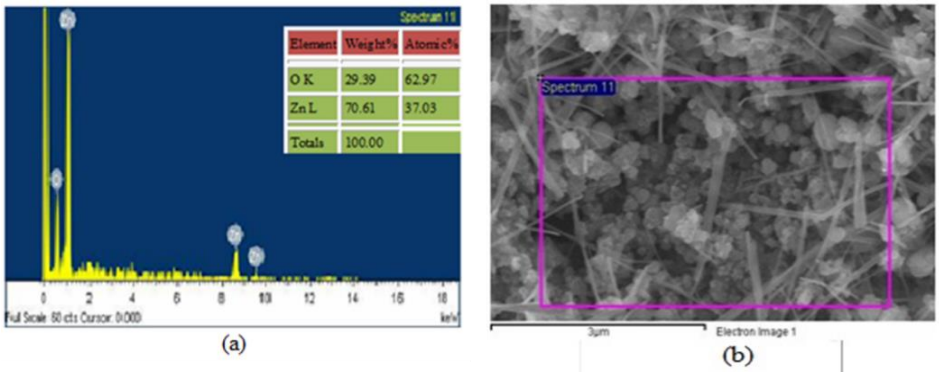
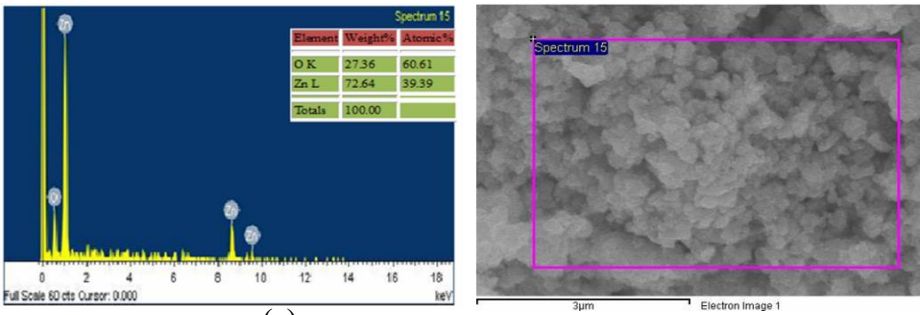
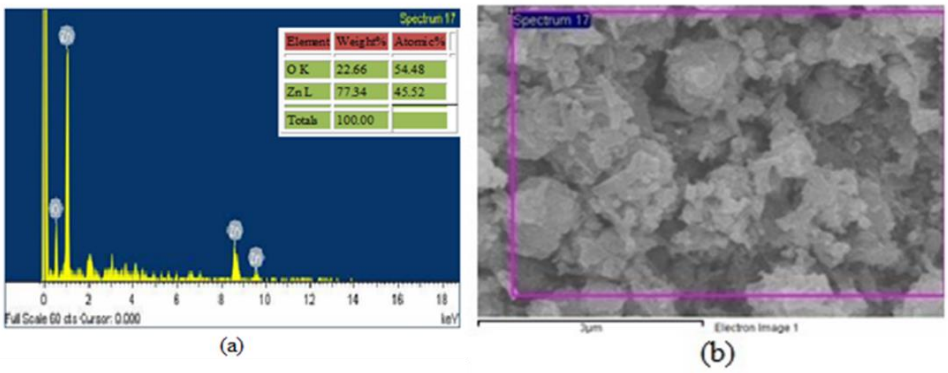



**Figure 10.** (a) EDX Spectrum and quantitative analysis of 1:1 ZnO (b) SEM images of selected area for EDX spectra of 1:1 ZnO.




(a) (b)

**Figure 11.** (a) EDX Spectrum and quantitative analysis of 3:2 ZnO (b) SEM images of selected area for EDX spectra of 3:2 ZnO.




(a)

(b)

(a) (b)

**Figure 12.** (a) EDX Spectrum and quantitative analysis of 9:1 ZnO (b) SEM images of selected area for EDX spectra of 9:1 ZnO.

## 4.6. FT-IR Analysis

FTIR gives information on vibrational and rotational modes of motion of a molecule and hence an important technique for identification and characterization of a substance. By means of this technique, it is possible to identify biomolecules found in plant extracts which play crucial role in the processes of reduction and stabilization of the green synthesis of nanoparticles (Senthilkumar and Sivakumar., 2014). Possible biomolecules responsible for the reduction and capping agent of bio-reduced ZnO NPs through particular bond vibrations peaks coming at defined wavenumbers was identified. The results of FTIR for uncalcinated and calcinated ZnO are shown in Figure13.

