CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Pharmacological Evaluation of Zinc Oxide Nanoparticles Synthesized by using Leaf Extract of *Bryophyllum pinnatum*.

by

Asma Ayub

A thesis submitted in partial fulfillment for the degree of Master of Science

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I dedicate this thesis to Allah Almighty, Prophet Muhammad (SAW) my Parents, and my supervisor Dr. Erum Dilshad who are the source of inspiration and motivation for me.

CERTIFICATE OF APPROVAL

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Abstract

In recent years, green synthesis of metal oxide nanoparticles using plant extracts has become the most emerging field of nanotechnology. Plant extract contain various constituents that act as an effective reducing agent for metal oxides. The aim of this study is to synthesized the Zinc oxide nanoparticles (ZnONPs) by using leaves of *B. pinnatum* and zinc acetate salt that used as a precursor. Zinc oxide nanoparticles were characterized by UV-visible spectroscopy, X-ray diffraction (XRD), Scanning electron microscope (SEM) and Energy-dispersive X-ray spectroscopy (EDX). Different biological assays such as antibacterial, antifungal, antioxidant and cytotoxic assays were used to confirm the therapeutic significance of ZnO nanoparticles. UV-vis spectrum indicated the maximum absorption peak of ZnO nanoparticles at 370nm. SEM images of the sample showed triangular shape and 80nm size. The synthesized ZnO nanoparticles XRD results indicated their hexagonal crystalline nature. EDX confirmed that the synthesized nanoparticles were ZnO. ZnO nanoparticles exhibited potential antibacterial, antifungal, antioxidant and cytotoxic activities as compared to plant extract. This study indicated that the ZnO nanoparticles will be used as an effective therapeutic agent.

Keywords: Green synthesis, Zinc oxide nanoparticles, *B. pinnatum*, Characterization, antioxidant, antibacterial, antifungal, cytotoxic.

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Abbreviations

As	Alive Shrimps
Ca	Control Absorbance
DPPH	(1,1-diphenyl-2-picrylhydrazyl)
EDX	Energy Dispersive X-Ray Spectroscopy
Lg	Linear Growth
Nsa	Nanopartical sample Absorbance
SEM	Scanning electron spectroscopy
UV-vis	Ultra Violet Visible Spectroscopy
XRD	X-ray Diffraction
ZnONPs	Zinc oxide nanoparticles

Chapter 1

Introduction

1.1 Background

Excessive use of antimicrobial drugs results in the development of resistance in microbes against these drugs, which is a health and life-threatening issue. To cope with this problem, it is necessary to develop new effective and potential drugs from the natural resources. Modern techniques along with knowledge of natural medicines are key principles for the development of new drugs. It was reported that plant extracts have potential antimicrobial and antioxidant compounds that have been used for the synthesis of nanoparticles as an effective therapeutic agent [1]. Nanotechnology is new field arisen from the chemical, physical, biological and engineering sciences. In this latest field of science new techniques are established to manipulate single atoms and molecules for the development of various applications in many areas of scientific community. In nanotechnology, the term nanoparticle was defined as a minute substance which act as entire unit with reference to its transportation and characteristics [2]. Because their unique surface chemistry and morphological properties, it is being used in different areas of science such as electronics, medicine, nutrition, designing, optics and microscopy [3]. Nanoparticles attain much attention in technological advancements because of their adjustable biological, physical, and chemical properties with improved performance over their

bulk counterparts. Nanoparticles are categorized on the basis of their composition, size, origin and shape [4].

Metal oxides play a vital role in different field of science (such as chemistry, physics, material sciences and biology) [5]. Metal oxide nanoparticles have novel physical and chemical properties because of their small size and a high density of edge or corner surface sites [6]. Metal oxide nanoparticles are used in fuel cells, fabrication of microelectronic circuits, sensors, piezoelectric devices and in the optoelectronics. There are many type of metal oxide nanoparticles, the most common are ZnO, MgO, TiO_2, CuO, NiO, ZrO_2 nanoparticles etc [5, 7].

ZnO is one of the most frequently synthesized metal oxides after TiO_2 and SiO_2 [8], because of its non-toxic properties, ZnO is recommended as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration (FDA 21CFR182. 8991) [9]. It was reported that zinc oxide has gained much importance among metal oxide particles because of its novel physical and chemical characteristics including mechanical and high chemical stability, nontoxic nature, high catalysis activity, wide applications in radiation absorption, electro chemical coupling coefficient etc.

Due to its unique properties, its excessive use increased in different areas including pharmaceutical, rubber, textile, cosmetic, electrotechnology and electronics industries [10]. The strong UV absorption properties of ZnO has enhanced its use in personal care items, including cosmetics and sunscreen [11]. ZnONPs exhibited strong UV-blocking, antibacterial and antimicrobial properties. ZnONPs is used as in ending of fabrics in the textile industry, because it has attractive properties for visible light and ultraviolet resistance, deodorant and antibacterial properties [12].

Two approaches are used to synthesize nanoparticles such as top down or bottom-up approaches [13]. Top-down method is used to break down large material and convert it into small substances. In top down method, mostly use physical method to prepare nanoparticles which includes laser ablation, sputtering deposition, lithography, vapor deposition and pulsed electrochemical etching. In

bottom-up method, commonly used techniques are plasma or flame spraying synthesis, chemical vapor deposition, sol-gel processing, microemulsion and laser pyrolysis associated with the formation of structure, atom by atom, molecule by molecule, or cluster by cluster [14].

Synthesis of nanoparticles by biological method involve the manufacturing of nanoparticles with help of microbes and plants with therapeutic properties. Biosynthesis was an eco-friendly, safe, cheap, biocompatible, green approach [15]. In another study, it was reported that biological method includes synthesis of nanoparticles with the help of plants, bacteria, fungi, algae etc. Biosynthesis is helpful for synthesis of ZnONPs on large scale production, free from other impurities [16].

Recently, green synthesis of NPs by plant-based method has attained more interest because of its easy handling, simplicity, environmental-friendly and high antimicrobial property [17]. Phytochemical studies reported that the plant and their metabolites possess significant phytochemicals including rutin, oleanolic acid, lupeol, sitosterol, ursolic acid, glycosides of kaempferol, quercetin, leucocyanidin, anthocyanins and proanthocyanidins [18]. Pharmacological investigations reported that the plants have antibacterial, hepatoprotective, anti-oxidative, anti-eoplastic, anti-nociceptive, gastro protective, anti-mutagenic anti-diarrheal and chemo preventive effects [18]. It was reported that different plant extracts used for nanoparticles synthesis such as *Nephelium lappaceum, Pongamia pinnata, Agathosma betulina, Plectranthus amboinicus, Calatropis Gigantea* and *Moringa oleifera,* exhibit agglomeration [19]. It was reported that the antibacterial property of ZnONPs may be because of the formation of free radicals on the surface of the NPs, and these free radical damage the lipid present in bacterial cell membrane which cause leakage and breakage of bacterial cell membrane [20].

The strong antibacterial property of ZnONPs is reported against *P. mirabilis* MTCC 3310 (*P. abilis*), *S. pyogenes* MTCC 1926 (*S. pyogenes*), *E. coli* MTCC 40 (*E. coli*), *P. aeruginosa* MTCC 424 (*P. aeruginosa*), *B. cereus* MTCC 430 (*B. cereus*), and *S. aureus* MTCC 9760 (*S. aureus*) [21]. ZnONPs have significant antifungal property against pathogenic fungal including *B. cinerea* and *P. expansum*

strains. It was studied that if the concentrations of ZnONPs were higher than 3 mmol per liter then it can inhibit the growth of *B. cinerea* and *P. expansum* [22]. ZnONPs of the *Cassia fistula* exhibited potential antioxidant activities by scavenging of 1, 1-diphenyl-2-picrylhydrazil (DPPH) radicals [23]. ZnONPs have cytotoxic activity against cancerous cells and normal cells such as lung epithelial cells [24], breast cancer cell line [25] and human lens epithelial cells [26].

Bryophyllum pinnatum plant is member of family Crassulaceae, it is a perennial herb commonly known as Zakhm-e-hayat, pattharcatta and parnabija. It is a perennial herb which grows up to 0.9-1.5 m tall, succulent dark green leaves and bell like pendulous flowers [27, 28]. Many categories of compounds were isolated from extracts of *B. pinnatum*. Sterols were found in *B. pinnatum* including stigmasterol [29] and bryophyllol, bryophollone and bryophollenone [30] which possess anti-inflammatory and analgesic properties. While the leaves have class of steroid known as bufadienolides which exhibited antibacterial and antitumorous actions [31].

The leaf is commonly used in traditional medicines as carminative, disinfectant, astringent, emollient, tonic, and hemostatic. It is also applicable for the treatment of wounds, scalds, menstrual pain, ophthalmic, hemorrhoids, boils, diarrhea, dysentery, burns and sloughing ulcers [31]. The leaf juice is effective for hepatoprotective property and also applicable to cure jaundice. Plant was effective in order to treat kidney stones in India. The plant was helpful for treatment of uterine contraction and also for menstrual problem. The leaves of *B. pinnatum* was effective for dysentery when used with ghee. The leaves extract of plant is also helpful for cholera, in toothache and wound healing. The fatty acids reported from *Bryophyllum pinnatum* have immunomodulatory activity [32]. Leaves were used for treatment of pains and inflammations, for many viral, fungal and bacterial infections, flu, leishmaniasis, fever, earaches, stomach ulcers and upper respiratory infections [33]. It was studied that leaves of *Bryophyllum pinnatum* plant exhibit anthelmintic, antifungal, antileishmanial, antihistaminic, analgesic, anticancer, antiulcer, antihypertensive, immunosuppressive, anti-inflammatory, antimutagenic,

antihypertensive and antidiabetic properties along with antibacterial and insecticidal actions.The nephrotoxicity in rats may be because of its antioxidant and oxidative radical scavenging properties [32].

1.2 Problem Statement

Many problems are associated with available conventional drugs such as efficacy, safety, availability and solubility. To overcome this issue, we synthesized the ZnONPs as therapeutic agent with enhanced efficacy and reduced side effect drug by using green synthesis approach.

1.3 Aims and Objective

The present study was design to synthesis of stable ZnO nanoparticles with less effort and more economic values by using *B. pinnatum* leaf extract and their characterization, evaluation of antioxidant, cytotoxic and antimicrobial activities. Following are the main objective of this study:

- Synthesis of ZnO nanoparticles by using extract of *Bryophyllum pinnatum.*
- Characterization of prepared ZnO nanoparticles by UV-Vis spectroscopy, SEM, XRD and EDX analysis.
- Evaluation of therapeutic potential of Zinc nanoparticles by performing antimicrobial, antifungal, antioxidant and cytotoxic assays.

Chapter 2

Literature Review

Nanotechnology achieved more importance due the to unique physicochemical properties of the nanoparticles including catalytic activity, melting point, electrical conductivity, wettability, thermal conductivity , light scattering and absorption outcomes with improved results over their bulk counterparts [4]. Nanotechnology has a wide range of applications in different fields such as cooling of transformer oil nuclear reactors, transportation industry, cooling of microchips, electrical energy, magnetic, biomedical fields and solar absorption [34, 35, 36]. In the field of medicine and biology, nanotechnology has various roles such as in separation and refining of biological molecules and cells, fluorescent biological labels, gene and drug delivery, bio-detection of pathogens, tumors destruction via heating (hyperthermia), tissue engineering, MRI contrast enhancement and phagokinetic studies [37].

