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Synthesis and Evaluation of ZnO Nanoparticles of *Trigonella foenum* for their Biological Activities

by

Huma Aslam

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Dedicated to Allah Almighty, Hazrat Muhammad (SAW) and to my father (Muhammad Aslam), who taught me that the best kind of knowledge to have is that which is learned for its own sake.It is also dedicated to my mother (Naila Aslam), who taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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Abstract

In the current research work, Zinc oxide nanoparticles are synthesized by using seed extract of *Trigonella foenum-graceum*. Its common name is fenugreek that is a natural herb and used as spices in our food. It is also used in different applications like medical field, pharmaceuticals and others. Plant seeds eradication also plays a very significant role in the production of ZnO nanoparticles. Within permanent ratio, Zinc acetate salts and *Trigonella foenum* seeds extract were added. Synthesis of ZnO nanoparticles confirmed when its color change. Nanoparticles can be formed by using different approaches that are chemical, physical and biological. Chemical method produce toxicity so to escape from this toxicity we have chosen the green or biological approach. Biosynthesized ZnO nanoparticles were resistant, ecological in environment and more easy to produce. The biosynthesized nanoparticles were characterized by performing UV-Vis spectrophotometer, SEM, EDX and XRD. UV-Vis spectrophotometer showed peak within 360nm to 380nm. SEM showed size of 30nm and triangular shaped. To check the therapeutic effectiveness of ZnO nanoparticles many other biotechniques were performed such as anti-fungal, anti-bacterial, anti-oxidant and cytotoxicity. Biologically synthesized nanoparticles of Trigonella foenum seeds extract revealed significant anti-bacterial anti-fungal, antioxidant and cytotoxic potential. In many biomedical applications these biosynthesized ZnO nanoparticles can used.

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Abbreviations

DPPH	(1,1-diphenyl-2-picrylhydrazyl)
SEM	Scanning Electron Spectroscopy
T. foenum	*Trigonella foenum*
UV-Vis	Ultra Violet Visible Spectroscopy
XRD	X-ray Diffraction
ZnO NPs	Zinc Oxide Nanoparticles

Chapter 1

Introduction

Nanotechnology is a vast field incorporating itself in other fields like physics, chemistry, pharmaceutical sciences, material sciences, medicine and agriculture thus it is interdisciplinary. Its phenomenal results in other fields have let it to open its vast scope in agricultural field as well. According to the Directorate-General for internal policies of the European Union; precision agriculture is a farm management approach in which inter and intra-field effects on crops are measured to garner maximum productivity from available resources and to ensure sustainability, aptness and protection to the environment. Broadly nanotechnology is used in the latest agriculture to materialize the concept of precision agriculture. Nanotechnology incorporates the particles on nanoscale having diameter of 100nm [8]. Due to the larger surface area and little size of nanoparticles and distinctive optical properties they have vast applications in plant protection, nourishment and farm practices [25].

The particulate diffusions or solid particles in size range of 10-1000nm are regarded as nanoparticles. To a nanoparticle matrix a drug is solvated, ensnared or cohered. It depends on method of preparation to obtain nanoparticles, Nano spheres or Nano capsules. Nano capsules are the colloidal nano bubbles in which the drug is encased to a cavity circled by a unique non-toxic polymer membrane, while Nano spheres are matrix systems of spherical shape in which physical and

even distribution is ensured. Because of the potential of circulation for an elongated time period, targets a specific organ, as carriers of DNA in the gene therapy technique, and the potential to bear proteins, genes and peptides, the biodegradable polymeric nanoparticles, significantly those coated with hydrophilic polymer such as poly (ethylene glycol) (PEG) known as long-circulating particles, are used as potential drug delivery devices, over the recent years [46]. The vast diversity of applications of Nano sized metal oxide particles have dwelled them in significant consideration. In toothpaste, beauty products, sunscreens, and textiles, Titanium dioxide (TiO2) and zinc oxide (ZnO) nanoparticles (NPs) are incorporated. Ceramic Nano-powders of metal oxides such as ZnO manifest noticeable antibacterial activity [55]. The use of metal oxides has an advantage over organic antimicrobial agents due their enhanced safety and stability [5].

To manufacture metal nanoparticles of preferred characteristics various physical and chemical processes are presently extensively used. Nevertheless, these production methods are typically cost inefficient, laborious, arduous and are significantly hazardous to the environment and living organisms. Therefore, there is a dire need for an alternative, cost-efficient as well as environmental-friendly and sound nanoparticle production method. Over the past decade, it has been noticed that many biological systems such as plants and algae can transmute inorganic metal ions into metal nanoparticles by reduction capacities of their proteins and metabolites. It is notable that nanoparticle production using plants described in present review exhibits significant advantage over biological systems.

Plants are attractive platform for nanoparticle synthesis because of their low-cost cultivation, short production time, safety and the ability to produce increased volumes [61]. Nanoparticles have been produced physically and chemically over the past years but contemporary developments depict the significant role of microorganisms and biological systems in metal nanoparticles synthesis. Due to growing success and ease of formation of nanoparticles, the use of organisms in this area is rapidly developing. Furthermore, metal nanoparticles biosynthesis is an environmental-friendly method (green chemistry) without incorporating hazardous, toxic and expensive chemicals [34]. With acute morphology, microbes

produce inorganic materials either intra or extracellularly often in nanoscale dimensions. Due to their chemical detoxification as well as due to energy-dependent ion influx from the cell by membrane proteins that function either as ATPase or as chemiosmotic cation or proton anti-transporters microbes resist toxic heavy metals. Solubility has major role in microbial resistance [16][14].

Consequently, microbial systems are effective in detoxifying the metal ions by either reduction or precipitation of soluble toxic inorganic ions to insoluble non-toxic metal nanoclusters. Microbial detoxification can be made either by extracellular bio mineralization, bio sorption, complexation or precipitation or intracellular bioaccumulation. Extracellular metal nanoparticles production has vast commercial applications in many fields. As the polydispersity is of main consideration, it is essential to optimize the conditions for mono-dispersity in a biological process. The accumulated particles are of certain dimension and with less polydispersity, in case of intracellular production.

Fungi are more significant and advantageous over other microorganisms in several ways. In comparison to plant materials and bacteria, fungal mycelial mesh can tolerate flow pressure and agitation and other conditions in bioreactors or other chambers compared to plant materials and bacteria. These are assiduous to grow, easy to handle and easy to manufacture. In downstream processing, the extracellular secretions of reductive proteins are more and can be easily handled. Moreover, the nanoparticles precipitated outside the cell are without unnecessary cellular components, thus can be directly used in various applications.

Fenugreek (*Trigonella foenum-graecum L. Leguminosae*) is one of the oldest medicinal plants, springing in India and Northern Africa. An annual plant, fenugreek stands an average height of two feet. To prepare extracts or powders for medicinal use, the leaves and seeds, which mature in long pods, are used.

Spices are natural food supplements that are used for thousands of years to intensify the flavor and sensory quality of foods. Distinct flavor, aroma, zest and color to foods are imparted by spices. Fenugreek spice also changes the consistency of food. Fenugreek (*Trigonella foenum-graecum*) is a leguminous herb cultured

in India and North African countries. It is the member of family Fabaceae and has many names in different languages i.e., Fenugrec in French, Methi in Hindi, Bockshorklee in German, Fieno greco in Italian, Pazhitnik in Russian, Alholva in Spanish, Koroha in Japanese, Hulba in Arabic, Halba in Malayan and K'u-Tou in Chinese. The seeds and leaves of fenugreek have widespread uses, like seeds are used as spices globally and leaves are used in various dishes in diet. Fenugreek seeds are pungent to taste and are known for a long time for their medicinal qualities. The history of use of Fenugreek seeds is traced back 2500 years ago. Its major producer is India and its main consumer for culinary and medicinal uses. It has vast use in cookery like dressing, additive and large quantities in soaps, curries, sauces, vegetable dishes and pancakes. It is effective against anorexia, and is a gastric stimulant, in the aboriginal system of medicine in India [24].

1.1 Problem Statement

Nanoparticles have been successful with enhanced therapeutic effects and reduced side effects. So in that context, we necessitate ourselves to prepare new agents or drugs that are much more effective and long-lasting from natural resources with much less adverse effect. For that purpose, we are going to synthesize Zinc nanoparticles using Fenugreek (*Trigonella foenum-graecum*) seed extract to prepare authentic agents for therapeutic usage.

1.1.1 Aims and Objectives

Nanotechnology has the potential to be used in many processes and products. The nanoparticles synthesis is one of the important areas of modern bionanotechnology. The present study is aimed to identify the synthesis, characterization, antioxidant, cytotoxic and antimicrobial activities of zinc oxide nanoparticles using Fenugreek (*Trigonella foenum-graecum*) extract. The main purpose of this study is to generate stabilized nanoparticles with much less effort and economic value.

1.1.2 Objectives

This study entails the following objectives:

1. Synthesis of ZnO nanoparticles by using extract of *Trigonella foenum-graecum.*
2. Physical characterization of nanoparticles by UV-vis, XRD, SEM, FTIR.
3. To determine the therapeutic potential of synthesized nanoparticles by antimicrobial, antioxidant and cytotoxic assays.

1.2 Scope

Advancements in ZnO nanotechnology helps us to design and synthesize Zn Nanoparticles. Because of the nontoxic nature of biosynthesis this is the best method to synthesis ZnO nanoparticles. The peculiar properties of ZnO nanoparticles like optical, physical, and antimicrobial ZnO NPs wide spread usage in medical and different sectors like would healing of wounds, food industry as sanitation, drug delivery etc. Investigations regarding the preparation of ZnO NPs aims at designing effective drug delivery agent, diagnosing and treating fatal diseases besides ensuring higher safety and efficacy.