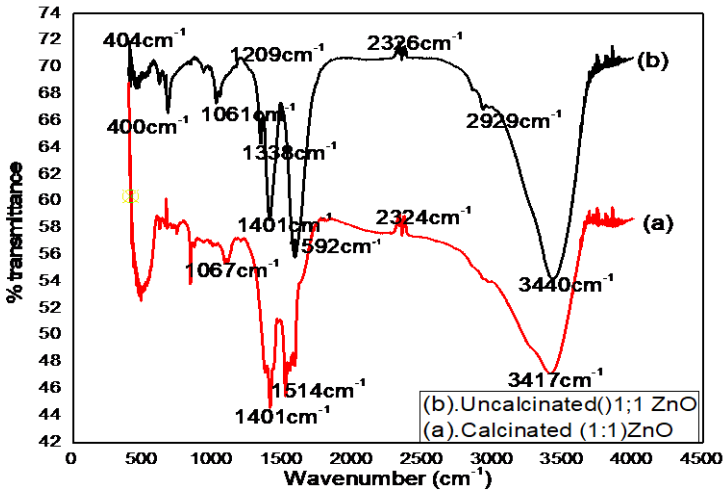



**Figure 13.** FTIR Graph of Uncalcinated (UZnO) and calcinated ZnO NPs synthesized from *Lippia adoensis* leaf extract with ratio 1:1.

From Figure 13, different bands were observed at 3440 $cm^{-1}$, 2929 $cm^{-1}$, 2326 $cm^{-1}$, 1591 $cm^{-1}$, 1401 $cm^{-1}$, 1338 $cm^{-1}$,1209 $cm^{-1}$, 1061 $cm^{-1}$, 404 $cm^{-1}$ and 3417 $cm^{-1}$, 2324 $cm^{-1}$, 1514 $cm^{-1}$,1401 $cm^{-1}$, 400 $cm^{-1}$, for uncalcinated and calcinated ZnO nanoparticle respectively. The peak in the region between 400 and 600 $cm^{-1}$ is assigned to Zn–O (Yuvakkumar *et al*., 2015). Also, the band located at 404 (for uncalcinated) and 400 $cm^{-1}$ (for calcinated) is assigned to ZnO stretching vibration. The FT-IR spectra exhibited the broad peak in higher energy region due to O–H stretching, H–bonded presence of alcohols and phenols around 3440 and 3417 $cm^{-1}$ (Awwad *et al*., 2014).

Absorption at 2326 and 2324 $cm^{-1}$ is because of the presence of $CO_2$ molecules in the environment (Maddahi *et al*., 2014). The peak around 1591 $cm^{-1}$ are due to C-C stretching aromatic ring. Strong intensity at 1401 $cm^{-1}$ is due to $\alpha$-$CH_2$ bending vibrations of aldehydes and ketones. The peak at 1338 $cm^{-1}$ result from aromatic amines and the peaks at 1061 and 1067 $cm^{-1}$ result from C–N stretch of aliphatic amines (Jamdagni, *et al*., 2018). Peaks observed at 1209 $cm^{-1}$ is due to C–O stretch from alcohols.

Current findings are supported by previous reports on phytochemical constituents of *Lippia adoensis* leaf extract (Berhanu *et al*., 2001). Generally, the overall observation from FTIR

proved the presence of ZnO nanoparticles and some organic compounds from the leaf extract such as: phenolic compounds, terpenoids or proteins that are bound to the surface of ZnO NPs.

## 4.7. UV-Visible Spectral Analysis

Figure 14, shows UV-Vis absorption spectrum of zinc oxide nanoparticles. Absorption spectrum was recorded for all samples in the range of 200 - 800 nm. Strong absorption bands were observed from UV–visible spectroscopy in between 360 and 363 nm for all samples, except for uncalcinated 1:1 ZnO nanoparticles (234 nm), which corresponds to characteristic band of ZnO nanoparticles. The adsorption edges of the NPs were effectively blue shifted when compared to the wavelength of bulk ZnO appeared at 385 nm (Senthilkumar and Sivakumar, 2014). This blue shift might be attributed to the smaller size of nanoparticles and due to the quantum confinement effect (Ullakko *et al*., 1996).