2.1 History of Nanotechnology

Nanotechnology is an emerging field of science. In 1857, the first scientific description on characteristics of nanoparticles was given by Michael Faraday while he was working to find relations of gold with light. In 1959, Richard Feynman described nanotechnology by giving concept to manipulate and control things at

atomic level in his publication entitled "There's plenty of space at the bottom". In 1950's to 1960's biotechnology gained more interest of the world, especially in the area of delivery of drugs. In late 60s, Professor Peter Paul Speiser and his coworkers were pioneers in the discovery of polyarcylic bead for oral administration, after this focus was shifted towards microcapsulation and synthesis of first nanoparticle for vaccine sand drug delivery, this led to the major achievement in drug delivery such as using nanoparticles to create a system that transport drugs across the blood brain obstacles. In 1974, for the first time term "nanotechnology" was used by Professor Noro Taniguchi of the Tokyo University of Science. Later K. Eric Drexler and co-researcher in 1981 published their first article on nanotechnology with title "An approach to the development of general capabilities for molecular manipulation". In 1996, the first scientific conference was held on the "Biological approaches and molecular nanotechnology applications". In 2007, Keruter reported that intraperitoneal injection of the nanoparticles increases life span of Ehrlich ascites carcinoma-bearing mice. Nanotechnology gain much importance after the invention of various characterization techniques such as TEM, AFM, DLS, etc [17].

2.2 Nanoparticles

The word "nano" is derived from Greek word "nanos" which mean dwarf or tiny. Size of a nanoparticle is one billionth of a meter, it is equals to 1/75,000th of the human hair size. Size of the atom is one third of the nanoparticle width [38]. Small size of the nanoparticles leads to formation of structures, devices and systems with innovative properties and functions. NPs exhibits unique physical and chemical characteristics because of increased surface area as compared to volume as particles are smaller in size and this is known as Quantum size effect [39]. Nanoparticles varies in shape, size and structure. Nanoparticles may be spherical, conical, tubular, spiral, flat, hollow core etc. or irregular. Surface may be uniform or irregular with surface modifications. Nanoparticles have crystalline or amorphous structure having single or multi crystal solid with loose or agglomerated

particles. Many methods for the synthesis of nanoparticles were used to achieve enhanced properties and minimize the production expense. Some methods were improved to enhance the mechanical, optical, chemical and physical properties of the nanoparticles. Advancement in field of instrumentation can lead to enhancement in nanoparticles characterization and consecutive application. Nanoparticles are used in variety of objects ranging from cooking vessels, electronics to renewable energy and aerospace industry [40, 41].

2.3 Nanoparticles Classification

Nanoparticles can be classified into four material based classes.

2.3.1 Carbon Based Nanoparticles

Nanoparticles that have carbon and exist in ellipsoids, spheres or hollow tubes morphological form. Carbon based nanoparticles contain carbon nanotubes (CNTs), carbon onions, carbon black, fullerenes (C60), carbon nanofibers and graphene (Gr). Different methods are used to synthesize carbon based nanoparticles such as chemical vapor deposition, laser ablation and arc discharge [4].

2.3.2 Inorganic Based Nanoparticles

Inorganic nanoparticles consist of metal and metal oxide nanoparticles. These nanoparticles combined with metals e.g. Ag or AuNPs, metal oxides such as TiO_2 and ZnONPs, and semiconductors such as ceramics and silicon [4].

2.3.3 Organic Based Nanoparticles

It consists of nanoparticles usually from organic compounds, except of inorganic and carbon based nanoparticles. Self-assembly in these nanoparticles were due to

usage of noncovalent interaction and structure of the molecules give favor to modified organic nanoparticles in desired designs such as micelles, polymers, liposomes, micelles and dendrimers nanoparticles [4].

2.3.4 Composite Based Nanomaterials

These were multiphase nanoparticles. In these nanoparticles one phase of nanoscale dimension can bind nanoparticle with another nanoparticles or bind nanoparticle with larger or bulk materials such as hybrid nanofiber or bind with more complex structures, for example metalorganic framework. The composite nanoparticles can be in any combination of carbon-based, organic-based or metal-based (any form of such as metal, ceramic, or polymer bulk materials) [4].

2.4 ZnO Nanoparticles

Zinc oxide (ZnO) is an inorganic compound, present in the form of white powder and is almost insoluble in water [42]. Zn is 23rd most abundant element on earth and also an essential nutrient that is necessary for all living organisms [43] and after iron it is the second most commonly found transition metal [44]. Zn is mandatory element in six different categories of enzymes, such as lyases, transferases, ligases, oxidoreductases, isomerases, and transferases, hydrolases [45]. Zinc has been the essential micronutrient element for metabolic activities in plants, animals and also in humans [46]. The nanoparticles synthesized from ZnO have gained much attention in recent years, because of their unique thermal and chemical stability [47]. Zinc oxide belong to the group of inorganic metal oxides which show large number of nanostructures, with photo oxidizing and photocatalytic property [48]. The nanoparticles of ZnO are versatile semiconductors that exhibits unique luminescent and optical transparent properties in UV–Visible (UV–Vis) regions [49]. Their large surface area, UV blocking properties, cheap, white appearance and high catalytic activity and wide range of application in agriculture and medicine,

ZnO particles have gained more interest in modern researches [50, 51]. Nowadays, ZnO was excessively used in antibacterial activity and environmental remediation [52].

2.5 Applications of ZnO Nanoparticles

ZnO was excessively used in many products as an additive in materials such as in cement, glass, paints, rubber (e.g., car tyres), ceramics, lubricants, foods (source of Zn nutrient) ointments, plastics, adhesives, sealants, fire retardants, pigments, batteries, and ferrites [42]. In food industry, source of Zn was Zinc oxide and it was an essential trace element [53]. While it acts as an invisible hindrance which shattered UV radiation away from the skin relatively than permitting its destructive energy to be absorbed, so it was excessively used in many cosmetic products like sunscreen products [54, 55]. But, through oral ingestion of zinc, absorption rate was low and large quantity or size of ZnO reduce its ability to block UV radiation, dispersion property, low transparency on skin, and hard agglomeration as compared to small-sized of ZnO. The nano-sized ZnO currently gained more interest due to its improved zinc uptake and cosmetic clarity which enhanced it UV filtering efficiency [56]. ZnO was nontoxic compound and has potential antibacterial property. ZnO nanoparticles exhibit stronger effect against microbes and due formation of reactive oxygen species (ROS) that occur on the surface of nanoparticles.

Nanoparticles of ZnO are used in many industrial areas such as food packaging, cosmetics and synthetic textiles (Fig2.1) [58]. Major advantages of ZnO were antibacterial activity, low price, sensing properties, ability to generate materials or structures with novel photocatalytic activities, optical properties, such as photonic crystals, catalytic materials, good gas [59]. Zinc oxide particles have a wide range of applications including in vulcanization process used as an activator of accelerator, in white stocks as reinforcement filler, as a form control in lattices, in lubricating oil used as additive and a high degree of transparency was exhibited

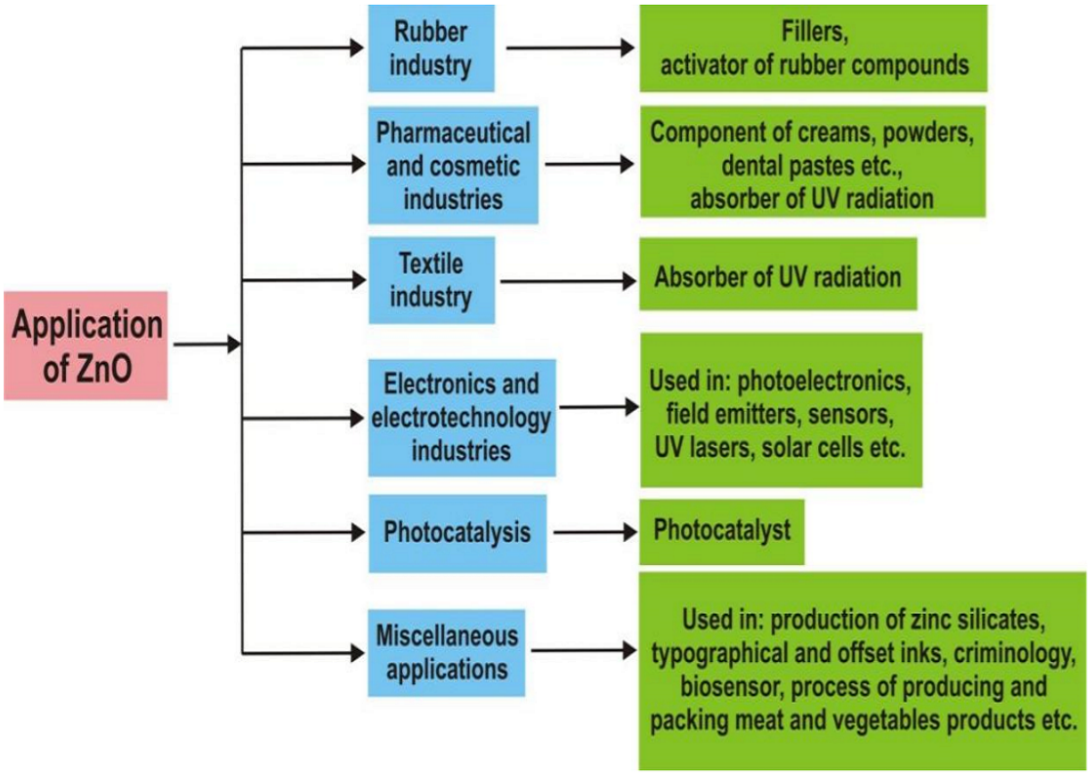

FIGURE 2.1: Application of Nanoparticles [57].

by catalysts Ultrafine ZnO materials. It is also helpful in manufacturing of paints, sunscreens, cosmetics, varnishes and plastics especially for broad UV-A and UVB blocking [60]. It was reported by in vitro cell experiments that free radical produces by ZnO and TiO_2 may initiate formation of free radical in the presence of light and the formation of these free-radicals cause the cell damage. Zinc oxide is a II–VI semiconductor,that is very necessary because of their novel optical or electronic properties and wide applications in many areas including light emitting diodes, solar cells, photonic catalysis, field emission and gas sensors [61].

2.6 Importance of Green Synthesis

In recent years 'green synthesis' approaches have gained great attention for their use in the development of materials in science and technology (Fig 2.2). Nanoparticles by green synthesis are produced by control, regulation, clean up and remediation procedures, which was an environmental friendly process. Few basic concepts

of "green synthesis" can be described by different parts like minimization of waste or prevention, reduction of pollution and used as renewable feedstock and for safer (or non-toxic) solvent. 'Green synthesis' was prescribed to keep away the synthesis of undesirable or hazardous by-products through generation of suitable, environment friendly and reliable synthesis procedures. The natural resources (such as organic systems) usage and ideal solvent systems were essential to get desired goal. Green synthesis of metallic nanoparticles utilized different biological materials (e.g., algae, bacteria, fungi, and plant extracts). Green synthesis by plants is most favorable mechanism for synthesis of metal or metal oxide nanoparticles among all the present green methods, because plants offer simple and easy process to synthesize nanoparticles at large scale as compared to fungi and bacteria mediated synthesis [62].