Chapter 2

Literature Review

2.1 ZnO Nanoparticles

ZnO is considered as a useful, tactical, favorable and adaptable inorganic material with vast applications. IIVI semiconductor is its other name [59]. Zinc and oxygen are classified as group two and six elements of periodic table. Distinctive optical, chemical sensing, semiconducting, electric conductivity, and piezoelectric properties are possessed by ZnO [42]. Nanoparticles of Zinc oxide (ZnO NPs), poses unique physical and chemical properties thats why they are used in many fields and thus are most important ones [75, 89]. While ZnO NPs supply water proof to the rubber composite, so these being the most important ones are firstly employed in rubber industry. By providing toughness, intensity and antiaging it also enhances performance of high polymer [44, 78].

ZnO high and strong UV absorption properties has make its use in personal care and beauty products like cosmetics and sunscreens [60]. Moreover, outstanding antibacterial, antimicrobial and excellent UV blocking properties are also possessed by ZnO NPs. The stunning functions of UV and visible light resistance, antibacterial and deodorant properties in finished fabric in textile industry is regarded to ZnO NPs [31]. Nano-ZnO because of its small size easily gets absorbed by body thus ZnO is added in various food products. In contrast to other metal oxide,

ZnO is low-cost, biocombinatoric, biocompatible, less hazardous and less toxic. ZnO is the class of the inorganic metal oxide present in a wide range of nanostructures. These metal oxides are characterized on the basis of photolytic and photo oxidizing ability in correspondence to chemical and biological species [93]. Different analytical techniques are used to identify the synthesized products like ultraviolet visible spectroscopy, X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) e.t.c [28][81].

2.2 Synthesis of ZnO Nanoparticles

Before the advancement of the nanoscience, difficult time was confronted in synthesis of nanomaterials with simple, cost efficient and highly productive method. The techniques for the synthesis of nanoparticles are divided in three processes i.e, solid-phase, liquid phase, gas phase. Mechanical ball milling and mechanochemical techniques are included in solid-phase processes, laser ablation, exploding wire, solution reduction, and decomposition process are included in liquid-phase processes and gas evaporation, exploding wire, laser ablation process and green synthesis comprises gas-phase processes. ZnO NPs can also be synthesized by chemical, physical and biological means.

2.2.1 Physical Method

The physical techniques by which ZnO NPs can be synthesized are vapor deposition, plasma and ultrasonic irradiation [38]. However, mighty equipment and excessive amount of energy is required in these techniques thus these techniques are considered to be costly and are not used massively in production. The physical methods uses vaporization and their deposition, plasma production and the use of ultrasonics. The physical methods utilizes the physical means of developing the ZnO nanoparticles.

2.2.1.1 Laser Ablation Method

Laser ablation Pulsed laser ablative deposition (PLD) is an obvious synthetic method because it synthesizes nanoparticles with a narrow size distribution and impurities of low level. There are three main steps in nanoparticles synthesis by laser ablation synthesis and nanoparticle formation by an objective immersed in liquid. It was found by Yousitake Masuda and colleagues in their work that in liquid phase the morphology of ZnO crystals can be controlled by simple aqueous solution method. Nanowires of ZnO were outstandingly synthesized at temperature of 50 °C having length and width of 50m and 100 nm respectively. There were no branches and aggregations of the synthesized nanowires [63].

2.2.1.2 Vapor Transport Method

Vapor transport method is the most typical method of synthesis of ZnO nanoparticles. On the basis of nano structure formation mechanism, it can be singed either by catalyst free vapor-solid (VS) process and catalyst-assisted vapor-liquid-solid (VLS) process. VS process has natural ability to produce a wide range of nanostructures like nanowire, nanorods, nanobelts etc. Kong et al and coworkers found that ZnO such as nanohelixes and nanobelts nanostructures synthesized by complex process of VS has a belt shape with width and thickness of 10-60nm and 5-20nm respectively and length of several hundreds of micrometers [45]. Excited Zinc, Oxygen and Oxygen vapors produced in this complex process react with each other and form ZnO nanoparticles. Zn and Oxygen vapors generation is done in many ways. Although, ZnO decomposition is restricted to high temperature though its decomposition is simple and direct [10]. Nanostructures are synthesized directly by condensing from vapor phase in VS process.

2.2.2 Chemical Method

Precipitation, microemulsion, chemical reduction, sol-gel and hydrothermal techniques are incorporated in chemical methods. In high pressure or temperature

conditions they may require high energy consumption [15]. Among the typical chemical methods the most important one is sol-gel synthesis which was developed by Spanhel and Anderson(1991), in concern to regulate the pH of solution and to keep away from precipitation of Zn(OH)2 it uses a zinc precursor salt and a chemical reagent. The solution is exposed to 1000 C temperature after that to obtain the ZnO NP [12]. To control size of nanoparticles and avoid particle agglomeration chemical stabilizers like citrates or polymers such as polyethylene glycols, polyvinyl pyrrolidone and amphiphilic block copolymers are added in ZnO NPs synthesis process [47]. Moreover, in chemical synthesis the notable factor is the concentration of chemicals used which will influence the size and shape of particles greatly using different concentrations and ratios of chemicals it is feasible to obtain particles ranging from nanometers (5-10nm) to micrometer size using the alike process [58].

2.2.2.1 Sol Gel Method

The sol-gel synthesis method of nanoparticles was devised to make inorganic compound with the help of chemical reaction of a specific solution. Sol-gel method is advantageous because it generates a sound amount of thermal stability, high mechanical stability, fair solution resistance, and likelihood to agitate transformation. For synthesis of ZnO NPs Agustinaa et al. used sol-gel and calcination methods effectively. Nano ZnO produced in these circumstances can be identified on the basis of framework and particle size. The result showed that prime conditions for nano Zinc oxide synthesis were realized at ultrasonic time of 60 minutes and pH of 10. The synthesized nano zinc oxide crystal has a homogeneous morphology having size of 45.35 nm. The zinc concentration in nano zinc oxide is 87.31% while having size about 50 nm. The ZnO crystals which are produced by this methods are morphologically homogeneous and have approx size of 45.53 nm. For this production the ultrasonic time of the production in 60 minutes and the pH is about 10 [63]. This method is used for the production of inorganic compounds by the help of chemical process.

2.2.2.2 Hydrothermal Method

In hydrothermal method, the use of organic solvents or additional processing of the product (grinding and calcination) thus it is easy, straightforward and eco-friendly technique. In autoclave this process of synthesis occurs, the mixture of substrates is heated gradually with a range of temperature of 100300 °C and was left spare over a few days. Crystal nuclei are formed by heating sequentially followed by cooling, then they grow. This process has many advantages, counting the prospect of carrying out the synthesis at low temperatures, this process is advantageous because it put through the synthesis at low temperatures, it depends on starting mixture, temperature and pressure of the mixture that will result in different distinct shapes and dimensions of the crystal. Using Zinc nitrate as a precursor Nehal A.Salahuddin and coworkers prepared zinc oxide nanotubes by hydrothermal synthesis. The length of 2.4 m and average outer diameter of 200nm of the ZnO nanotubes was observed [63].

2.2.2.3 Precipitation Method

To obtain zinc oxide, controlled precipitation is commonly used, typically this is done to gain outcome with repeatable properties. Using a reducing agent, solution of zinc salt is reduced spontaneously and rapidly it is to limit the growth of particles with certain dimensions which is followed by precipitation of a precursor of ZnO from the solution. The precursor goes through thermal treatment, then grinding is done to carry off impurities. The calcined powders have a high level of agglomerates of particles so it is difficult to bream them down. Precipitation process is dependent on parameters such as pH, temperature and time of precipitation process. From aqueous solutions of zinc chloride, zinc sulphate and Zinc acetate, the solution of Zinc oxide is also precipitated.In this process controlling factor are the concentration of the reagents, the rate of addition of substrates and the reaction temperature ZnO NPs of average size of 30nm were obtained by William-Hal method and was used by Sadraei et al [76].

2.2.3 Biological Method

Over the past decade the use of biological methods for synthesizing ZnO NPs are in particular consideration. The biological synthesis is cost-efficient and ecofriendly because of absence of toxic chemicals or high energy applied thats why the development of this new approach is significant and of main interest [40]. It has been reported in literature that in contrast to conventional chemical or physical methods used in present that synthesis of metallic and metal oxides nanoparticles biologically is more environmental or eco-friendly [43]. Accordingly, these biological synthetic methods are commonly known as green synthesis.

2.2.3.1 Synthesis of ZnO Nanoparticles by Bacteria

By utilizing microbial culture or biomass the biosynthesis of metal or metal oxides nanoparticles can occur in extra or intracellular environment [62]. It has been revealed by studies that enzymes and proteins produced and released by microorganisms can reduce metal ions and stabilize the particles in case of extracellular synthesis. The enzyme secretions produced by bacteria cell (Bacillus licheniformis can stabilize ZnO nanoparticles). $Zn(OH)_2$ is produced by reaction of zinc acetate and sodium bicarbonate which is then degraded thermally to form ZnO nuclei. To avoid agglomeration and particle growth assurance of the nanoscale size of the metal oxide, the ZnO NPs are stabilized by enzymes produced by bacteria.

Moreover, it is identified that for formation of ZnO NPs enzymes produced by microorganisms are responsible. However, authors state that the solution pH and the electro kinetic potential of the bacteria may play a role in the synthesis route by reducing the metal ions and consequently triggering the biosynthesis of the nanoparticles rather than forming $Zn(OH)_2$ Instead of forming Zn(OH)2, by activating biosynthesis of nanoparticles. In an identical study, extracellular biosynthesis of ZnO NPs by Staphylococcus aurens thus same approach was stated [83]. The efficient utilization of activated ammonia from ureolytic bacteria (Serratia ureilytica) for ZnO NPs synthesis has been reported in additional work.

It is stated in the study that the synthesis route for nanoparticles is based on reaction of zinc (II) ions with culture media profuse with microorganism, profuse in ammonia producing $Zn(OH)_2$ and $[Zn(NH3)4]_2$. These substances are then subjected to thermal decomposition at temperature of 50 °C to gain the crystalline ZnO NPs powder [73].