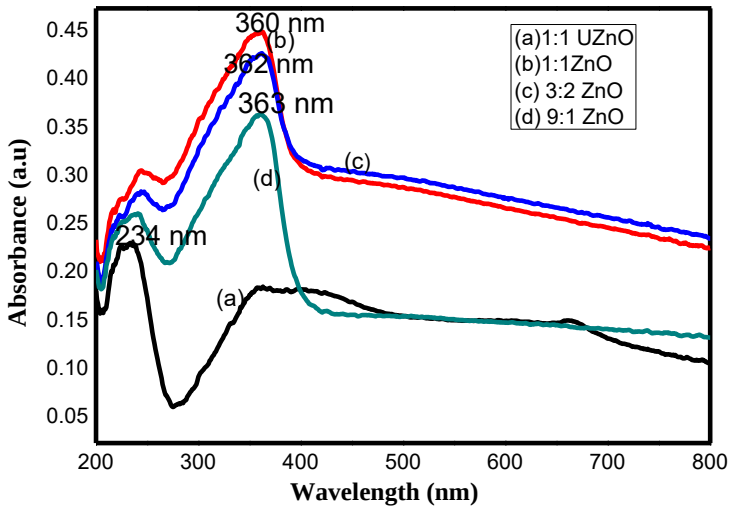



**Figure 14.** UV-Vis absorbance results of ZnO NPs synthesized using zinc acetate dihydrate precursor and *Lippia adoensis* leaf extract, at different reactant proportion.

## 4.8. Antibacterial Activity of ZnO Nanoparticles

Antibacterial activity performance of ZnO nanoparticles was done by using disc diffusion method. The effects of particle size and concentration on the antibacterial activity of ZnO nanoparticles were studied using this method. This method is well documented and standard

zones of inhibition have been determined for susceptible and resistant selected bacterial strains. Nanoparticles of ZnO exhibit attractive antibacterial properties due to increased specific surface area as the reduced particle size leading to enhanced particle surface reactivity and contact with the bacterial pathogens. The smaller size of nanoparticles facilitates easy entry into the bacterial cell membrane and enables inhibition mechanisms to occur inside the cell. Increasing zinc oxide nanoparticle concentration and the smaller the particle size increases zone of inhibition. The values of zone of inhibition obtained from the assay are also presented in Table 2.

**Table 2.** Zone of inhibition (mm) of ZnO Nanoparticles against gram-positive and gram-negative bacterial strains.

| Samples | Conc. (mg/µL) | Zone of inhibition(mm)(Diameter) | | | |
|---|---|---|---|---|---|
| | | Gram-positive bacteria | | Gram-negative bacteria | |
| | | *S. aureus* | *E. feacalis* | *E. coli* | *K .pneumonia* |
| 1:1 UZnO | 1:2 | 14 | 10 | 9 | 12 |
| | 1:4 | 12 | 8 | 12 | 11 |
| | 1:8 | 12 | 8 | 9 | 10 |
| 1:1 ZnO | 1:2 | 8 | 6 | 6 | 6 |
| | 1:4 | 8 | 6 | 8 | 6 |
| | 1:8 | 9 | 6 | 6 | 6 |
| 3:2 ZnO | 1:2 | 8 | 6 | 6 | 6 |
| | 1:4 | 8 | 6 | 6 | 6 |
| | 1:8 | 8 | 6 | 6 | 8 |
| 9:1 ZnO | 1:2 | 6 | 6 | 8 | 8 |
| | 1:4 | 7 | 6 | 6 | 6 |
| | 1:8 | 8 | 6 | 8 | 6 |
| DMSO ($N_C$) | | **6** | **6** | **6** | **6** |
| Carbenicilin ($P_C$) | | - | - | **6** | **8** |
| Vacomycin ($P_C$) | | **14** | **10** | - | - |
| ZnO NPs & antibiotic | | 16 | 14 | 13 | 10 |

Antibacterial activity results showed that green synthesized ZnO nanoparticle using *Lippia adoensis* leaf extract has exhibited strong antibacterial activity against both gram-positive (*S. aureus, Enterococcus feacalis)* and gram-negative (*Escherichia coli, Klebsiella pneumonia)* bacteria strains. As shown in Figure 15, the uncalcinated ZnO nanoparticles performed better antibacterial activity on gram-positive than the gram-negative bacteria. Previous research report

also indicated antibacterial activity of ZnO nanoparticles were greater on gram-positive than gram-negative bacteria (Tayel *et al*., 2011). The reason for the difference in sensitivity between gram-positive and gram-negative bacteria might be attributed to the differences in morphological constitutions between these microorganisms. Gram-negative bacteria have an outer lipopolysacharide membrane and this makes the cell wall impermeable to antibacterial chemical substances. Gram-positive bacteria on the other hand, are more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier. Therefore, the cell walls of gram-negative bacteria are more complex in lay out than the gram-positive ones acting as a diffusion barrier and making them less susceptible to the antibacterial agents (Alonso *et al*., 2000).