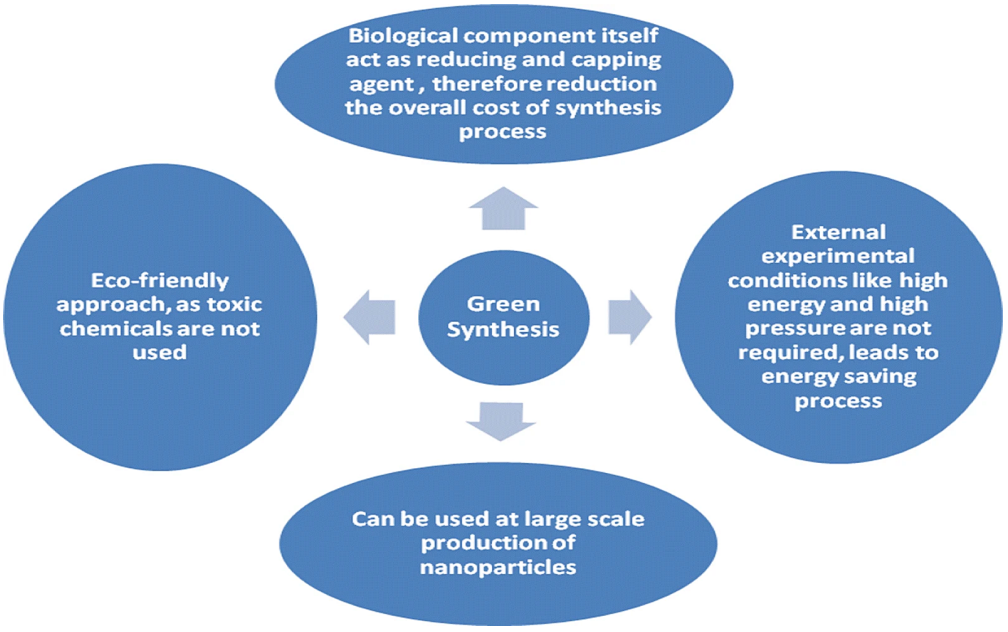

FIGURE 2.2: Importance of Green Synthesis of Nanoparticles [62].

2.7 Synthesis Techniques for ZnO

Various approaches are used for synthesis of ZnONPs (Fig 2.3). There were two different approaches for synthesis of nanoparticles such as top-down and bottom-up method. The top-down method includes physically cutting or breakdown larger

structure into nano-sized particles. In contrast, the bottom-up method used atoms and molecules to create materials by biological or chemical synthesis, or managed growth and deposition [63, 64]. Biological synthesis, also known as "green synthesis", was fascinating because of the most efficient, ecologically important, simplest and reproducible privilege. In spite of that, the mechanism of green synthesis is not still completely known [65].

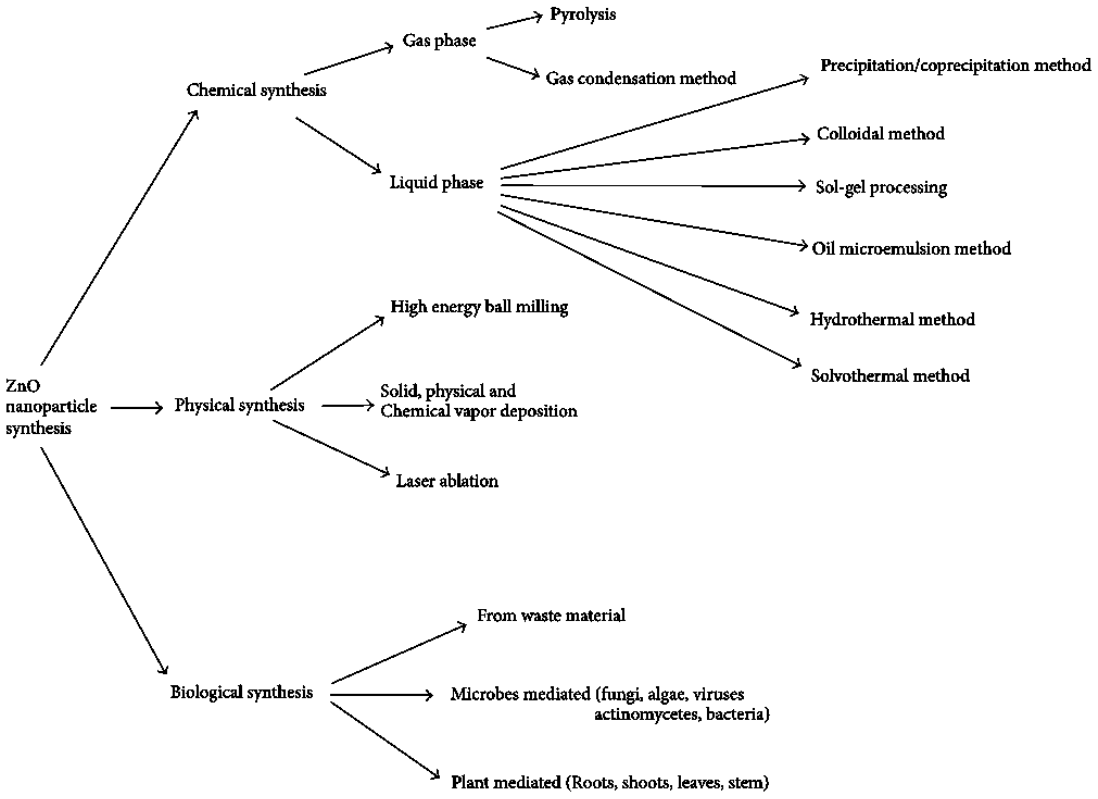

FIGURE 2.3: Major Techniques for Synthesis of Nanoparticles [66].

2.7.1 Physical Approaches

Synthesis of ZnO nanoparticles by mechanical or physical methods involve laser ablation, high energy ball milling, electric arc deposition, melt mixing, ion implantation, physical vapor deposition and sputter deposition. Usually physical methods were used in industrial processes due to higher production rates of ZnO nanoparticles by physical methods. In 1961, Salah and his coworker developed high energy ball milling that was a non-equilibrium process. In high energy ball

milling, process powdered substance is put in a ball mill that is exposed to higher energy collision from balls. It is studied that high energy ball milling method was very simple techniques, cost-effective and very efficient approach for the formation of ZnO nanomaterials. In this method, 15 balls were used having 20mm diameter restricted in a bowl of 500ml. XRD and field emission scanning electron microscopy (FESEM) revealed that powder of ZnO particles have 15nm crystallite size, about 60nm of particle size, and 0.67% of lattice strain. Similar technique was studied and used for antimicrobial activity ZnO [67].

In Laser ablation method, a laser beam was used to eliminate particle from a liquid or a solid surface. Ismail and his coworkers reported that spherical ZnO have average diameter of 35nm. In their study pulsed laser ablation method was used in double distilled water [68]. Materials were heated at lower reflux and the energy absorbed by laser and evaporates, while at higher refluxes, materials may change into plasma. Most commonly used methods were chemical vapor deposition (CVD), physical vapor deposition(PVD) and vapor solid liquid (VLS) [69]. In physical vapor deposition (PVD) methods, surfaces were coated by deposition of the metals. In PVD, two types of techniques were used such as sputtering and evaporation. In Sputtering technique, the particle leaves the surface when they were colliding with high energy particles. Plasma provided the ions for sputtering method [70].

2.7.2 Chemical Approaches

Chemical approaches for the synthesis of nanoparticles involves solvothermal, precipitation, microemulsion, hydrothermal, chemical vapor deposition and sol–gel. Usually nanoparticles were formed by wet chemical synthesis method, that consists of the physical states of the solid and liquid phases [71]. Wet chemical synthesis is used on industrial level, it was extensively used as capping agents or stabilizers instead of its poisonous nature to prevent the agglomeration and to control particle size. Most common capping agent or stabilizers were thioglycerol, oleic acid, polyethylene glycol and Triethylamine (TEA), although they have apoptotic or

necrotic and immunogenic potential [66]. In microemulsion process, stabilizers utilized immiscible phases of water and hydrocarbon to generate thermodynamically stable fluid droplets. It was reported that ZnO NP synthesis by mini-emulsion-based method was done by using TEA to control the shape (hexagonal wurtzite crystal) and size(<200nm) of ZnONPs [72]. Valdez et al. in 2014 reported that the utilizing sol–gel synthesis to form dodecylamine (DDA)-capped ZnO nanocrystals possess low surface density of DDA (25%) because ZnONPs have the hydroxide groups (protons) on the surface. Precipitation approach includes a reaction started by a source of alkali and zinc to stimulate aggregation. After centrifugation or filtration the precipitates are collected [73]. Oliveira et al. reported that the controlled precipitation of ZnONPs through zinc sulfate and zinc nitrate with sodium hydroxide [74]. In acid-catalyzed esterification of zinc acetate in a mixture of m-xylene and l-pentanol is used to precipitate of ZnO nano crystals [75]. It was reported that the gallium–indium ZnONPs for electrolyte-gated transistors were generated by solvothermal technique. It was suggested that chemical vapor deposition was effective and simplest methods that has also used for synthesis of ZnONPs. Although chemical vapors deposition method has been reported to form heterogeneous growth [76]. It was studied that the vapors deposition method used for synthesis of ZnO nano walls at high temperature on a silicon (Si) substrate [77].

2.7.3 Biological Approaches

The traditional physical and chemical methods used for synthesis of ZnONPs have major limitation. The physical approach require use of pressure, high temperature and energy consumption and whereas in the chemical method toxic and hazardous chemicals are used which result into contaminating the environment and are also risky to the individual working on it [66]. Biological or green methods are the replacement of the conventional physical and chemical synthesis approaches due to eco-friendly property of biological methods [78]. Biological synthesis of ZnONPs involves microbes (bacteria, fungi, yeast, algae, and phage), proteins,

DNA, proteins and plant extracts [79, 80]. Parameter for biological methods includes selection of the organisms that were the most suitable (with respect to their biochemical pathways and enzyme activities) and optimal conditions for enzyme activity (such as buffer, temperature, medium, pH) or cell growth. To control the morphology of ZnO nanoparticles, it was necessary to have optimizations of all these parameter. Among biological methods, we can differentiate synthesis with fungi, yeast, bacteria and extracts of different part of plant [81-84].

2.7.3.1 Synthesis of ZnO Nanoparticles by Using Bacteria

B. licheniformis was used to synthesize nanoflowers of ZnO by an environment friendly approach which degraded methylene blue dye, exhibits photocatalytic activity. The nanoflowers exhibit more improved photocatalytic activity, then already existing photocatalytic materials and it has been considered that higher oxygen vacancy in the synthesized nanoparticles is directly related to the improved photocatalytic activity. By absorption of light, active species generate photo catalysis which decomposed the unwanted organic material and used as a potential tool for bioremediation. Synthesized nanoflowers from *B. licheniformis* were 400 nm in height and 40 nm in width [85]. *Rhodococcus* was capable to sustain in harsh situations and it was able for the metabolism of the hydrophobic compounds so it was used in biodegradation [86].

NPs synthesized by *Rhodococcus pyridinivorans* were spherical shaped and as a substrate zinc sulphate was used which exhibits 100-130nm of size range confirmed through XRD and FE-SEM analysis. That the existence of amine salt, alkane, mononuclear benzene band, secondary sulphornamide, phosphorus compound, monosubstituted alkyne, enol of 1-3-di ketone, β-lactone, amide II stretching band, amide I bending band, and hydroxyl aryl ketone was confirmed by FTIR analysis [87]. ZnONPs were synthesized by *A. hydrophilic* and as a substrate ZnO was used. Synthesized NPs exhibited 42-46nm size range, which was confirmed through XRD and AFM analysis with variations in shapes like spherical and oval

[88]. It was studied that ZnONPs have antioxidant activity and NPs were stabilized by *P. aeruginosa* rhamnolipid and it was also reported that rhamnolipid stabilizes the ZnONPs as is hard to form micelle aggregates on surface of carboxymethyl cellulose [89] and it perform as a better capping agent due to its long carbon chain [90].

Bacteria including lactic acid bacteria (LAB) possess non-pathogenic properties has increased interest in bacteria mediated synthesis of NPs. While, lactic acid bacteria also known as the health beneficial bacteria, are also frequently present in the food products [82]. In the previous study, the intracellular synthesis by using *L. plantarum* VITES07 was reported which generate a spherical and pure crystalline shape of ZnO NPs with the 7 to 19 nm size range. It was studied that NPs synthesized by LAB were intermediately stable because LAB secreted biomolecules in synthesis process which act as a capping agent [91]. Synthesis of NPs by bacteria was a green approach but it has many limitations such as the whole process was necessary to avoid the contamination, screening of microbes was time taking process, lack of control on NP size, shape, careful monitoring of culture broth and expenses in term of the media used to grow bacteria was also very high [85].