Concerning the intracellular synthesis, the mechanism of synthesis of nanoparticles is quite exigent because of complication of cell compositions and processes. Nevertheless, many studies surmise that the cells incorporate the metallic ions which will be reduced by the proteins and enzymes within the cell to form the nanoparticles [35].

2.2.3.2 Synthesis of ZnO Nanoparticles by Fungi

The formation of metal and metal oxide nanoparticles by fungal biomass or culture has alike synthetic route as the green synthesis in which biosynthesis of ZnO NPs is done by bacteria but in fungal biomass, Aspergillus fumigatus cell culture is used to synthesize ZnO NPs [39]. It is suggested in the studies that proteins and enzymes secreted by Aspergillus fumigatus is mainly responsible for synthesis and encapsulation of nanoparticles.

The fungus has excellent potential for secretion of higher concentration of metabolites to the media culture as compared to the bacterial cells thus fungus is superior to green synthesis. Moreover, fungus cells are more resistant to process conditions, subtilities and variations such as pressure, flow rate and stirring which intensify their potential use for large scale synthesis [105].

2.2.3.3 Synthesis of ZnO Nanoparticles by Algae

Since algae is a simple organism yet the phytochemical composition of algae is identical to plant extract. In various species of algae many active compounds possessing functional groups like hydroxyl and carboxyl groups are found and their antioxidant activity can also be figured [9]. By Fourier Transform Infrared Analysis

(FTIR), the presence of such active compounds in algae extract is validated which utilize them as substrate to green productionof ZnO NPs. Consequently, the biosynthesis mechanism of ZnO NPs by algae substrates is identical to mechanism of plants in which reducing and stabilizer agents or chelating substances are active compounds like polyphenols, flavonoids.

2.2.3.4 Synthesis of ZnO Nanoparticles by Plants

Due to unique phytochemical production of plant parts such as leaf, stem, root, fruit and seed they are used for ZnO NPs synthesis. It is ecofriendly, sustainable and cost efficient to use natural extracts of plant parts and it does not involve incorporation of any intermediate base groups.

It is time efficient, does not involve arduous machinery or equipment and gives highly pure product and quantity enrich impurity free product [32]. Due to wide ranging production and stable, diverse in shape and size NPs plants are much favored source of NPs production [67]. Bioreduction is the phenomena in which the phytochemicals secreted by plants like polysaccrides, polyphenolic compounds, vitamins, amino acids, alkaloids, terpenoids reduce metal oxides or metal ions to 0 valence metal nanoparticles [32, 67].

The preparation of ZnO NPs from leaves or flowers is the simplest and most typical method in application in which plant part is washed entirely in running tap water and sterilized by double distilled water (use of tween 20 to sterilize is also common). The plant part is dried at room temperature, which is succeeded by weighing and then mashing it with the help of mortar and pestle. Then Milli-Q water is added to plant part in accordance to the required concentration and then mixture is brought to boil by continuous stirring with magnetic stirrer [71]. Then the obtained solution is filtered by Whatman filter paper clear solution of plant extract (sample) was acquired by filtering the obtained solution by Whatman filter paper. 0.5 mm of hydrated Zinc nitrate or zinc oxide or zinc sulfate was added to certain volume of sample and then the mixture was boiled at required temperature and time to have efficient mixing [71]. Optimization is also performed

preferentially by various temperature, pH, concentration of sample and time at this stage of the process. The indication of synthesis of NPs is visual confirmation by variation of color of mixture in incubation period. The corroboration of NPs synthesized is done by UV-Vis spectrometry after then centrifugation of mixture is done succeeded by drying the pallet under hot air oven after which crystals of NPs will be attained [101].

Synthesized nanoparticles are further validated using following techniques: Energy Dispersion Analysis of X-ray (EDAX), Field Emission Scanning Electron Microscopy (FESEM), Fourier Transform Infrared Spectroscopy (FTIR), UV-Visible Diffuse Reflectance Spectroscopy (UV-DRS), Atomic Force Microscopy (AFM), X-ray diffractometer (XRD), Thermal-gravimetric Differential Thermal Analysis (TG-DTA), Photoluminescence Analysis (PL), Raman Spectroscopy, Attenuated total reflection (ATR), X-ray Photoelectron Microscopy (XPS, and Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) [7].

2.3 Need for Green Synthesis

The classical methods of synthesis of nanoparticles were cost-inefficient and demanded use of toxic chemical compounds/organic solvents as reducing agent, thats why green synthesis method for nanoparticles has become topic of concern in recent years [50]. Green synthesis is also eco-friendly and it alleviate pollution risk at begetter or source scale in addition to it does not produce waste to there is no need to scavenge waste after the synthesis.

The prime importance in green synthesis is given to the reagents which are eco and environmental-friendly. Biogenic technique of synthesis has an advantage over chemical and physical method because it is eco-friendly and non-hazardous even though physical and chemical methods are speedy and easy for nanoparticle synthesis [6, 74]. During synthesis of nanoparticles using biological organisms in

green synthesis, new sources of dynamic materials that are stable, nontoxic, non-hazardous, cheap, eco and environmental-friendly are produced. The properties of nanomaterials are intensified because of gained small size, shape and certain properties of biological substrate used in the green synthesis of nanoparticles.

2.3.1 Genus *Trigonella*

Fenugreek (*Trigonella foenum-graecum L. Leguminosae*) is one of the oldest medicinal plants, springing in India and Northern areas of Pakistan. It is the member of family Fabaceae and has many names in other languages i.e, Fenugrec in French, Methi in Hindi, Bockshorklee in German, Fieno greco in Italian, Pazhitnik in Russian, Alholva in Spanish, Koroha in Japanese, Hulba in Arabic, Halba in Malayan and Ku-Tou in Chinese [26].

2.3.2 *Trigonella foenum-graecum*

The seeds of *Trigonella foenum* are used as spices (flavor enhancer) globally whereas its verdant vegetables are used in edibles. The seeds of Fenugreek have bitter and pungent taste and are long acknowledged for its medicinal attributes.

The history of use of Fenugreek seeds is traced back 2500 years ago. Its major producer is India and its main consumer for culinary and medicinal uses. It has vast use in cookery like dressing, additive and large quantities in soaps, curries, sauces, vegetable dishes and pancakes. It is effective against anorexia, and is a gastric stimulant to cure gastroesophageal reflux, in the aboriginal system of medicine in India and also very effected against the gastric juices and esophageal junction juices [24, 26].

Taxonomic classification of *Trigonella foenum-graecum* is given below:

Class : Magnoliopsida

Order : Fabales

Family : Fabaceae (Peas or Legumes)

Genus : *Trigonella L.* (fenugreek)

Specie : *Trigonella foenum-graecum L. (T. Foenum-graecum)*

-Sickle Fruit fenugreek

2.4 Application of ZnO Nanoparticles

Zinc oxide has diverse properties like physical and chemical. It is extensively used in different areas (Fig. 2.1).

ZnO plays a vital role in a very broad range of characteristics:

1. from pharmaceuticals to agriculture.
2. from tyres to ceramics.
3. from paints to chemicals.

2.4.1 Agricultural Use

To enhance the yield and growth of food crops Zinc oxide nanoparticles are used. Different concentrations of zinc oxide nanoparticles were treated with seeds to boost up following characteristics of food crop.

These characteristics are:

1. Seed germination
2. Seedling vigor
3. Plant growth
4. Seed proliferation
5. Effective in increasing stem and root growth in seeds [66].

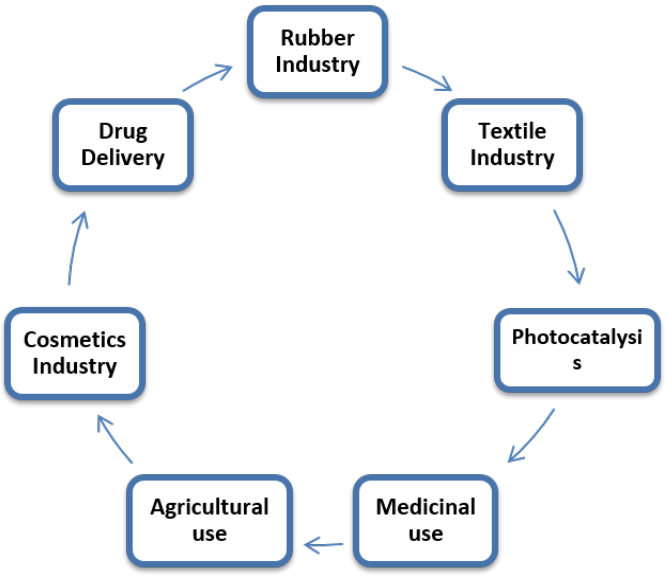

FIGURE 2.1: Applications of ZnO nanoparticles [44]

2.5 Characterization of ZnO Nanoparticles

2.5.1 UV.VIS Analysis of Extract

We can check the reduction of zinc oxide from the plant extract (*Trigonella Foenum*)by the optical density through the UV-Vis analysis. Only a small part of reaction mixture was taken and observed spectras that was set between 320nm to 440 nm after diluted the mixture with distilled water [17].

2.5.2 SEM Analysis of Extract

By using the SEM (Scanning Electron Microscope) we observed the morphology of the zinc oxide nanoparticles formed by the seed extract of fenugreek. Slides of SEM were prepared by coating of gold on the ZnO nanoparticles to particularized the conductivity. Then under the 20KV voltage sample was observed in SEM [2]. The SEM analysis enables to study the morphology, shape, outer surface structures and the diameter of the structure. The ZnO nanoparticles which are made were

scanned by the electron beem of SEM and then the SEM produces a picture on the screen which shows the morphological structures of the nanoparticles.

2.5.3 XRD Analysis of Extract

To figure out the size and phase variety of synthesized nanoparticles we used X-Ray Diffraction spectroscopy. We can find out the size by the size-strain plot method [104].