In comparison with the standard drug, Vacomycin and Carbenicilin, strong bactericidal potential is exhibited by1:1 UZnO and 1:1 ZnO against both gram-positive (*S. aureus* and *Enterococcus feacalis)* and gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) bacterial strains. This is due to the fact that; the larger surface area to volume ratio of grain size with smaller particles size, the more will be its antibacterial potential (Nair *et al*., 2011). Furthermore, due to alcohols found in *Lippia adoensis* leaf extract present in uncalcinated ZnO NPs which are efficient in cell walls degradation and cause polyphenols to be released from cells (Gemechu *et al*., 2015) showing synergetic effect. ZnO nanoparticles synthesized using *Lippia adoensis* leaf extract were better against *Klebsiella pneumonia* compared to standard antibiotics which were used as positive controls (Carbenicilin).

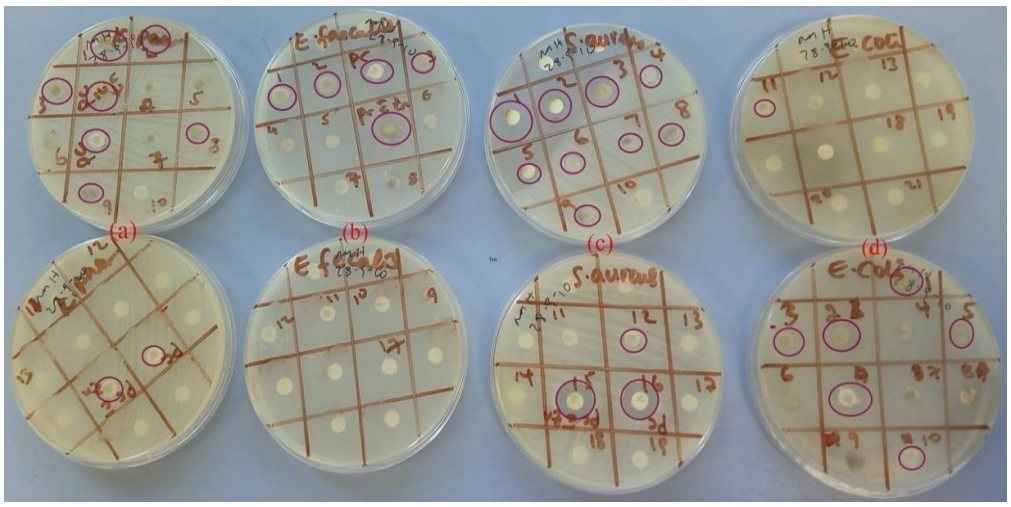



**Figure 15.** Zone of inhibition (mm) of ZnO Nps against: (a), *Klebsiella pneumonia* (b) *Enterococcus feacalis* (c) *Staphylococcus aureus* and (d) *Escherichia coli.*

Generally, antibacterial activities of ZnO NPs depend on their morphology, particle size, powder concentration, specific surface area, etc. Previous literature implies that the green synthesized ZnO NPs reported by (Sangeetha *et al*., 2012) showed more biocidal activity against various microorganisms when compared to chemically synthesized ZnO NPs.

# 5. CONCLUSIONS

It is known that the green synthesis of ZnO nanoparticles is much safer and environmentally friendly as compared to chemical synthesis. In response to this assumption, this study demonstrated the green synthesis of ZnO nanoparticles using *Lippia adoensis* leaf extract. The synthesized product was characterized and confirmed to be ZnO NPs using UV–Vis spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and Energy dispersive X-ray analysis (EDX). As confirmed from XRD, the size of ZnO nanoparticle was found to be the in ranges of 9 – 26.7nm which was obtained by varying reactant ratio by volume.

The uncalcinated ZnO nanoparticles performed better antibacterial activity against both gram-positive and gram-negative bacteria. Hence, the smaller size of ZnO nanoparticles facilitated easy entry into the bacterial cell membrane and enabled effective inhibition of the bacterial strains. Generally, the result in this study indicates that ZnO nanoparticles are both effective against gram-positive (*S. aureus*, *Enterococcus feacalis)* and gram-negative (*Escherichia coli*, *Klebsiella pneumonia)* suggesting that strong action of green synthesized ZnO nanoparticle against biological systems.