2.7.3.2 Synthesis of ZnO Nanoparticles by Fungi

Fungus was used for the ZnONPs synthesis by biological method. In extracellular synthesis method of NPs from the fungus it was found most helpful for production on large scale, with favorable downstream processing and economic viability [92]. The biological synthesis of ZnO NPs from fungi was a beneficial method because of the high binding ability, their ability in bioaccumulation of the metals and high resistance to higher metal concentration than bacteria [93]. However, the fungi have capability to produce a huge number of extracellular enzymes and redox proteins. This lead to the decrease metal ions into NPs in huge number, which was useful for the production on large-scale [94]. In medium, fungi secreted larger number of protein, these proteins act as a capping agent that bound with and

encapsulated on the surface of NPs and give the stability. It is reported that the ZnONPs was synthesized from *A. fumigates*, TFR-8 that lead to the generation of NPs with the 3.8 nm average size of diameter and high monodispersity particles (uniformly distributed) without any agglomeration. It was studied that fungi secreted the protein that was encapsulated and bound with the spherical NPs that prohibited the NPs to from agglomerate. After 125 days the stability of NPs was evaluated through the size with the help of particle size analyzer. The results indicated that NPs were stable after day 90 and the size was enhanced because of the agglomeration. This demonstrated that fungi secreted protein that performed as a capping agent that stabilize the NPs up to 90 days [85]. It is reported that, synthesis of ZnO NPs from *Alternaria alternate* was done with help of filtrate-cell free supernatant (FCF). While on other hand, Zinc Sulphate solution precipitate than the FCF was found to produce NPs with 75 $\pm$ 5 nm size. The FTIR and analysis of absorption spectra indicated that synthesized ZnONPs have the protein and other organic compounds. These results were correlated to the past study and concluded that the fungi have secreted a high level of extracellular protein, in order to stabilize the NPs, they bound on the surface of NPs, which prohibited the formation of the aggregation [95].

2.7.3.3 Synthesis of ZnO Nanoparticles by Algae

Algae belong to group of photosynthetic organisms including from single-cell forms (ex. Chlorella) to multicellular ones (ex. Brown algae). Algae lack structures that were present in plant including roots and, leaves. Marine algae were classified on the basis of presence of pigments in them such as red color pigment which was present in Rhodophyta, brown pigment was present in Phaeophyta and green pigment was present in chlorophyte. Algae were useful for the production of nanoparticles of Ag and Au but application of algae for the synthesis of nanoparticles of ZnO was less and studied in very few papers [96].

Microalgae attain more interest due to its capability to degenerate poisonous metals and change them into less toxic forms [97]. ZnONPs were synthesized from

family Sargassaceae members *Sargassum muticum* and *S. myriocystum*. *Sargassum muticum* reported size of NPs with the help of FESEM and XRD which exhibited equal ranges of hexagonal wurtzite structure having the hydroxyl group and sulfated polysaccharides. *S. myriocystum* size was compared by using AFM and DLS which exhibited many size ranges with the existence of carbonyl and hydroxyl stretching in NPs which varied greatly in shape [98].

2.7.3.4 Synthesis of ZnO Nanoparticles by Plants

Biological synthesis of ZnONPs mediated by plants was beneficial and it was presumed that plants were capable to reduce metal ions and therefore a convenient alternative to synthesize nanoparticles. In addition, some plants have ability to accumulate heavy metals and have the capability to convert such substances into no or less toxic form. There were many procedures of metal resistance in plants including metal ions active transport into the vacuole, chelation of metals and cell wall binding [71]. ZnONPs were synthesized by different parts of plant such as fruit, leaf, root, seed and stem because they produced phytochemicals. Synthesis of NPs by extracts of plant parts was very cheap, environment friendly procedure. It was time saving, did not demand any requirement of expensive tool and precursors and it gives highly pure, quantity and enhanced product without adulteration or impurities. Plants were found very favorable resource of NPs synthesis due to their production on largescale and formation of stable NPs, that were differ in shape and size. Bio-reduction includes metal ions or metal oxides reduction into 0 valence metal NPs through phytochemicals like amino acids, terpenoids, polyphenolic compounds, polysaccharides, vitamins and alkaloids secreted by the plant [83, 98].

It was reported that ZnONPs were synthesized by using plant extract of red tomato fruit (*Lycopersicon esculentum*), olive leave (*Olea europaea*) and chamomile flower (*Matricaria chamomilla*) and these NPs act as biocontrol agents that can increase resistance in rice against rice diseases such as bacterial leaf blight disease [99].

2.8 Genus Bryophyllum

The genus Bryophyllum belongs to family Crassulaceae. Adanson (1763) first established the genus Bryophyllum. It was native to Madagascar and consists of almost 25 perennial succulent species [100]. While, mostly species were introduced in other tropical areas where they have sometimes become invasive plants. Bryophyllum species have a particular mode of vegetative reproduction, while young plantlets grow on the edges of leaves before being shed for propagation. It was reported that Bryophyllum species have various secondary metabolites. Peculiar interest was gained by the bufadienolides, due to their toxicological application for grazing animals and many other bioactivities [101]. Genus Bryophyllum was used to treat inflammation, abscesses, rheumatism, burns, and wounds [100].

2.8.1 *Bryophyllum pinnatum*

Bryophyllum pinnatum (Fig 2.4) is the member of the family Crassulaceae and the common names of *B. pinnatum* is Canterbury bells, life plant, miracle leaf and love plant. It was widely distributed in Madagascar America, China, Hawaii, tropical Africa, Australia, and India [102]. Few biological active compounds were isolated from plant such as flavonoids, alkaloids, lipids, triterpenes, bufadienolides, glycosides, kaempferol, rhamnoside, organic acids and phenols. It was studied that the leaves of *B. pinnatum* have antineoplastic, hepatoprotective, anti-asthmatic, antihypertensive, antitussives, antidiabetic, antimicrobial, anti-inflammatory, antiulcer and analgesic activities [103]. Taxonomical classification of *B. pinnatum* is given below:

Kingdom: Plantae – Plants
Sub kingdom: Tracheobionta – Vascular plants
Division: Spermatophyta – seed plants
Subdivision: Magnoliophyta – Flowering plants
Class: Magnoliopsida – Dicotyledons
Subclass: Rosidae

Order: Rosales

Family: Crassulaceae – stonecrop

Genus: *Bryophyllum*

Species: *Bryophyllum pinnatum* (lam.) Oken

The word Bryophyllum was derived from Greek word Bryo means to sprout and phyllon mean leaf. It has ability to propagate through leaf cutting, pinnatum derived from Latin which mean feathered, winged [104].

FIGURE 2.4: *Bryophyllum pinnatum* [104].

2.8.2 Medicinal Importance of *Bryophyllum pinnatum*

2.8.2.1 Hepatoprotective Property

Many researchers reported the hepatoprotective activity of *B. pinnatum*. It was demonstrated that *B. pinnatum* have effective hepatoprotective activity as it decreased the level of the enzymes SGPT, SGOT, SBLN and SALP as their increased

level was directly associated with sensitive indicators of liver injury.

It was studied in rats that the *B. pinnatum* leaves juice and the ethanolic extract were used in rats against CCl4 induced hepatotoxicity and it has been found as an effective hepatoprotective agent. In another histopathological studies, hepatoprotective activity of *Bryophyllum pinnatum* was reported [105].

2.8.2.2 Antidiabetic Activity

It was reported that *B. pinnatum* aqueous extract decrease the level of glucose in blood in both normal and STZ-treated induced diabetic rats with help of obscure mechanism [106].

2.8.2.3 Antimicrobial Activity

It was reported that *B. pinnatum* have two unique flavonoids including 4,3,5,7 tetrahydroxy 5 methyl 5 propenamine anthocyanidines and 5 methyl 4,5,7 trihydroxyl flavones which exhibited the strong antimicrobial property against *K. pneumonia, P. aeruginosa, Staphylococcus aureus, E. coli, Aspergillus niger* and *Candida albicans* [107].

It was studied that 60% methanolic extract of leaves of *Bryophyllum pinnatum* exhibited the antibacterial activities and at a concentration of 25 mg/ml it exhibited strong antibacterial property [108].

It was reported that the *Bryophyllum pinnatum* have phenolic compounds that was useful for the cure of typhoid fever and many other bacterial infections, mainly due to the *P. aeruginosa, S. typhi, B. subtilis, K. aerogenes, S. aureus, E. coli and K. pneumoniae* [107, 109]. Some studies reported that *B. pinnatum* have the active constituents bufadienolides including bryophyllin A and bryophyllin C, which exhibited potential insecticidal property against third instar larvae of the silkworm [110]. It was investigated that *B. pinnatum* have fungitoxic and phytotoxic effects against fungal pathogens [111].

2.8.2.4 Anticancer Activity

Anticancer property of ethanolic extract of *B. pinnatum* was reported by pre-screening method for cytotoxic effect [112, 113]. It was demonstrated that the ethanolic extract exhibits cytotoxic property against the brine shrimp nauplii by brine shrimp lethality (BSL) bioassay. At different concentrations, it exhibited different mortality rate [114].

The leaves of *B. pinnatum* have five bufadienolides, which exhibited inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells induced by the tumor promoter, 12- Otetradecanoylphorbol-13-acetate. All bufadienolides exhibited inhibition, and bryophyllin A showed more inhibition among all compounds that are zinc tested [110].

2.8.2.5 Nephroprotective Activity

It was studied that the aqueous extract of *B. pinnatum* leaves have potential nephroprotective activity in Gentamycin-induced nephrotoxicity in rats [115]. It was reported that plant extract have potent antiurolithitic and diuretic property when hydroalcoholic extract from leaves of *B. pinnatum* were given to male wirstar rats through oral and intraperitoneal route [115, 116].

2.9 Characterization of ZnO Nanoparticles

2.9.1 Ultra Violet Visible Spectroscopy(UV–Vis)

UV-Vis spectrophotometry was used to examine maximum absorbance of the zinc oxide nanoparticles of *B. pinnatum*. For the UV–Vis analysis, from purified sample at the end of reaction suspension of the 1 mL was collected and sonicated for 15 min at 4000 rpm. The UV–Vis spectra was used measured over the range of 200–800 nm [117].

2.9.2 X-ray Diffraction (XRD)

For analyzing the size, shape and crystal structures of nanoparticles X-ray diffraction was used, which is a well-known technique [118]. X-ray crystallography used for the identification of nanoparticles size, purity and crystal density of nanoparticles [119].

2.9.3 Scanning Electron Microscopy (SEM)

Scanning electron microscopy is a high-resolution approach for measuring shape, size, crystallinity (electron backscattering detection), size distribution, aggregation, and dispersion (cryo-SEM) [118].

The synthesized ZnO nanoparticles from *B. pinnatum* plant extract was subjected to Scanning Electron Microscope. SEM slides was formed by preparing a smear of the solutions on the slides.

Platinum thin layer will coat to make the samples conductive. Then the samples was characterized in the SEM at an accelerating 20 KV of voltage [120].

2.9.4 Energy Dispersive X- Ray (EDX)

Energy Dispersive X ray (EDX) technique was used for analyzing the purity of ZnO particles. Powder of ZnONPs was used for EDX analysis [121].

2.10 Bioassay

Bioassay is used to identify the concentration of physical, chemical or biological elements through measuring and comparing the magnitude of the results of the test with that of standard over a suitable biological system under standard set of conditions [122, 123].

2.10.1 Bioassay Importance

Bioassay helps to identify toxicity of developing drugs, the concentration of pollutants from specific sources, pharmacological activity of substances and measure the concentration of unknown particles [124].