2.5.4 EDX Analysis of Extract

EDX analysis basically operated with XRD and is used to find out the chemical composition of the ZnO Nps in the mixture [18].

2.5.5 Bioassays

Under labortary condition, test are performed on living organisms and exposed to different concentration of toxic [18].

Types of Bioassays

TABLE 2.1: Types of Bioassays

Qualitative Assays	**Quantitative Assay**
These do not present any statistical analysis	These are analyzed by biostatistical methods. These are used to determine the efficiency of sample

Purpose of Bioassays

1. To check the specificity.

2. To measure the pharmacological activity.

3. To check the potency of drug.

4. To check the effectiveness of complex compound like B-12.

2.5.5.1 Antibacterial Assay

Anti-bacterial are used to control the growth of pathogenic bacteria. It resists the disease and kill bacteria. Many antibiotics are developed from the plants and other compounds to cure the disease. Bacterias are the infectious agents that cause toxicity.

In this Assay we used five pathogen strains. A disc diffused method was determined to check the bacterial activity [82].

Bacillus subtilis *Vibrio susbtilisearlier* is discovered in 1835 by the person named as Christian Gottfried Ehrenberg then after some time in 1872 its name was changed that was *Bacillus subtilis* by the scientist named as Ferdinand Cohn.

Morphology *Bacillus subtilis* is the gram positive specie and appearantly rod-shaped bacteria.

Occurrence This bacteria present on the surface of soil and in the gut area of mammals.

It helps to treat the gut tract infections. It is not a pathogenic bacteria. During cooking this bacteria can easily survive. It cause contamination in the laboratory. Bacteria settle on the plate. It cause many diseases like ovine abortion and bovine mastitis [95].

Micrococcus luteus *Micrococcus luteus* is gram negative and non-motile bacteria. It was discovered by Alexander Flemming in 1928 and firstly named as *Micrococcus lysodeikticus*. It is present in soil, water, dust and on the skin layer of human. It form yellow colonies. It cause infection due to no proper hand washing. It also found in laboratory and cause contamination [68].

Enterobacter aerogenes This bacteria is the gram negative, motile and rod shaped. It is present in different parts of human body like uniray tract, respiratoty tract and blood system [22].

Salmonella typhi *Salmonella typhi* is bacteria which mostly infect the blood and intestinal tract and it infect the humans. Infection caused by this bacteria is lethal for human health. It is gram-negative and rod-shaped bacteria.

Main causes included are:

1. Headache
2. Cough
3. High Fever

Mostly it spread by oral fecal route. Contaminated food or water can cause infection. These bacteria present in canned food. Its treatment is very difficult [51].

Staphylococcus aureus Sir Alexander Ogston developed *S.aureus* in 1880. It is gram positive bacteria. It cause skin infection and food poisoning. It cause serious infections in the body.

It can be transferred in many different ways:

1. Sneezing
2. Contact directly with contamination which may be a carrier.

A syndrome known as Toxic Shock Syndrom is due to S.aureus bacteria [11].

Agrobacterium tumefaciens *Agrobacterium tumefaciens* is a gram-negative, motile and rod shaped bacteria. It causes disease of crown gall. It is mostly used for plant breeding. It is nitrogen fixing bacteria and found in the soil. It is a plant pathogen that cause tumor and help in the plant breeding. It cause changing in different plants and this change is good for several plants like apple, pear, raspberry and rose [33].

2.5.5.2 Antifungal Assay

Antifungal assay is basically for the killing of microorganisms specifically fungi. Fungi is pathogenic and can cause many diseases. To detect the infections that are due to the fungus we use antifungal assay. Fungi can destroy fruits and vegetables as well as plants. Many plants are used and show antifungal activities. Poor diet and unhygiene environment become the cause of weak immunity and thus it can easily spread fungal disease. So we need antifungal drugs for this type of fungal dieseases. The agar tube dilution method used for the antifugal activity of synthesized ZnO NPs [57].

Aspergillus fumigates It is the specie of the fungus. It causes infection of skin, eyes and other organs. It cause several diseases that are dread full like it produce fungus ball in lungs. It exists as molds. It founds in soil. It produce conidia that are airborne spores. It play important role in carbon recycling. Its various species used at commercial level for the manufacturing of alcohol in industry. People who have weak immune system are at high risk for this infection [37].

Mucor indicus It is the important member of zygomycetes. It is dimorphic. It present in soil and in decayed fruits and vegetables etc. It used in the production of beers. The disease caused due to this fungi called zygomycosis [64].

Fusarium solani *Fusarium solani* is the specie of fungus and associated with Ascomycota divison. It mostly present in soil. It form colonies. It causes infection in plants as well as in human. In plants it cause several infection and symptom of the infection is wilting of plant. It also detoriate the roots. In human weak immune system can cause infection of skin. It used in the food industry [54].

Aspergillus niger *Aspergillus niger* is the specie of Aspergillus. It produce infection in various fruits like apricot and grapes etc. That are called black mould. It present in soil. It form black colonies. It also used at industrial level. *Aspergillus niger* produce many enzymes then these enzymes help in the food industries [27].

2.5.5.3 Antioxidant Assay

Antioxidant are mixture that prohibit the process of oxidation. To find out the antioxidant activity DPPH (2, 2 diphenyl-1-picrylhydrazyl) is used. This process is basically to examine the scavenging activity of the plant extract against the free radicals. As DPPH is very stable free radical and it donate hydrogen ion easily. The color of DPPH change if it shows some activity against the radicals. We can examine this activity through spectrophotometer. In this process methanol and ethanol can be used as solvent. Antioxidant activity of ZnO NPS was determined by means of the DPPH method [19].

2.5.5.4 Cytotoxicity Assay

Brine shrimp assay is the other name for the cytotoxic assay. In this assay we monitor the toxicity of synthesized nanoparticles.
The viability depend on:

1. The healthy shrimp cells in the sample.
2. Gene and protein that play major role in cell division.
3. Alarming condition when exposed with toxicity.

In plants toxicity that is produced may be due to the secondary metabolites which are present in it. This assay help to check the toxicity of drug screening. However this assay is effective as:

1. Rapid
2. Relible
3. Cost Effective

The cytotoxic assay of ZnO nanoparticles produced by the fenugreek can be determined by the brine shrimp toxicity assay [52].

Chapter 3

Methodology

3.1 Materials And Methods

The research was conducted in the well-equipped biological laboratory of the Department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad.

3.1.1 Materials

Materials that were used during research are listed below:

Table 3.1: Materials and Chemicals.

Materials	**Company Names**
Petri dishes	Sigma-Aldrich
Micropipette	Sigma-Aldrich
Test tubes	Sigma-Aldrich
Glass vials	Sigma-Aldrich
Aluminium foil	Sigma-Aldrich
Eppendorf tubes	Sigma-Aldrich
Micropipette tips	Sigma-Aldrich

Continuation of Table 4.1

Materials	**Company Name**
Glass vials	Sigma-Aldrich
Falcon tubes 50ml	Sigma-Aldrich
Cotton plugs	Sigma-Aldrich
Cotton swabs	Sigma-Aldrich
Test tube racks	Sigma-Aldrich
Forceps	Sigma-Aldrich
Beakers 100ml, 500ml	Sigma-Aldrich
Test tube racks	Sigma-Aldrich
Para film or masking tape	Sigma-Aldrich
Discs	Sigma-Aldrich

Chemicals

TABLE 3.2: Materials and Chemicals.

Chemicals	**Company Names**
Zinc acetate	Sigma-Aldrich
Luria broth	Sigma-Aldrich
Nutrient Agar	Sigma-Aldrich
Sea salt	Sigma-Aldrich
Brine shrimps egg	Sigma-Aldrich
Sabourad dextrose agar (SDA)	Sigma-Aldrich
Ascorbic acid	Sigma-Aldrich
Terbinafine	Sigma-Aldrich
Streptomycin	Sigma-Aldrich
DPPH reagent (2,2-diphenyl-1-picrylhydrazyl)	Sigma-Aldrich
Ethanol	Sigma-Aldrich

3.1.1.1 Microorganisms Used

Fungal Strains

1. *Mucor species*
2. *Flavius*
3. *Fumigatus*
4. *Niger*
5. *Fusarium solani*

Bacterial Strains

Gram Positive

1. *M. luteus*
2. *S. aureus*
3. *B. subtilis*

Gram Negative

1. Tumefaciens
2. S. Setubal
3. E. aerogenes

3.1.2 Methodology

Overview of Methodology:

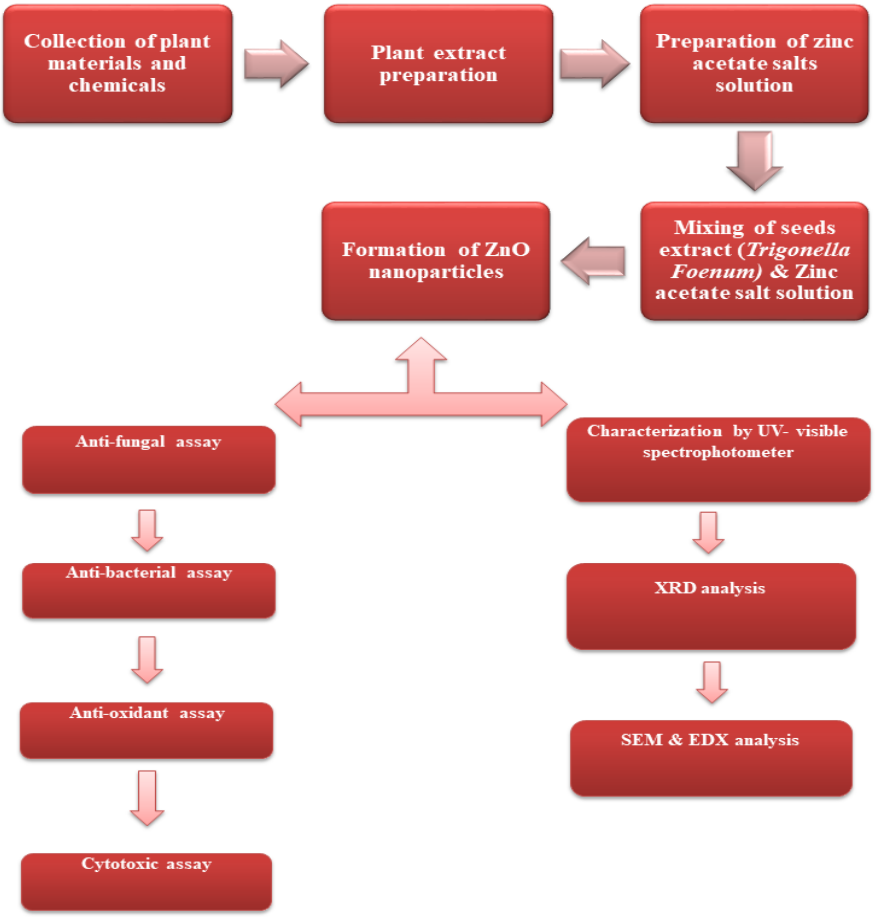

FIGURE 3.1: Overview of Methodology.