# 6. RECOMMENDATIONS

Despite the environmental advantages of using green chemistry based biological synthesis over traditional methods as discussed in this study there are some unresolved issues such as particle size, shape consistency and understanding of the mechanisms involved in producing metallic nanoparticles using biological entities. In the case of plant extracts, nanoparticle formation mechanisms vary between different plants species. Therefore, there is a need for more studies to evaluate and understand the actual plant dependent mechanisms. This is a clearly unexplored field and requires much more research investment to fully utilize green synthesis of metallic nanoparticles using biological entities.

In particular, ZnO reduces the bacteria viability; however, the exact mechanism of its antibacterial activity has not been well understood so far. Further investigations are essential in order to improve the antibacterial activity mechanism of ZnO nanoparticles.

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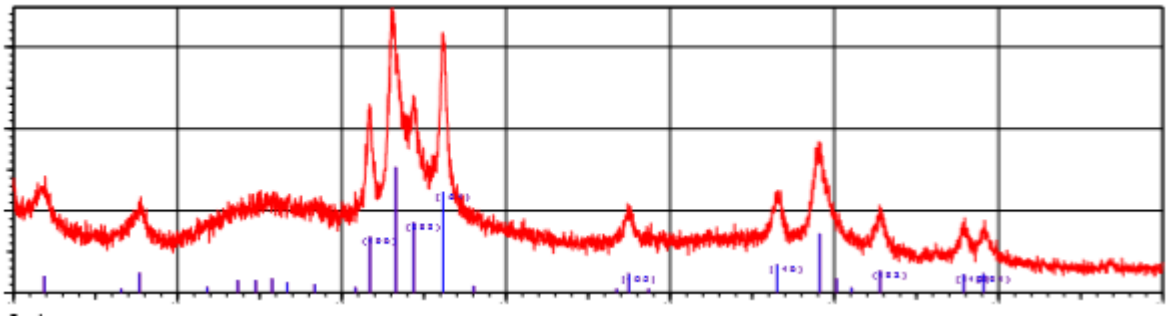
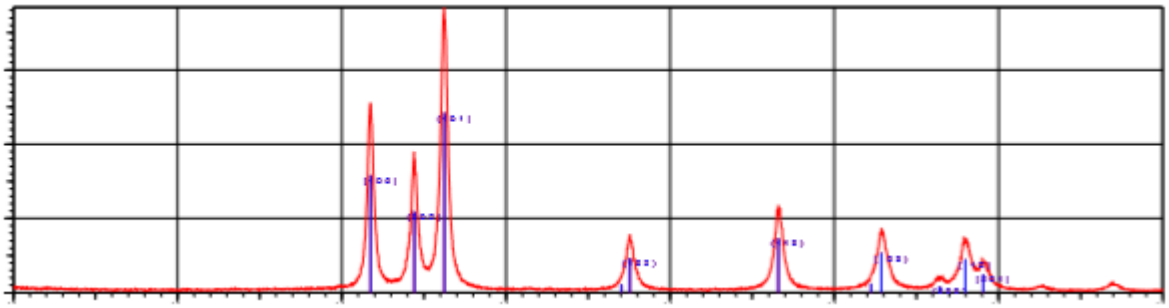
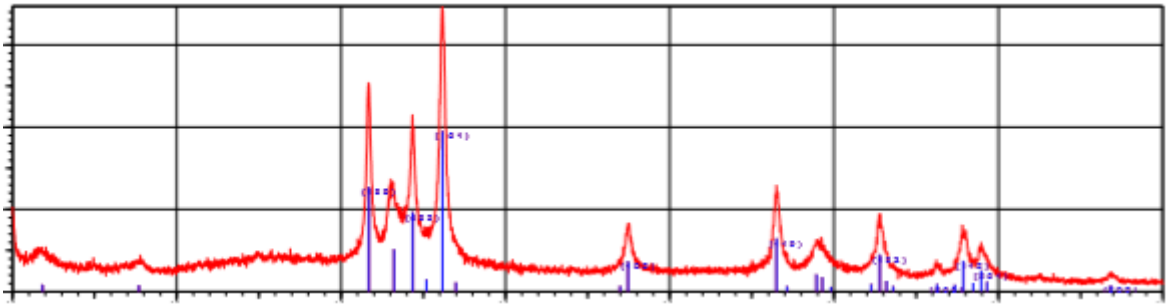
# APPENDIX