2.10.2 Antibacterial Activity

Antimicrobial compounds have the capability to induce the antimicrobial effect against the microbes. Two types of antimicrobial compounds were present particularly based on natural synthetic and semi-synthetic nature. They are used to inhibit the growth of bacteria and fungi [125]. About 17million deaths were reported worldwide annually, because of bacterial most commonly in the elderly and children. Although advancement in antimicrobial chemotherapy but the morbidity and mortality rate related to bacterial infections still significant. As bacterial strains exhibit enhanced resistance to available antibiotics due to it situation were more critical. The emerging bacterial pathogens show intrinsic resistance to antimicrobial drugs [2]. An appropriate technique is required to overcome the issue related to available antibiotic drugs against multiple drug-resistant bacteria by enhancing drugs efficacy [126, 127]. Six pathogenic bacterial strains, *Micrococcus luteus, Enterobacter aerogenes, A. tumefaciens, S. aureus, S.typhi* and *B. subtilis* were used in this assay. Antimicrobial activity was investigated by disc diffusion method [80].

2.10.2.1 *Enterobacter aerogenes*

Enterobacter genus is the member of the family Enterobacteriaceae, those are Gram-negative, non-spore-forming, rod-shaped, and facultative anaerobic non-spore-forming. *Enterobacter aerogenes* was first term as *Aerobacter aerogenes* but later on, in 1960 it was included in to the genus Enterobacter [128, 129]. *E.aerogenes* obtained from clinical humans specimens such as blood ,respiratory, gastrointestinal tract or urinary [130].

2.10.2.2 *Salmonella typhi*

It is a gram-negative, flagellated, rod-shaped bacterium and it is found in human body. *Salmonella typhi* causes many serious diseases in the human body such as typhoid, cystic fibrosis, bradycardic, and abdominal pain and it has been considered to be a huge problem for developing countries [131-134].

2.10.2.3 *Agrobacterium tumefaciens*

It is Gram-negative bacteria and it has applications in agriculture used as a tool for horizontal gene transfer to induce tumors in many plant species and have economic importance such as fruit trees (cherry, berry, walnut), woody ornamental shrubs (rose), vines (grape), herbaceous perennials and shade trees. *A. tumefaciens* causes disease in the plants known as crown-gall. Due to this at the junction of the root and shoot a tumor-like growth or gall usually appear [135].

2.10.2.4 *Staphylococcus aureus*

Its Gram-positive bacteria and in spherical shape. After Gram staining under light microscopes observed that they are in cluster form that look like a bunch of grapes. It causes many diseases such as skin and soft tissue infections, bacteraemia, osteoarticular infections pleuropulmonary infections, and infective endocarditis. Some other clinical infections such as toxic shock syndrome, epidural abscess, urinary tract infections and meningitis [136-138].

2.10.2.5 *Micrococcus luteus*

Its Gram-positive cocci, the diameter is about 0.5 to 3.5 micrometers and organized in the form of tetrads or irregular clusters. It can be found in soil, air, water and on the human skin [139]. It causes many diseases such as endocarditis, pneumonia, septic arthritis, septic shock, meningitis and intracranial suppuration [140].

2.10.2.6 *Bacillus subtilis*

It is ubiquitous bacteria usually found in air, water, soil, and decomposing plant residue. The endospore produced by *B. subtilis* that enables it to survive in the harsh conditions such as heat and desiccation in the environment. It secretes many proteases and other enzymes that help it to degrade many natural substrates and play role in nutrient cycling [141]. It is studied that *B. subtilis* act as active members in food poisoning especially in bakery products [142].

2.10.3 Antifungal Activity

Fungal diseases have significant fatal complications related to the usage of the drugs that are immunosuppressive nature such as drugs of anticancer and so, it is necessary to develop antifungal drugs that are safe for clinical usage and have potent effect on patients. The incidence of fungal infection increasing, as the currently available antifungal drugs have many side effects, fungus exhibited resistance to these drugs and ineffective for new emerging strains, so it is necessary to develop the next generation antifungal drugs [143].

To cope with this problem needed to make alternative new drugs that have the potential to overcome the harmful effects of fungal pathogens. The antifungal activity of synthesized ZnO NPs was definite by using the agar tube dilution method [144].

2.10.3.1 *Aspergillus*

Aspergillus genus is belonging to the division Ascomycota. It is usually present in decaying organic matter such as fruits and vegetables and soil. *A. fumigatus* is ubiquitously distributed worldwide due to the production of the small spores known as conidia, conidia dispersing through the air, and remaining in the environment for longer period [145, 146].

The Aspergillus genus caused many diseases such as aspergilloma, invasive aspergillosis, allergic bronchopulmonary aspergillosis, ocular infections, sinusitis, cutaneous aspergillosis, otomycosis, CNS infection, osteomyelitis, endocarditis, and urinary tract infection [147].

2.10.3.2 *Mucor*

It is a filamentous fungus, commonly found in the digestive system of mammals, soil, rotten vegetables, and fruits, it also has various amphibians and vertebrates host. Many Mucor species are heat sensitive and cannot grow in a warm environment because of this they cannot cause infection in the endothermic animal and humans. Zygomycosis is most common infection caused by Mucor species [148].

2.10.3.3 *Fusarium*

Species of *Fusarium* mostly found on subterranean, soil, and aerial plant parts, plant debris, and other organic substrates. Usually, it is cause of many diseases in the plants but sometimes it also causes the severe eye infection in the human eye and leads to the damaging of the cornea. Some other diseases such as skin infection, endophthalmitis, are caused by *Fusarium* [149, 150].

2.10.4 Antioxidant Property

An antioxidant is a substance that causes inhibition of oxidative damage to a particular molecule. The trapping of free radicals is the main property of an antioxidant. Antioxidant compounds such as polyphenol, flavonoids and phenolic acids can scavenge free radicals including hydroperoxide, lipid peroxyl or peroxide and result in inhibition of the oxidative mechanisms that cause degenerative diseases[151, 152]. Different assays are used to evaluate the antioxidant activity such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, superoxide radical scavenging assay, the hydrogen peroxide assay, reducing power assay and nitric

oxide scavenging activity [153]. In an antioxidant assay DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method the electron-transferred that results in the formation of a violet solution in ethanol. At room temperature free radicals are stable and reduced in the presence of an antioxidant molecule and result in the formation of colorless ethanol solution. The DPPH assay is a simple, easy and rapid method to investigate antioxidants property through [154]. Antioxidant activity of the nanoparticles of ZnO was evaluated by scavenging free radicals of 2,2–diphenyl–1–picrylhydrazyl hydrate (DPPH) with different concentration of nanoparticle as reported earlier [155].

2.10.5 Cytotoxicity

Any chemical or physical substance that can affect the health and metabolism of the cell. These substances induce toxicity in cells by different methods including; cell membranes destruction, prevent the synthesis of protein, inhibition of poly-deoxynucleotide elongation, irreversible binding to receptors, and other enzymatic reactions [156]. Brine shrimp lethality assay is used to evaluate the cytotoxic effect of the bioactive substance. Cytotoxic assay is a preliminary toxicity screening of fungal toxin, cyanobacteria toxins, plants extracts and heavy metals [157]. Brine shrimp lethality assay is used to evaluate the toxicity of biologically synthesized nanoparticles and plant metabolites. Toxicity in plants is due to many active secondary metabolites which are important components of plant. This assay evaluate the toxicity of medicines, drug screening, and various plant extracts [158]. Cytotoxic activity of ZnONPs was evaluated by using the brine shrimp lethality assay [159]. It was reported that the nanoparticles of ZnO synthesized using *Alstonia scholaris* that was used to evaluate cytotoxic activity by Brine shrimp lethality with different concentration of nanoparticle having LC_250 value with 95% confidence intervals [160].

Chapter 3

Material and Methods

This research work was conducted in wet lab of department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad. Different physical characterization techniques are used to the analysis of nanoparticles. Different physical techniques were performed for sample analysis. UV-vis spectroscopy and X-Ray diffraction analysis were performed in the lab of Nanosciences & Technology Department (NS&TD) of National Center of Physics (NCP), Islamabad. SEM/EDS analysis was performed in the lab of U.S.-Pakistan Centre for Advanced Studies in Energy, NUST, Islamabad.

3.1 Material

The list of chemicals,instruments and equipments used in the research work are given in Table 3.1 and 3.2.

Table 3.1: List of Equipment

Equipment	Sources
UV-Vis Spectroscopy	NCP, Islamabad
XRD	NCP, Islamabad

Table 3.1: List of Equipment

Equipment	Sources
SEM/EDX	NUST, Islamabad
Spectrophotometer	CUST, Islambad
Centrifuge	CUST, Islamabad
Incubator	CUST, Islamabad
Autoclave	CUST, Islamabad

Table 3.2: List of Chemicals and Materials

Chemicals and Instruments	Company
Zinc acetate	Sigma Aldrich
Sabourad dextrose agar	Sigma Aldrich
Nutrient agar	Sigma Aldrich
Luria broth	Sigma Aldrich
Ascorbic acid	Sigma Aldrich
DPPH reagent(2,2-dipheny l-1-picrylhydrazyl)	Sigma Aldrich
Sea salt	Sigma Aldrich
Streptomycin	Sigma Aldrich
Ethanol	Sigma Aldrich
Terbinafine	Sigma Aldrich
Aluminum foil	Sigma Aldrich
Para film or masking tape	Sigma Aldrich
Cotton plugs	Sigma Aldrich
Cotton swabs	Sigma Aldrich
Forceps	Sigma Aldrich
Test tubes	Sigma Aldrich
Test tube racks	Sigma Aldrich
Falcon tubes 50ml	Sigma Aldrich

Table 3.2: List of Chemicals and Materials

Chemicals and Instruments	Company
Micropipette	Sigma Aldrich
Micropipette tips	Sigma Aldrich
Eppendorf tubes	Sigma Aldrich
Glass vials	Sigma Aldrich
Petri dishes	Sigma Aldrich
Discs	Sigma Aldrich
Beakers 100ml, 500ml	Sigma Aldrich

3.2 Microbes and Brine Shrimp

Following are the microbes that were used in the biological evaluation.

Bacterial Strain

(Gram positive: *M. luteus, S. aureus, B. subtilis*) (Gram negative: *A. tumefaciens, S. setubal, E. aerogenes*)

Fungal Strains

(*Mucor species, A. flavis, A. fumigatus, A. niger, Fusarium Solani*)

Brine Shrimp

Brine shrimp eggs were used and it was obtained from Sigma Aldrich.

3.3 Methodology

Overview of methodology was shown in Figure 3.1.