3.1.2.1 Preparation of Plant Extract

About 20 g of fresh leaves/seeds of fenugreek were taken and washed with distilled water. Then leaves were cut down into pieces and crushed with 100mL distilled water using mortar and pastel. Then boiling was done for 10 minutes. After that all contents were cooled for some time and then filtered using Whatmann No. 1 filter paper. The extracts thus obtained was used as reducing and stabilizing agents [4].

3.1.2.2 Synthesis of Zinc Oxide Nanoparticles

0.5M zinc acetate solution was prepared. 90 ml solution of zinc acetate was taken and 10 ml of fenugreek extract was added in the solution to make final volume 100ml. The solution was kept at room temperature for approximately 5 hours. After 5 hours the suspension was centrifuged at 3000 rpm for 20 minutes. Supernatant was discarded and pellet obtained was added in distilled water. This process was repeated several times about 2-3 times [72].

3.1.3 Characterization of Zinc Oxide Nanoparticles

3.1.3.1 UV-Vis Analysis

Sample preparation When our process of centrifugation completed than pallet was collected and superannuated present in test tube was expelled out. After this, distilled water was added in pallet. Stock solution formed. Then this solution was used for characterization purpose [90].

Experiment Method Stock solution was used to perform UV-Vis spectrophotometer analysis. Through these analysis, optical properties of zinc oxide nanoparticles were observed and identified. Firstly, water was taken as blank reference. Almost 4ml of biological synthesized ZnO NP suspension was taken and fit it into spectrophotometer. Light was produced in UV-Vis spectrophotometer and spectra was observed at 320nm 440nm [3].

3.1.3.2 SEM Analysis

Procedure Morphology of zinc oxide nanoparticles synthesized from fenugreek was determined by using Scanning Electron Microscope. SEM slides were prepared and thin layer of gold was coated on the smears to make them conductive. Already formed sample of ZnO NPs was observed to determine magnification power and resolution. Then the samples were monitored in SEM at voltage of 20 KV [30].

3.1.3.3 X-Ray Diffraction Spectroscopy

Procedure To examine the structure of zinc oxide nanoparticles, X-ray diffraction spectroscopy was used. In this technique, X-ray radiations were used. Through the cathode ray tube that is present in spectroscope, X-ray radiations were formed. The structure of zinc oxide nanoparticles was evaluated when these radiations were bombarded on the sample. Diffractometer was used to produce the spectrum [36].

3.1.3.4 Energy Dispersive X-Ray Spectroscopy

Procedure For chemical characterization (composition and proportion) of zinc oxide nanoparticles, energy dispersive X-ray spectroscopy was used. On the surface of zinc oxide nanoparticles beam of electrons collide (10-20KeV). After collision of beam of electrons on the surface of biosynthesized zinc oxide nanoparticles, image was formed [102].

3.1.4 Biological Evaluation of Synthesized Nanoparticles

3.1.4.1 Antibacterial Assay

Six strains were used for the identification of antibacterial activity of biosynthesized zinc oxide nanoparticles. Assay was performed by using disc diffusion method [13]. These six bacterial assays consists of gram positive and gram negative bacteria.

Three gram positive and three gram negative strains were used.

- **Gram Positive Strains**

1. *Bacillus subtilus*
2. *Salmonella aureus*
3. *Micrococcus letus*

- **Gram Negative Strains**

1. *Salmonella setubal*
2. *AT-10*
3. *Enterobacter aerogenes*

Sample Preparation After the process of centrifugation, to make the final 1000 ppm concentration, 25ml distilled water was added into 25mg pallet of biosynthesized zinc oxide nanoparticles by using Trigonella foenum. Different dilutions were made during this assay as follows:

1. 10 ppm
2. 20 ppm
3. 30 ppm
4. 40 ppm
5. 50 ppm
6. 100 ppm

Media Preparation To perform this method, petri plates were used. For culturing Luria broth was used:

Table 3.3: Chemicals and Concentrations.

Chemicals	**Concentrations**
NaCl	5g/ 500ml
Bacto-tryptone	5.5g / 500ml
Yeast	2.5g / 500ml
Agar	7.5g / 500ml

Method for Culturing Firstly, petri plates were autoclaved at 121°C for 20 min for the purpose of sterilization. Similarly, Luria broth was prepared and autoclaved. After this Luria broth agar was added into petri plates and left for some time to solidify. When process of solidification was done then streaking was performed with the cotton swab. In petri plates, discs loaded with different concentration of sample were placed. 2 discs were used as negative and positive control. Streptomycin and distilled water were used for positive and negative control respectively.

After this petri plates were sealed and incubated in dark environment for a day at room temperature. After 24 hours, discs were checked and measured for zone of inhibition [65].

3.1.4.2 Antifungal Assay

Antifungal activity of synthesized zinc oxide nanoparticles was determined by using agar tube dilution method [56]. Different fungal strains were used for this purpose.

1. *Aspergillus flavis*
2. *Aspergillus fumigatus*
3. *Aspergillus niger*
4. *Mucor species*
5. *Fusarium solani*

Sample Preparation After centrifugation, 25ml distilled water and 25mg of ZnO nanoparticles pallet were mixed up to obtain final concentration i.e. 1000 ppm.

Growth Media Preparation Growth media was prepared for the growth of fungi. Fungal growth media was prepared according to this composition:

Table 3.4: Chemicals and Concentrations.

Chemicals	Concentrations
Sabouraud dextrose agar	26 g
Distilled water	400 ml

Procedure Test tubes were used for this process. For sterilization, we autoclaved it for 20 minutes. Then we marked tubes as 10cm. After this, all the process was performed in laminar flow hood to avoid any impurity. Then 4ml of autoclaved sabouraud dextrose agar was added into test tubes. After that, sample was added in test tubes and slants were made, it took few minutes at room temperature to solidify. Then inoculation of fungal cultures was done in the middle of slant. One positive and negative control were used. In positive control, terbinafine was added and in negative control distilled water was used. After this, incubation was done for almost 96 hours at 28 °C. The formula for Percentage Inhibition is as follows:

$$\frac{\text{Linear growth in negative control-Linear growth in samples}}{\text{Linear growth in negative control}} \times 100$$

For the identification of fungal growth, negative control reading was taken. To find out the percentage inhibition we used formula as mentioned above.

3.1.4.3 Antioxidant Assay

Antioxidant activity of zinc oxide nanoparticles was determined by using DPPH method (2, 2-diphenyl-1-picryl-hydrazyl-hydated) [41].

Preparation of Sample To acquire 1000 ppm stock concentration, 25 mg pallet was mixed in 25 ml distilled water and further dilutions were produced as follows:

1. 100 ppm

2. 200 ppm

3. 300 ppm

Table 3.5: Chemicals and Concentrations.

Chemicals	**Concentrations**
DPPH	12mg
Ethanol	100ml

DPPH

Experimental Process For this experiment, Glass vials were taken. In every vial, we added 200 μl solutions (sample and control) with 2.8 ml DPPH reagent. In this procedure negative and positive control taken were ethanol and ascorbic acid respectively. Mixture was incubated in dark at room temperature for half an hour. Experiment was done in triplicate. Ethanol was used as reference. To find the scavenging percentage.

This formula applied:

$$\frac{\text{Control absorbance} - \text{Nanoparticle sample absorbance}}{\text{Control absorbance}} \times 100$$

3.1.4.4 Cytotoxic Assay

Cytotoxic activity of the Zinc oxide nanoparticles was determined by using Brine lethality assay [1].

Sample Preparation Stock solution was prepared by adding 25mg of pallet in 25ml of distilled water to obtain final concentration of 1000ppm. Different dilutions were produced by using different concentrations:

1. 100 ppm

2. 200 ppm

3. 300 ppm

Preparation of Sea Salt Water

Sea Salt 34g/L

Eggs Hatching In Sea salt water, brine shrimp eggs were hatched.

Procedure This experiment was performed in glass vials. Biosynthesized nanoparticles were added in vials at different concentrations (100 ppm, 200 ppm, 300 ppm) with sea salt to form the final volume 5ml. Distilled water was used as negative control. After a day, when maximum shrimps were hatched, 15 were added in each vial. After this process, glass vials were put at 25°C. Then sterilized pipet was used to count the shrimps. Formula for percentage mortality is given below:

$$\frac{\text{No. of alive shrimps in negative control-No. Of alive shrimps in test}}{\text{No. of alive shrimps in negative control}} \times 100$$

Chapter 4

Results and Discussion

This chapter covers all the aspects that are related to current research which is based on green synthesis of ZnO NPs of *Trigonella Foenum* seeds extract and their physical and biological characterization as follows:

Physical Characterization

1. UV-Vis (ultra violet visible) spectrophotometer analysis
2. SEM (Scanning electron microscope)
3. EDX (Energy dispersive X-ray spectroscopy)
4. XRD (X-ray Diffraction spectroscopy)

Biological Characterization

1. Antibacterial
2. Antifungal
3. Antioxidant
4. Cytotoxic

Results of synthesis of nanoparticles and their characterization are mentioned below:

4.1 Synthesis of ZnO Nanoparticles

This process was performed in the existence of sunlight at room temperature. Dilution (9:1) was prepared for zinc acetate salt solution and seeds extract of *Trigonella foenum* for the biosynthesis of ZnO NPs. Manually 5ml of seeds extract was added into the 45ml of zinc acetate salt solution and then for 25 minutes it was put on to the magnetic stirrer for mixing purpose. Zinc acetate reduced into ZnO NPs after proper mixing and color of solution changed into light brown to yellow. Change of color is the sign of formation of ZnO NPs and after 10 minutes of dissolving, this color change was observed. Solution color change is the recognition point of metallic nanoparticles. After this zinc oxide nanoparticles dried via hot air (oven) up to 48 hours at temperature of 40°C [86].