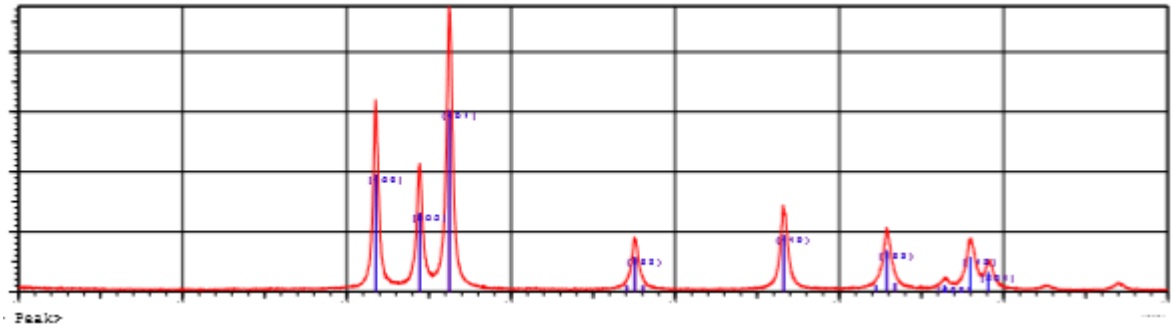
Appendix 1: XRD pattern for 1:1 Uncalcinated ZnO Nanoparticles.



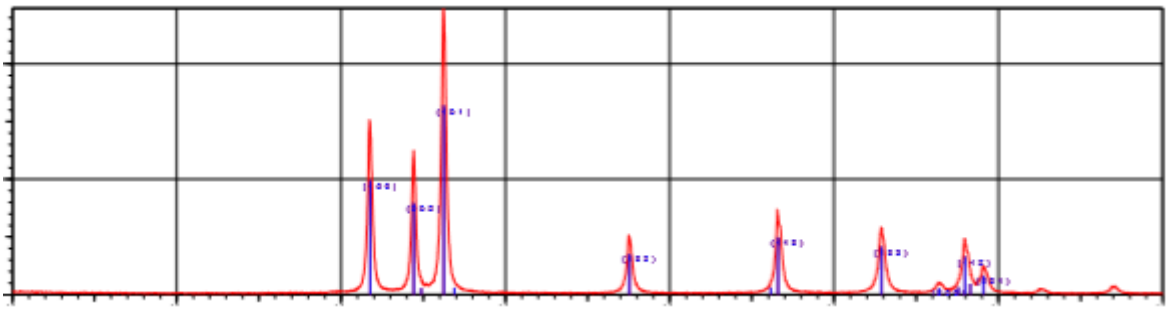
Appendix 2: XRD pattern for 1:1 ZnO Nanoparticles.



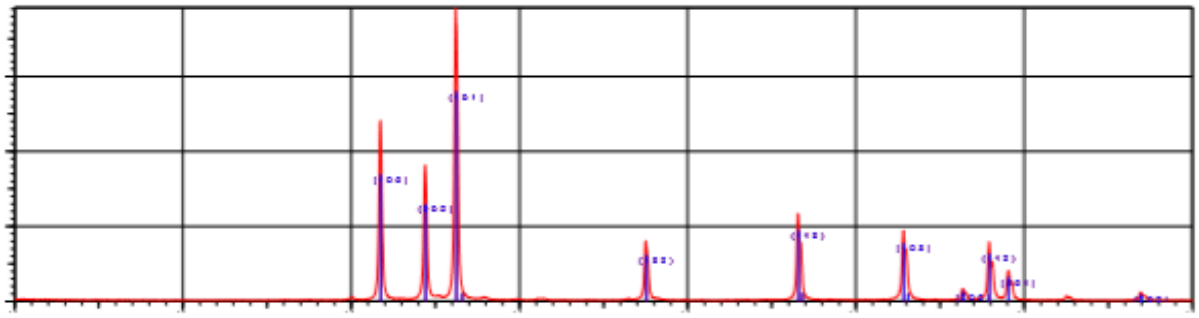
Appendix 3: XRD pattern for 3:2 Uncalcinated ZnO Nanoparticles.




Appendix 4: XRD pattern for 3:2 ZnO Nanoparticles.



Appendix 5: XRD pattern for 9:1 ZnO Nanoparticles calcinated at 400 $^{0}$C.



Appendix 6: XRD pattern for 9:1 ZnO Nanoparticles calcinated at 450 $^{0}$C.