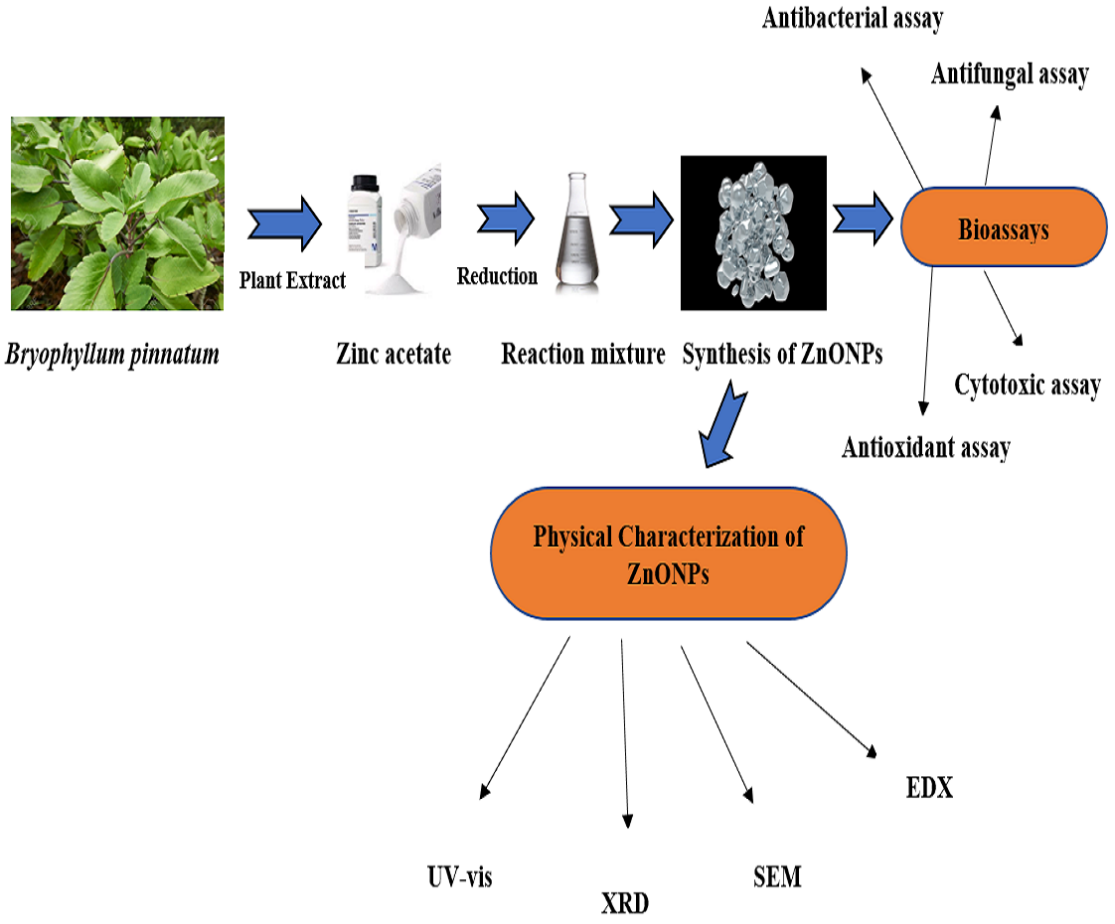

FIGURE 3.1: Overview of Methodology

3.4 Synthesis of Zinc Oxide Nanoparticles

3.4.1 Preparation of Plant Extract from *Bryophyllum pinnatum* leaves

Fresh leaves of the *B. pinnatum* were collected from Rawalpindi, Pakistan. 100g of fresh leaves of *B. pinnatum* were taken and washed with tap water then followed by distilled water to remove dust and impurities. Leaves were dried and then chopped by using mortar and pestle. This extract was put into a flask added with 100ml of distilled water and boiled for about 15-20 minutes. The leaf extract was allowed to cool at room temperature and after that filtration was done by using Whatmann No. 1 filter paper. Then stored the leaf extract at 4 ℃ and used it as a reducing agent in the experiment [161].

3.4.2 Synthesis of Zinc Acetate Salt Solution

Zinc acetate Zn ($C_4H_6O_4$) (99.9%) was purchased from Sigma-Aldrich was used as Zinc precursor. 22.92-gram zinc acetate salt was taken and put into the flask that has 500 ml of distilled water in it. The suspension was put on a magnetic stirrer for about 30 min at 600rmp. Then salt was completely dissolved in water.

3.4.3 Synthesis of ZnO Nanoparticles

ZnO nanoparticles were synthesized by zinc according to a previously reported method [162] with few modifications. 45ml of zinc acetate solution and 5ml of *B. pinnatum* leave extract was mixed to make total volume 50ml in a falcon tube. The reaction solution was kept in incubator for 1 hour and appropriate color change indicated the formation of nanoparticles. The reaction mixture was then to centrifuged for 40 minutes at 3000rmp. The obtained pellet was washed to distilled water to remove debris or by-products and the supernatant was discarded, this process was repeated three time. When centrifugation was completed than for drying purpose the sample was placed in an incubator at 60℃ for 24 hours. Finally, nanoparticles were formed and powdered of nanoparticles were collected and stored in eppendorf tubes for further process.

3.5 Characterization of Zinc Oxide Nanoparticle

3.5.1 Ultra Violet Visible Spectroscopy(UV−Vis)

UV-Vis spectroscopy was used to examine maximum absorbance of the zinc oxide nanoparticles. Light source versatile lamps optimized for the visible-near infrared Vis-NIR (360-2000 nm), Ocean Optics, HL-2000 (tungsten halogen light sources) were used to record the UV-Vis spectra. The USB2000 plus detector (miniature fiber optic spectrometer) was used to collect Uv-Vis Spectra. The samples were diluted one hundred times before measurements [163].

3.5.1.1 Sample Preparation

When the sample was centrifuged, discarded the supernatant and collected pellet was used for sample formation. The stock solution was prepared by dissolving the pellet into distilled water (1000 ppm). Later this stock solution was used for UV-Vis analysis.

3.5.1.2 Experimental Procedure

UV-Vis spectroscopy was used to examine maximum absorbance of the zinc oxide nanoparticles of *B. pinnatum*. For the UV–Vis analysis, from the purified sample at the end of the reaction, 1 mL of the sample was taken and water was set as a blank reference and it was sonicating at 4000 rpm for about 15 min. The range of UV–Vis spectra were measured over the 200–800 nm [117].

3.5.2 X-ray Diffraction (XRD)

For analyzing the size, shape and crystal structures of nanoparticles, X-ray diffraction was used, which is a well-known method [118]. X-ray diffractometer (PAN analytical X-Pert PRO) was used for X-ray diffraction(XRD) analysis that was operating at 30 kV and 40 mA [119]. In this procedure firstly x-rays radiations were produced using the cathode ray tube. These x-rays were bombarded on the sample to analyzed the structure of ZnO NPs. X-ray diffractometer was used to generate x-ray spectrum [164].

3.5.3 Scanning Electron Microscope (SEM)

Scanning Electron Microscope (SEM) was used to examine the morphology of synthesized nanoparticles. In SEM, the sample of nanoparticles was observed with a frequency of 2838 cps (max) at the 20Kv of voltage. The smear of the slides was coated with gold to make them conductive. The slides were prepared

by making a smear of the solutions on it. At different resolution and magnification power, the size and shape of synthesized nanoparticles were determined [165].

3.5.4 Energy Dispersive X-Ray Spectroscopy (EDX)

EDX analysis was used to determine the purity of ZnONPs. EDX spectrum of ZnONPs was used to determine the composition of elements that were present in ZnONPs [166]. The drop of suspension of the nanoparticles was placed on a micro-glass slide that was connected to the metal grid coated by carbon film, and then at room temperature dried it. X-ray spectrometer (EDX) was operated at 10 KeV an accelerating voltage. The sample of nanoparticles was then sputter-coated with gold and visualized to determine the shape, particle size and percentage of synthesized particles [167].

3.6 Biological Assay

Different bioassays were performed for the biological evaluation of the synthesized ZnONPs by using *B. pinnatum*. Following assays were performed for biological evaluation.

3.7 Antimicrobial Assay

For the evaluation of antimicrobial activity, two types of antimicrobial assays were performed such as antibacterial and antifungal assay.

3.7.1 Antibacterial Assay

For the evaluation of antibacterial activity, six different bacterial strains were used, three of them were gram-positive and three were gram-negative stains. Disc

diffusion method was used for the evaluation of antibacterial activity reported by Bauer et al. [168] with slight modifications.

3.7.1.1 Bacterial Strains

- **Gram-positive:**
  Bacillus subtilus, *Staphylococcus aureus* and *Micrococcus luteus.*
- **Gram-negative:**
  AT-10, Enterobacter aerogenes and *Salmonella typhi.*

3.7.1.2 Sample Preparation

The obtained pellet(25mg) was dissolved in distilled water(25ml) and the final concentration of 1000ppm was prepared as a stock solution. Different dilutions from stock solution were made such as 100ppm, 50ppm, 40ppm,30ppm,20ppm and 10ppm and these were used for the antibacterial assay.

3.7.1.3 Bacterial Growth Media

Luria broth agar in petri plates was utilized as a bacterial growth media. It is composed of the following materials: Agar 7.5g / 500 ml, NaCl 5g/ 500ml, Yeast 2.5g / 500ml and Bacto-tryptone 5.5g / 500ml.

3.7.1.4 Procedure

For bacterial culturing, petri plates were washed and dried in the oven. Media, petri plates, tips, filter paper discs, cotton swab, forecep and distilled water were autoclaved at 121℃ for 20 minutes. All autoclaved material was placed in Laminar flow and labeled all the plates in triplet. For bacterial growth, Luria broth was poured in all sterilized plates with equal volume and let it to solidify. After the solidification, bacterial strains were streaked with the help of cotton swab smoothly

and carefully in all prepared petri plates. Then discs containing samples were arranged on the solidified broth plates with the proper sequence. In each petri plates, eight discs were placed, six discs for the different concentrations of sample and one for positive control that was streptomycin (100ppm) and one for negative control that was distilled water. Then sealed all petri plates and subjected for incubation for 24hours at 37℃. After 24 hours, measured the zone of inhibition for every disc with help of ruler [169].

3.7.2 Antifungal Assay

To assess the antifungal activity of ZnONP Synthesized using *B. pinnatum*, five different fungal strains were used to determine antifungal activity using tube dilution method was followed [170]. *Fusarium solani, Mucor species, Aspergillus fumigatus, Aspergillus flavis, and Aspergillus niger.*

3.7.2.1 Sample Preparation

The pellet (25mg) of ZnONPs were taken that were collected after centrifugation and dissolved in distilled water(25ml) and the final concentration of 1000ppm was prepared as a stock solution and used for the antifungal assay.

3.7.2.2 Media Preparation

Fungal growth media was prepared by using 26g Sabouraud dextrose agar in 400ml of distilled water.

3.7.2.3 Procedure

Test tubes, media, cotton plug, tips, loop and distilled water were autoclaved at 121℃ for 20 minutes. To prevent contamination next steps were performed in laminar flow, test tubes were marked to 10cm. Now poured 5ml of prepared

fungal media in each test tube and sample was added at different concentrations(100ppm,200ppm and 300ppm) and cotton plugs were used to cover these test tubes. Now test tubes were placed horizontally so that slant was form up to mark. Ten test tubes were taken for each fungal strain and three were labeled as positive control (Terbinafine), three for negative control (water) and three for different concentration of sample. After solidification, these test tubes were inoculated with fungal strains with the help of the inoculation loop. After completing these steps test tubes were covered with cotton plugs. Now incubated all these tubes in the incubator at 28℃ for two days. After two days' fungal growth was measured in slating position. Negative control was used to measure fungal growth in a linear position [170]. The proportion of fungal growth inhibition was calculated by the following formula.

$$\%age\ inhibition = \left[\frac{(Lg\ in\ \ -ive\ control) - (Lg\ in\ samples)}{Lg\ in\ the\ \ -ive\ control}\right] \times 100 \quad (3.1)$$

3.7.3 Antioxidant Assay

The antioxidant activity of ZnO nanoparticles and plant extract was evaluated by scavenging free radicals of 2,2–diphenyl–1–picrylhydrazyl hydrate (DPPH) [171].

3.7.3.1 Sample Preparation

After centrifugation, the obtained pellets(25mg) of ZnONPs was dissolved in water(25ml) and prepared the final concentration of 1000ppm as stock solution. Different dilutions were made from a stock solution such as 100ppm, 200ppm and 300ppm.

3.7.3.2 Preparation of DPPH Solution

The reagent (DPPH) solution was prepared by adding 12mg of DPPH in 100ml of ethanol.

3.7.3.3 Procedure

The glass vials were taken washed and dried. Eighteen glass vials of volume 10ml were used and 2.8ml of DPPH solution was added in each vial. In this assay nine vials were used for positive and negative control ascorbic acid in which three vials positive control 200µl of ascorbic acid was poured while in three vials negative control ethanol and in other three distilled water was added. In other nine vials sample of 25ppm, 50ppm and 100ppm concentration was added such that there were three vials for each concentration. To determine the antioxidant property of ZnONPs, vials were placed in a dark region for about 20 to 30 minutes. The absorbance of the sample was measured at 517nm and ethanol blank used as a reference. The free radical percentage scavenging was calculated by the following formula:

$$\%age\ scavenging = \left[\frac{(Ca) - (Nsa)}{Ca}\right] \times 100 \tag{3.2}$$

3.7.4 Cytotoxic Assay

Cytotoxic effect of ZnONPs was determined by using brine shrimp lethality assay [159].