4.2 Characterization of ZnO Nanoparticles

4.2.1 Analysis through UV-Vis Spectrophotometer

The synthesis of metallic ZnO nanoparticle analyzed when the color of mixture of zinc acetate and seed extract (*Trigonella foenum*) change the color of solution from colorless to light brown yellow. To check the ZnO NPs synthesis, we use UV-vis spectrophotometer technique. Through this technique we check optic properties and stability of nanoparticles. The spectra observed between 360 nm to 380 nm showed ZnO NPs.

This technique is used to analyze:

1. Surface chemistry
2. Optic properties
3. Size
4. Shape

5. Plasma resonance excitation [80]

In a study, ZnO NPs of *Moringa oleifera* leaf extract showed excitation absorption at 370 nm, revealed that at room temperature it has high binding energy [20]. In another study, biosynthesized zinc oxide nanoparticles of *Atalantia monophylla* leaf extract showed highest peak at 410 nm because at this point it showed highest excitation binding energy [97].

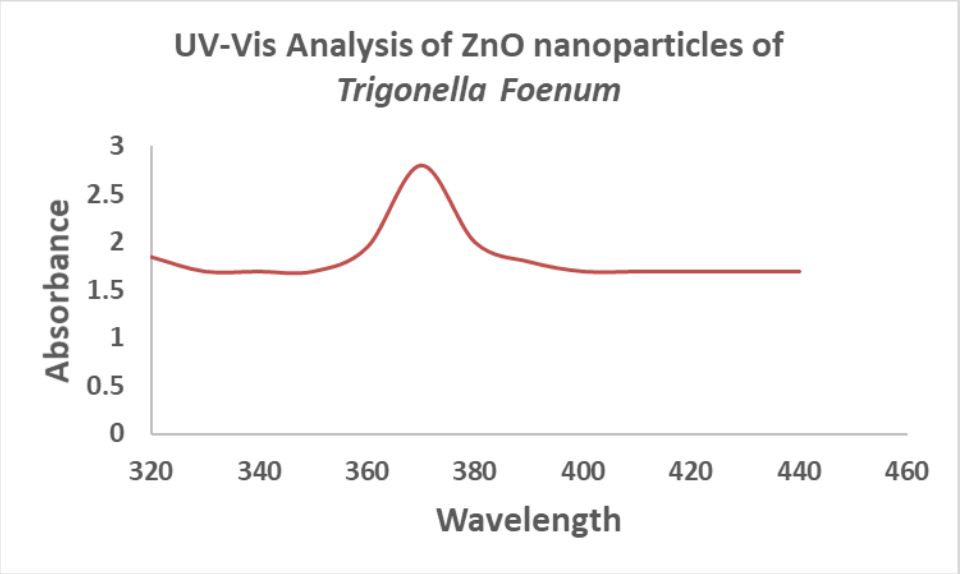

FIGURE 4.1: Uv-Vis Spectra of ZnO Nanoparticles [54].

Analysis through SEM We can analyze the morphology of biologically synthesized zinc oxide nanoparticles through SEM. SEM is the technique used to check morphology, size, chemical orientation, crystal structure of nanoparticles [91].

After analysis of SEM, the size of nanoparticles obtained was 30 nm with triangular shape (Fig 4.2). In this technique sample is subjected to beam of electron to produce magnified image for observance. This technique operated at high magnification to observe very small particle size. Magnification range from 20X to 30,000X and resolution is from 50 to 100m [98]. In a previous study, it was observed that biologically synthesized nanoparticles of Aleo vera showed size of 9 to 18 nm with some of the deviations. The shape of particles was hexagonal [49]. In another study, biosynthetic Zinc oxide nanoparticles from leaf extract of *Glycosmis pentaphylla* showed spherical shape [96].

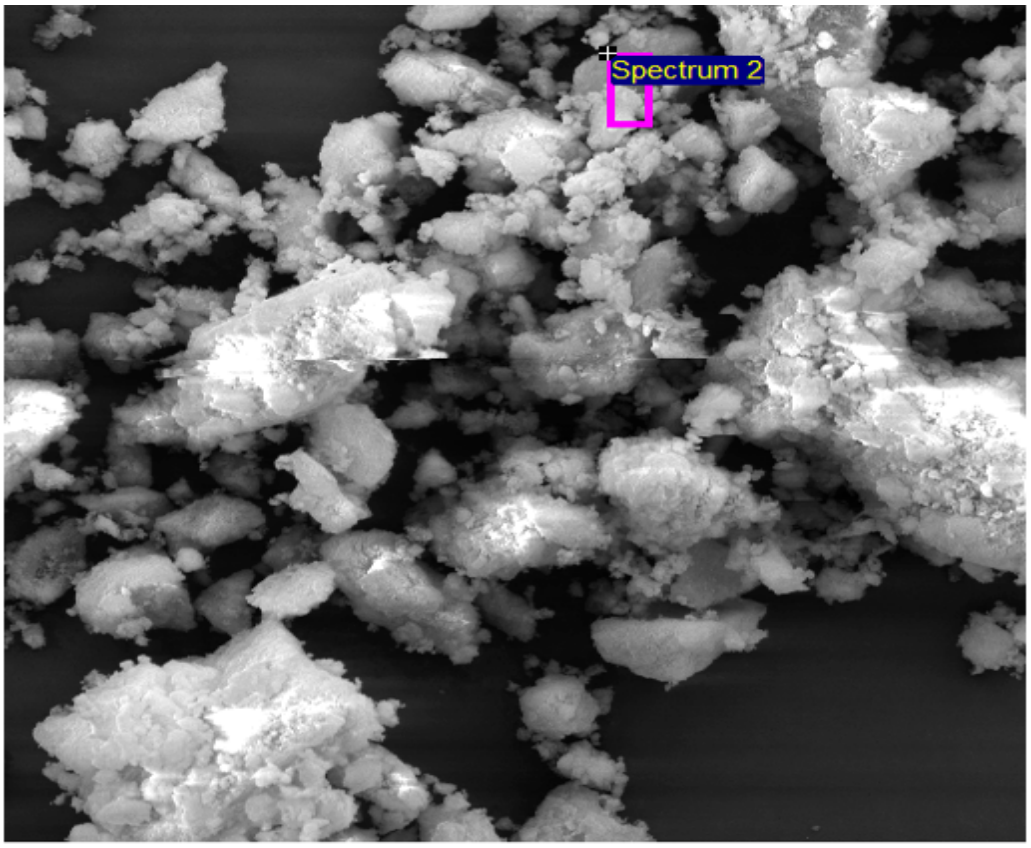

FIGURE 4.2: SEM Analysis of Synthesized ZnO Nanoparticles.

4.2.2 Analysis through EDX

EDX with SEM is the other technique to determine the arrangement and composition of biologically synthesized nanoparticles. In this spectrum, it is observable that important elements of biologically synthesized nanoparticles are zinc and oxygen (Fig 4.3). This spectrum also explains that these elements are without any impurity. Peak of carbon is also shown and it is only due to the reason because for SEM analysis carbon coated grid capping agent is used.

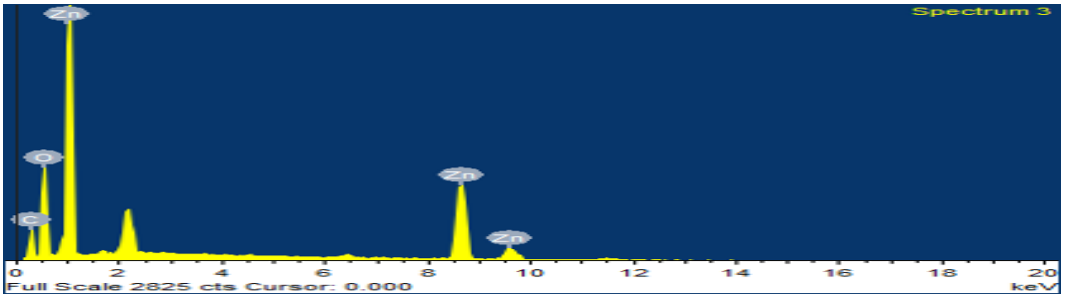

FIGURE 4.3: EDX Spectrum of Synthesized Zinc Oxide Nanoparticles.

In a previous study, it was revealed that existence of components like zinc and oxygen were proved in the biosynthesized ZnO NPs from *Ocimum basilicum L. var.* and *purpurascens Benth-Lamiaceae* leaf extract [79].

4.2.3 Analysis through XRD

X-Ray crystallography is the other technique used to check:

- Crystalline Phase composition
- Phase identification
- Crystalline structure [53]

Results showed that biologically synthesized ZnO nanoparticles of Trigonella foenum are of crystalline nature. XRD pattern of the ZnO sample was observed by using index POWDER-X software as well as matched with standard data (JCPDS, 361451). The result showed that synthesized ZnO nanoparticles hexagonal wurtzite structure crystalline nature with identification peaks having lattice parameter a=3.252(3) (A0), c=5.208(6) (A0). It was also observed that there is no impure peak in graph (Fig 4.4).

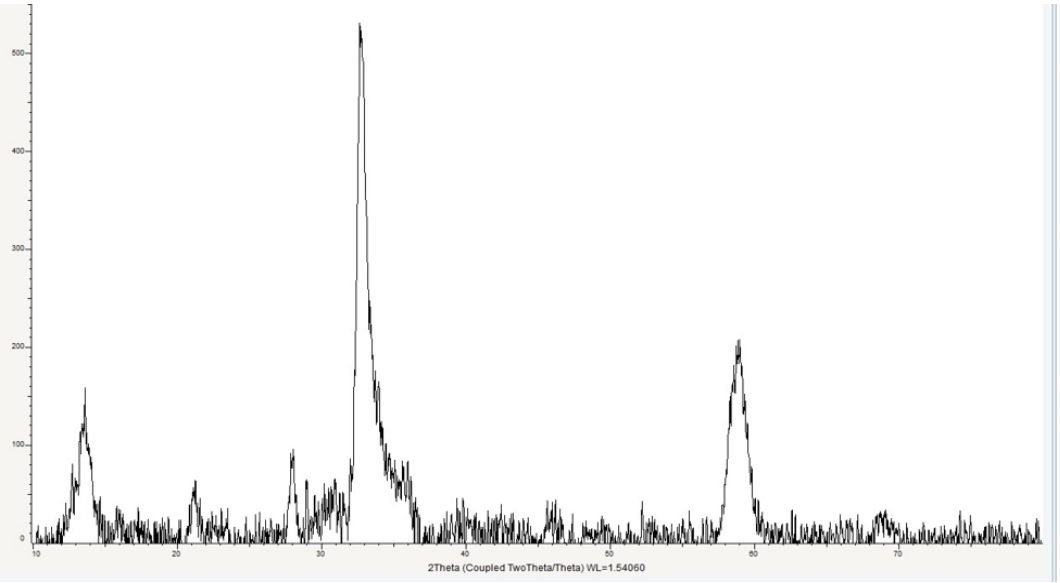

FIGURE 4.4: XRD Peak Diffractogram of ZnO Nanoparticles

In an earlier study, green synthesized nanoparticles using *Ocimum Tenuiflorum* leaves extract in this the peak of diffraction showed at 2 $\theta = 36.19°$ at lattice plane [77]. In another study, biological synthesized nanoparticles by *Aloe socotrina* leaf extract revealed that XRD analysis gave information about crystalline nature. To locate the lattice plane JCPDS was used for the peaks [23].

4.3 Biological Evaluation of ZnO Nanoparticles

4.3.1 Antibacterial Assay

To check the antibacterial activity of biosynthesized ZnO nanoparticles using *Trigonella foenum* seed extract, we used disc diffusion method. To check antibacterial activity, total 6 strains were used. Three gram positive (*M.luteus, S. aureus, B. subtilis*) and three gram negative (*A. tumefaciens, S. setubal, E. aerogenes*). Results are described in Table 4.2. Results against six strains were compared with the positive control which was streptomycin. Zone of inhibition was observed after 24 hours. Vernier caliper was used to check the inhibition zone.

The inhibition of bacterial growth increased with the increased concentration of the ZnO nanoparticles. In both types of strains, highest zone of inhibition was observed at 100 ppm and lowest was observed against 10 ppm. Synthesized particles were found more effective against the Gram negative bacteria than Gram positive bacteria as cell wall of Gram negative bacteria is thin as compared to that of Gram positive bacteria. Bacterial activity and concentration of ZnO NPs showed direct relationship. As concentration decreases the activity also decreased.

In a previous study it was revealed that biosynthetic ZnO nanoparticles using *Solanum nigrum* leaf extract showed that the bacterial activity depends on the size of the particles, smaller the size, the better is the antibacterial activity [72]. In another study, biologically synthesized nanoparticles using leaf extract of *Azadirachta indica* revealed that antimicrobial activity depend on following:

- Particle size
- Morphology
- Concentration
- Surface area

As the concentration of the nanoparticles increases so the antibacterial activity also increased [99].

TABLE 4.1: Basic differences between gram positive and gram negative are listed below [88].

Characteristics	**Gram-Positive**	**Gram-Negative**
Cytoplasmic membrane	1	2
Peptidoglycan	Many layers	Thin layer
Thickness	20-80nm	7-8nm
Teichoic acid	Present mostly	Not present

In a study, it was reported that by increasing the concentration of Zinc oxide nanoparticles, the size of inhibition zone also increased. In this study, inhibition zone was based on following:

1. Type of bacteria
2. Size of bacteria
3. Concentration of nanoparticles.

Staphylococcus aureus was used as gram positive and *Escherichia coli* as gram negative. Zone of inhibition for *Staphylococcus aureus* observed was maximum in both wells and disc diffusion agar method with increased concentration of ZnO NPs. By comparing, *S. areus* with *E. coli*, in this case *E. coli* showed less inhibition zone than *S. areus*. So, growth of gram-positive is not inhibited by maximum concentration of ZnO NPs [21].

Two possible methods are involved for the reciprocal action of nanoparticles and bacteria (positive or negative):

1. ROS (Reactive Oxygen Specie) increased production level
2. Aggregation of nanoparticles on the surface of bacteria [69]

Usually there are three main approaches that are adjacent to gram positive bacteria:

1. After the penetration, free radicals are present on the surface of Zinc oxide nanoparticles that produce oxidative stress in the cell.
2. Synthesized nanoparticles cause some chemical changes in the membrane (plasma lamella) of the bacteria which ultimately cause disturbance in some process like gas exchange and transport of material.
3. Free radicals that are produced are very lethal for the bacteria it may damage cell.

In a previous study, the antibacterial activity of zinc oxide nanoparticles on the *Campylobacter jejuni* was explored for inhibition zone. Experiment was performed on agar plate. Different concentration were taken for the determination of antibacterial activity of zinc oxide nanoparticles (0, 0.025, 0.03, 0.04, 0.05, and 0.10 mg/ml). 0.025 showed the moderate effects for cell growth [99].

4.3.2 Antifungal Assay

Antifungal assay was performed for biologically synthesized zinc oxide nanoparticles using *Trigonella foenum* seeds extract to check their antifungal activity against different fungal strains. Experiment was performed and results are mentioned in (Table 4.3). There were used 5 fungal i.e.,

1. *Mucor.sp*

Table 4.2: Antibacterial Activity of Synthesized ZnONPs using disc diffusion method

ZnO NPs	**Zone of inhibition (cm)±S.E**											
	Gram Positive Strains						Gram-Negative Strains					
Con (ppm)	*M. luteus*		*S. aureus*		*B. subtiles*		*A. tumefciens tumtumefaciens*		*S. typhi*		*E. aerogenes*	
	ZnO NPs	P. extract	ZnO NPs	P. extract	ZnO NPs	P. extract	ZnO NPs	P. extract	ZnO NPs	P. extract	ZnO NPs	P. extract
10	-	-	-	-	-	-	0.7 ±0.1	-	0.8± 0.15	-	0.4 ±0.1	-
20	0.7± 0.1	-	0.5 ±0.1	-	-	-	1± 0.1	-	1.0 ±0.1	-	0.4 ±0.1	-
30	1± 0.15	-	0.6 ±0.1	-	-	-	1.2 ±0.1	-	1.5 ±0.3	-	0.6 ±0.1	-
40	1.4 ±0.1	-	0.8 ±0.1	-	0.5 ±0.1	-	1.5 ±0.1	-	1.8 ±0.1	-	0.7 ±0.2	-
50	1.7 ±0.1	0.8±0.1	0.9 ±0.1	-	0.7 ±0.1	-	1.6 ±0.1	-	2.1 ±0.1	0.5 ±0.1	0.9 ±0.3	0.6 ±0.1
100	2± 0.1	1.5±0.1	1.5 ±0.1	1.1 ±0.1	0.8 ±0.05	0.4 ±0.1	2.1 ±0.15	-	2.9 ±0.1	1± 0.1	1.5 ±0.1	1.1 ±0.1
Negative control 1100ppm	0	0	0	0	0	0	0	0	0	0	0	0
Positive control 100ppm	2.5	2.3	2.9	2.8	3	3.2	2	2	3.1	3	3	3

M.luteus ;Micrococcusluteus; S.aureus Staphylococcus aureus; B.subtilis Bacillus subtilis; A.tumefaciens Aerogenes tumafaciens ; S.setubal Staphylococcus Setubal; E.aerogenes Escherichia aerogenes ZnONPs=zinc oxide nanoparticles, p.extract =plant extract

2. *F. solani*
3. *A. fumigatus*
4. *A. flavis*
5. *A. niger*

It was experimentally verified that synthesized ZnO NPs and seed extract (*Trigonella foenum*) show inhibitory effect against *Fusarium solani* 90% and 35% respectively. Minimum inhibitory effect was shown against *Mucor.sp* i.e ZnO NPs showed 5% and plant extract (*Trigonella foenum*) showed 0%. Noticeable inhibitory effects was also observed against other three strains showed i.e.

- *A. fumigatus* [ZnO NPs showed 70% and plant extract showed 25%]
- *A. Flavis* [ZnO NPs showed 70% and plant extract showed 15%]
- *A. Niger* [ZnO NPs showed 60% and plant extract 10%]

Positive control (Terbinafine) and negative control (Distilled water) used for comparison. It was verified that biosynthetic ZnO NPs revealed highest inhibition effect than plant seed extract because of the following reasons:

- Small size
- Large surface to volume ratio
- Connection with secendory metabolism

In a previous study, biologically synthesized zinc oxide nanoparticles using ***Camellia ceninsis*** showed antifungal activity against four fungal strains. Minimum inhibition was observed against *Aspergillus flavus* while highest inhibitory effect was observed against *Penecillium.sp* [100].