3.7.4.1 Sample Preparation

In cytotoxic assays, the prepared pellet of the ZnONPs was dissolved in distilled water and the stock solution was prepared (1000 ppm). The stock was used for the preparation of different dilutions such as 100ppm, 200ppm and 300ppm.

3.7.4.2 Sea Salt Water Preparation

Sea salt water was prepared using 17 g of sea salt was dissolved in 500ml of distilled water. Flask was kept open and stored at 4℃.

3.7.4.3 Hatching of Eggs

Brine shrimp eggs were hatched in sea salt water.

3.7.4.4 Procedure

Clean and dry twelve glass vials were taken, three of them labeled with negative control i.e. 5ml of sea salt water was added. In other nine vials the synthesized ZnONPs and plant extract sample were added with different concentration such as 50pm, 100ppm, 250ppm and also added sea salt water to make a final volume of 5ml. Most of the brine shrimp eggs were hatched after 24 hours and tiny shrimps were seen floating on the surface of the water. The shrimps were then added into the vials that containing sample solution along with sea salt, 15 shrimps in each vial. Then vials were placed in the light at room temperature for 24 hours. Finally, after 24 hours, alive shrimps were counted by using pasture pipette. The percentage mortality was calculated by following formula:

$$\%age\ Mortality = \left[\frac{(No.\ of\ AS\ in\ -ive\ control) - (No.\ of\ AS\ in\ test)}{No.\ of\ AS\ in\ the\ -ive\ control}\right] \times 100 \tag{3.3}$$

Chapter 4

Result and Analysis

In this chapter, the results obtained from UV-Vis, SEM, EDX, and XRD for the powdered samples of ZnONPs synthesized by leaf extract of *Bryophyllum pinnatum* was discussed. In addition to this, the biological activity of ZnONPs by various assays such as antibacterial, antifungal, antioxidant and cytotoxic assays will be discussed. Results of the present research are also given below:

4.1 Formation of ZnONPs

When the color of the solution was changed into off white colour it indicated the formation of ZnONPs. The reaction mixture of zinc acetate and aqueous leaf extract of *Bryophyllum pinnatum* were mixed then color change after 24 hours of incubation. The off white color is the preliminary confirmation of the formation of ZnONPs. When an aqueous extract of the *B. pinnatum* was added in zinc acetate solution, it can reduce the Zn ions into the Zn nanoparticles which can cause the color change of solution into off-white color. The excitation of Surface Plasmon Resonance of ZnONPs was the reason of color change [172]. In one of other study conducted by group of researchers, it was found that color change was observed when *C. fistula* and *M. azedarach* leaves extract were used for the synthesis of ZnO nanoparticles and color change from yellow to light brown and

red to off-white respectively indicated the formation of ZnONPs [162]. Results of these studies correlated with results of current research thus validated the present results.

4.2 Characterization of ZnO Nanoparticles

4.2.1 ZnO Nanoparticles Analysis through UV-Vis Spectrophotometer

UV-Vis spectroscopy was used to determine synthesis of nanoparticles, analyzed optical properties, and stability of nanoparticles. UV-Vis spectroscopy is used to confirm the synthesis of ZnONPs by leave extract of *B. pinnatum*. Because of Surface Plasmon Resonance effect conducting electron begins to oscillate at a certain wavelength range. The range of UV-visible wavelength was between 300 and 500 nm [119].

Properties of the material depend upon the size of nanoparticles and size plays a major role in changing the entire properties of materials. So, size measurement of semiconducting nanoparticles becomes necessary to explore the properties of the materials. UV-visible absorption spectroscopy is more frequently used for the analysis of the optical properties of particles [173]. The absorption spectrum of the synthesized ZnONPs of *B. pinnatum* was shown in Figure 4.1. It shows a high absorption peak at about 370nm. The absorption peak at 370nm confirmed the synthesis of ZnONPs by leaf extract of *B. pinnatum*. ZnO formation was the reduction of zinc ion due to the phytochemicals present in plant extracts such as alkaloids, flavonoids, terpenoids, and tannins and these phytochemicals act as bioreductant, stabilizing and capping agent to synthesized ZnO nanoparticles. The obtained absorption spectrum was highly related to the past studies on green synthesis of ZnONPs [174, 175]. When the concentration of leaf extract increased, it also enhanced the phytochemical content of the leaf extract and it can quickly reduce the precursor which can increase synthesis of nanoparticles rapidly and

improved the absorbance value [176]. In a previous study, ZnO nanoparticles synthesized using *Bryophyllum pinnatum* plant extract show maximum absorption at 307nm [177].

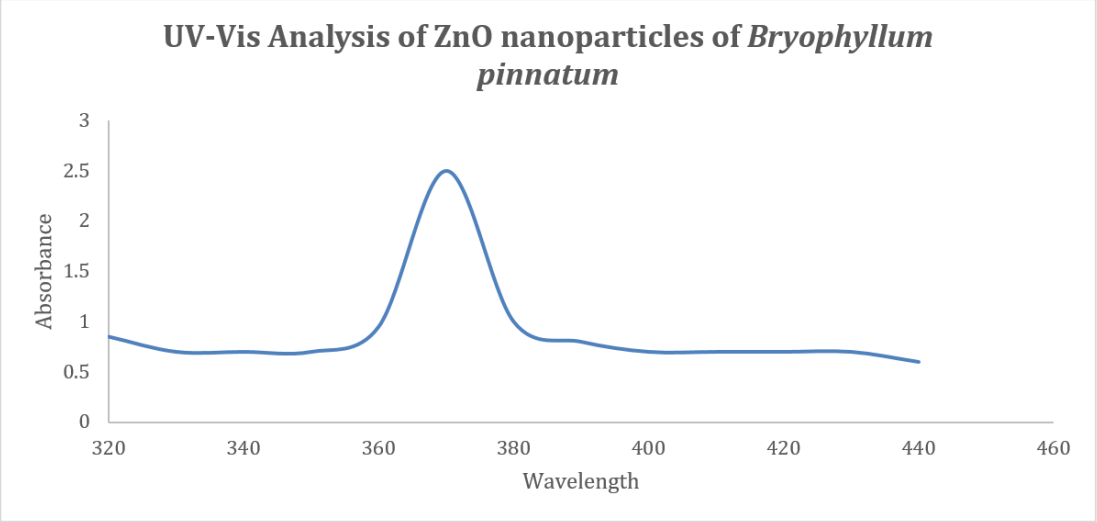

FIGURE 4.1: Uv-Vis Analysis of ZnO Nanoparticles

4.2.2 Zinc Oxide Nanoparticles Analysis through SEM

The Scanning Electron Microscope (SEM) is a highly used technique in many industries and laboratories to measure the microstructural morphology and chemical composition of specimens up to a nanometer scale. In SEM a beam of high energy electrons generated a variety of signals at the surface of the solid sample. These signals that due to interactions between electron and sample, possess information about the sample such as external morphology, crystalline structure chemical composition and orientation of the materials that make the sample [178, 179]. Scanning electron microscopy also confirmed the morphology and size of NPs. The scanning electron microscope images of ZnONPs showed size about 80nm (Figures 4.2), which demonstrated the presence of triangular-shaped ZnONPs.

An earlier study reported the synthesis of ZnONPs from *P. caerulea* leaf extract, which showed that the ZnONPs have spherical shape with an average diameter of 70 nm [119]. In another study, SEM analysis of ZnONPs synthesized by

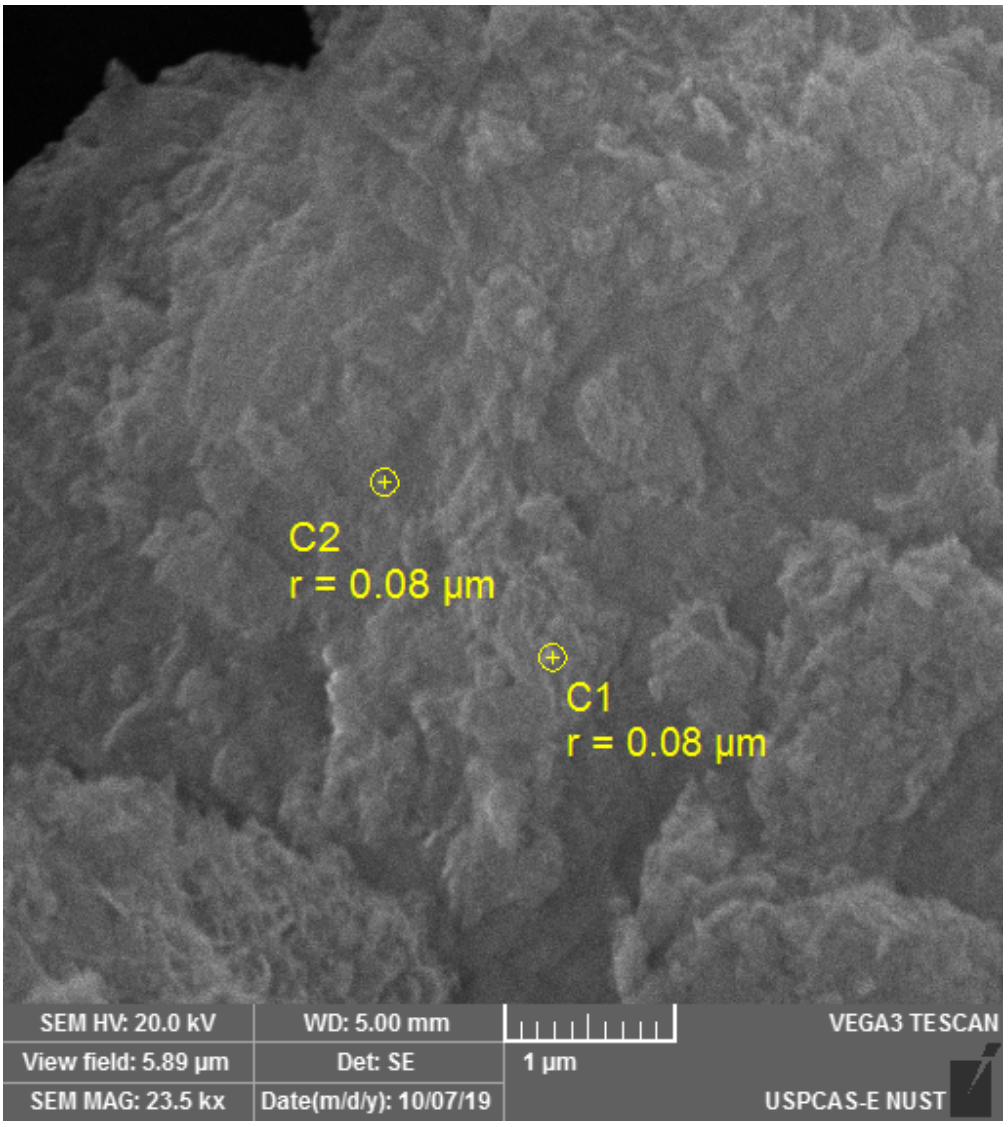

FIGURE 4.2: SEM Analysis of ZnO Nanoparticles.