In a previous study, antifungal activity of zinc oxide nanoparticles was observed against ***Candida albicans***. For this purpose disc diffusion method was used. Zone of inhibition was checked. No zone of inhibition was observed with 0 concentration of ZnO NPs. Through this we can analyzed that zone of inhibition totally depended on the concentration of synthesis of ZnO NPs. With maximum

TABLE 4.3: Percentage inhibition of ZnO NPs and plant seeds extracts (*Trigonella foenum*) against different fungal species

S.No	Samples	**Percentage inhibition against Fungal Species** (%)				
		Mucor *sp.*	*F. solani*	*A. fumigatus*	*A. flavis*	*A. niger*
1	ZnO NPs	5	90	70	70	60
2	Plant extract	0	35	25	15	10
3	Distilled water (-ve Cont)	0	0	0	0	0
4	Terbinafine (+ve Cont)	100	100	100	100	100

concentration, the zone of inhibition also increased. Different concentrations were 5mg/ml, 10mg/ml, 15mg/ml and 20mg/ml. In this, 5mg/ml showed less antifungal activity and 20mg/ml showed highest zone of inhibition and antifungal activity. Area is also a factor for inhibition zone [85].

In another study it was reported that antifungal activity of ZnO nanoparticles was observed against different fungal strains. Those fungal strains were *F. oxysporum* and *P. expansum*. It was reported that both are dependent on the concentrations. Highest zone of inhibition was observed at the highest concentration of ZnO NPs [103].

In another study, biologically synthesized zinc oxide nanoparticles by using ***L. aculeata*** leaf extract were checked for their antifungal activity against two different fungal strains. *Aspergillus flavus* and *Fusarium oxysporum*. Both showed the highest zone of inhibition and showed direct relationship with the concentration. *Fusarium oxysporum* showed minimum zone of inhibition at low concentration [103].

4.3.2.1 Antioxidant Assay (DPPH)

DPPHH (2, 2 diphenyl-1-1 picrylhydrazyl) assay is the method to check the antioxidant activity of biologically synthesized nanoparticles by using seed extract of *Trigonella foenum* and respective nanoparticles. Ascorbic acid was taken as positive control. Methanol was used as blank and negative control. Scavenging activity is shown in (Table 4.3). ZnO NPs showed more free radical scavenging activity than the seed extract. At highest sample concentration of ZnO NPs, free radical scavenging activity is 93.47% and seed extract of *Trigonella foenum* showed 64.13%. At 200ppm the free radical scavenging activity of ZnO NPs was 57.01% and while seed extract showed 47.12% activity. At 100 ppm the lowest concentration, the activity shown by ZnO NPs was 40.22% and for seed extract it was 28.73%. IC_50 is also shown in Table 4.2. IC_50 for ZnO NPs was found to be 172 ppm while for plant extract it was 223 ppm. It was proved that ZnO nanoparticles has shown more free radical scavenging activity than seeds extract. ANOVA test was used for statistical analysis. Results were found statistically significant ($p<0.05$) as shown in Table 4.4.

In a study it was shown that biologically synthesized nanoparticles using the olive (*Olea europaea*) were evaluated for their antioxidant activity by using DPPH analysis. In that study, the concentration and activity were found in a direct relationship. Highest concentration showed the highest free radical scavenging activity and vice versa [57].

In another study, biosynthesized nanoparticles by using leaf extract of *Tectona Grandis* revealed that DPPH colour was changed to yellow from purple after the process of reduction. Furthermore, increased concentration showed increased free radical scavenging activity (RSA). Moreover, in this study ascorbic acid was compared as a positive control and ZnO NPs showed significant results [29].

In an earlier study, biosynthesis of ZnO NPs by using *Pseudomonas aeruginosa* Rhamnolipids was reported and anti-oxidant activity was also determined. DPPH assay was used in this study to check the free radical scavenging activity. Different

concentrations were used for this purpose (0, 25, 50, 100, 150 and 200 mg/ml). Prepared nanoparticles were mixed with the solution of DPPH for one hour and 10 min at room temperature. Maximum scavenging activity was observed at the highest concentration [84].

In another study, biologically synthesized zinc oxide nanoparticles of *Ruta graveolens (L.)* were investigated for antioxidant activity by using the DPPH method. Methanol was taken as blank in this study and ascorbic acid was taken as positive control. Different concentrations were used (0, 2.5, 5.0, 7.5, 10, 12.5mg/ml) and highest free radical activity was shown at the highest concentration. It showed scavenging activity with IC50=9.34mg/ml [87].

In another study, biologically synthesized zinc oxide nanoparticles from the *Andrographis paniculata* leaf extract were used to check their antioxidant activity. Antioxidant activity was observed through DPPH assay. DPPH changes its color after mixing of solution with the plant extract.

The scavenging of free radical was observed by the given formula:

$$\%I = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

TABLE 4.4: %age scavenging and IC50 of zinc oxide nanoparticles and seeds extracts *Trigonella foenum* against DPPH

Samples	**Percentage scavenging**			
	150 ppm	200 ppm	300 ppm	IC50(ppm)
ZnO NPs	40.22	57.01	93.47	172
Plant extract	28.73	47.12	64.13	233

Ascorbic acid was taken as the reference antioxidant drug. Different concentrations were used and highest scavenging free radical activity was observed at the highest concentration. At 500 mg/ml almost 60% scavenging activity was observed and it was maximum. IC50 in this case observed was 75.42 μg/mL. In this study, it

was showed that high scavenging activity was observed at highest concentration of sample [48].

4.3.2.2 Cytotoxic Assay

Cytotoxic assay was used to check the toxicity of biologically synthesized ZnO NPs using seed extract of *Trigonella foenum* and assay was carried out on brine shrimps. Different concentrations were taken for ZnO NPs and seed extract. Results were shown in (Table 4.5).
Different concentrations were:

- 150 ppm [ZnO NPs revealed 26.6% mortality and seed extract (***Trigonella foenum***) revealed 10% mortality]
- 200 ppm [ZnO NPs revealed 40% mortality and seed extract (***Trigonella foenum***) revealed 20% mortality]
- 300 ppm [ZnO NPs revealed 60% mortality and seed extract (***Trigonella foenum***) revealed 36.66% mortality]

It was verified that high concentration revealed the highest mortality rate while lower concentrations showed lower mortality rate. IC50 for ZnO NPs was found to be 250 ppm while for seed extract it was found to be 533 ppm. So it is demonstrated that seed extract (*Trigonella foenum*) showed less mortality rate as compared to its ZnO nanoparticles. Statistically results were verified with two way ANOVA. All results were statistically verified and valid ($P<0.001$) (Table 4.6).
In an earlier study, it was revealed that biologically synthesized zinc oxide nanoparticles using ***Punica granatum*** (pomegranate) peel extract showed cytotoxity against different cancer cell line [70].

TABLE 4.5: Analysis of variance for factors affecting the free radical scavenging activity of Zinc oxide nanoparticles.

Source of the variation	**Df**	**% of the total variation**	**Sum of the squares**	**Mean square**	**F-Value**	**P-value**	**Significant**
Interaction	4	13.05	2542	635.5	96.39	< 0.0001	Yes
ZnO Nanoparticle	2	63.73	12410	6207	941.6	< 0.0001	Yes
Concentration	2	22.62	4406	2203	334.1	< 0.0001	Yes
Residual	18	-	118.7	6.593	-	-	-

In another study, biosynthesized zinc oxide nanoparticles by using ***Albizia lebbeck*** stem bark revealed the cytotoxity against cell lines MDA-MB 231 and MCF-7 cells. It was proved that green synthesized nanoparticles were effective against cancer cells and activity was directly related to the concentration of the nanoparticles [92].

TABLE 4.6: %age Mortality and IC50 of Zinc oxide nanoparticles and *Trigonella Foenum* seeds extract

Samples	**Percentage scavenging**			
	150 ppm	200ppm	300 ppm	IC50 (ppm)
ZnO NPs	26.66	40	60	250
Seeds extract	10	20	36.66	533

Previously, cytotoxicity was determined by using the MTT assay and neutral red uptake assay. At 14 and 20 g/ml for 24 hours, results were significant (¡0.005) [31]. In another study, it was reported that ZnO NPs caused toxicity in the many cell lines. MTT assay was performed for the cytotoxicity analysis. Different concentrations were taken to check the viability of cell and activity was observed in a concentration dependent manner. At maximum concentration of synthesized nanoparticles and with increased time period its cell viability % decreased [94].

TABLE 4.7: Analysis of Variance for Factor Affecting the Mortality of Brine shrimps

Source of the variation	**Df**	**% of the total variation**	**Sum of the squares**	**Mean square**	**F-Value**	**P-value**	**Significant**
Interaction	4	8.21	983.3	245.8	38.36	< 0.0001	Yes
ZnO Nanoparticle	2	77.55	9284	4642	724.5	< 0.0001	Yes
Concentration	2	13.27	1589	794.5	124.0	< 0.0001	Yes
Residual	18	-	115.3	6.407	-	-	-

Chapter 5

Conclusion and Future Prospects

Trigonella foenum-graecum is a herbaceous crop having a common name (**Fenugreek**). Seed extract of (*Fenugreek*) herb used for the biosynthesis of Zinc oxide nanoparticles by green chemistry approach which is a cost effective and ecofriendly procedure. UV-Vis spectra was observed around 370 nm which confirmed presence of ZnO nanoparticles. SEM revealed triangular shaped particles with size of 30nm, EDX along with SEM were also performed which confirmed the elemental composition to be of zinc metal. XRD confirmed the crystalline nature of the particles. Furthermore, these particles were also found antibacterial, antifungal, antioxidant and cytotoxic in nature.

Antibacterial analysis showed that it was concentration dependent and zone of inhibition was observed in a concentration dependent manner. Antifungal analysis also showed maximum inhibitory effects with the maximum concentration. Similar results were obtained for antioxidant and cytotoxic evaluation. So it was observed that biologically synthesized nanoparticles by *Trigonella foenum* can be used in different fields like medicinal, drug delivery, industrial and cosmetics industry in future.

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