Azadirachta indica leaf extract showed the synthesis of hexagonal wurtzite shaped ZnONPs and the size of the NPs was 20-45 nm [180]. In a similar study, synthesis of ZnONPs by *P. niruri* leaves extract, SEM analysis revealed that synthesized NPs have spherical and cylindrical shaped nanocrystals and size ranged to 5 μm in diameter [181]. It was reported that the green synthesis of ZnONPs by *Parthenium hysterophorus* leaf extract showed the ZnONPs have different shapes such as quasi-spherical, radial and cylindrical and with variations in size. These NPs were existed in small aggregate or cluster form [182]. It was reported that the ZnONPs was synthesized by *Moringa oleifera* leaf extract, it showed synthesized ZnONPs were spherical with crystalline morphology and with a diameter of 52nm [183].

SEM analysis of different ZnONPs synthesized by using different plants leave extract, demonstrated that the nanoparticles showed variations in morphology because each leaf extract has different bioactive elements.

4.2.3 Zinc Oxide Nanoparticles Analysis through EDX

Energy dispersive x-ray spectroscopy (EDX) is a widely used technique for the analysis of the elemental composition of biosynthesized ZnONPs. It gives a map of multiple specific chemical elements at particular assigned spot. It also gives a demonstration for the determination of the relative and absolute concentration of all elements [184]. EDX spectrum (Fig. 4.3) revealed the presence of zinc and oxygen signals in the ZnONPs prepared using *B. pinnatum* leaf extract. EDX analysis confirmed that the synthesized nanoparticles are ZnONPs.Elemental composition of the sample is shown Table (4.1).

Table 4.1: Elemental Composition of ZnONPs

Element	Weight%	Atomic%
C K	28.21	46.53
O K	33.90	41.98
Zn K	37.89	11.48
Totals	100.00	

From the figure, we can observe the peaks of zinc and oxygen along with carbon may be because carbon-coated gird capping agents utilized in scanning electron microscope measurements. We can clearly notice the major element is zinc along with oxygen, which confirms the formation zinc oxide nanoparticles [185]. Another group of researchers performed the synthesis of ZnO nanoparticles using garlic Skin and EDX analysis show 78% zinc and 22% oxygen signal which confirmed the elemental composition of ZnONPs [186]. In another study the green synthesis of ZnONPs using plant leaf extract, the EDX analysis confirm the elemental composition of ZnONPs, indicated that sample have 75.36% Zinc, 22.36%

Oxygen, and 2.29% Carbon [119], results which correlates with the present study.

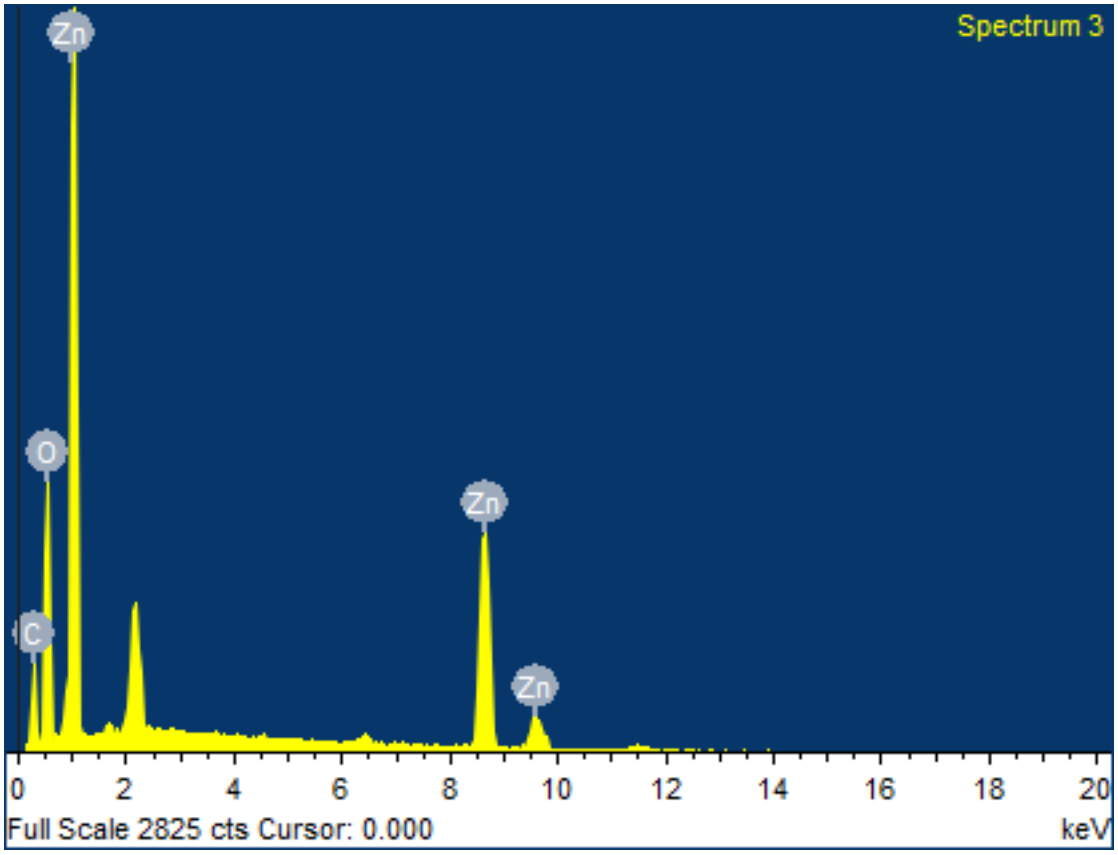

FIGURE 4.3: EDX Spectrum of Synthesized Zinc Oxide Nanoparticles.

4.2.4 ZnO Nanoparticles Analysis through XRD

X-ray diffraction (XRD) is the most commonly used technique for the characterization of ZnONPs. XRD identified the crystalline structure, lattice parameters, nature of the phase and crystalline grain size of the ZnONPs [187]. The broadening of the XRD peaks shows the presence of ZnO nanoscale particles. XRD pattern of the ZnO sample was observed by using index POWDER-X software as well as matched with standard data (JCPDS, 36–1451). The XRD pattern of powder ZnONPs was shown in Fig 4.5. All the peaks of the XRD patterns were identified as (002), (101), (102), (110), (200), (112), (103), and (004) indexed to ZnO with the hexagonal wurtzite structure shown in figure (4.4). Further analysis indicated that no other impurity or extra peak was detected which proved that

the obtained product is of high purity. The crystalline measurement was done by using Scherrer equation Dc=0.9 $\lambda/\beta\cos\theta$ [188].

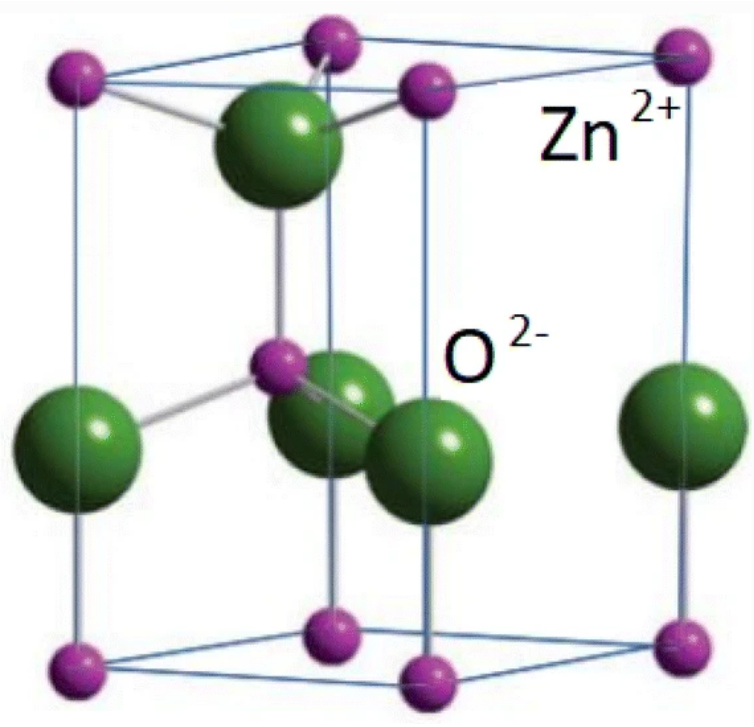

FIGURE 4.4: Hexagonal Wurtzite Structure of ZnO[189].

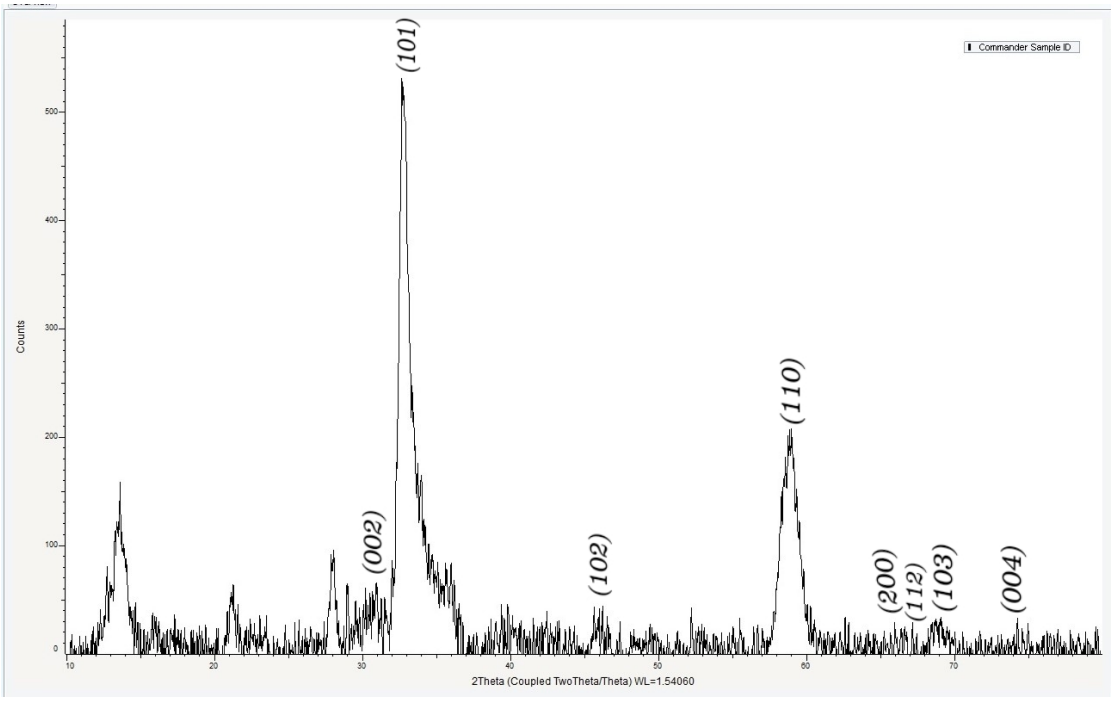

FIGURE 4.5: XRD Peak Diffractogram of ZnO Nanoparticles

In another study the green synthesis of ZnONPs using *Bryophyllum pinnatum*, the crystalline nature of ZnO nanoparticles was confirmed from X-ray diffraction (XRD) analysis [177] results which correlates with the present study.

4.3 Biological Assays of ZnONPs

4.3.1 Antibacterial Assays

Antibacterial activity of biologically synthesized ZnONPs was measured by using disc diffusion method. Antibacterial assay was performed on six strains of bacteria such as three-gram negatives strains *(A. tumefaciens, S. setubal, E. aerogenes)* and three gram-positive strains *(M.luteus, S. aureus, B. subtilis)* comparing with positive and negative control. The antibacterial activity of the synthesized ZnONPs is shown in Table (4.2). The antibacterial activity of ZnONPs depend on the surface area and concentration of the nanoparticles, while the crystalline structure and particle shape have less effect. Hence, increasing the concentration and surface area enhances the antibacterial activity of ZnONPs [190]. The toxic effect of ZnONPs against different bacterial strains was determined by comparison with control treatment. In antibacterial activity minimum zone of inhibition was observed at different concentration of ZnONPs such as 10ppm, 20ppm, 30ppm, 40ppm,50ppm and 100ppm. After overnight clear inhibition zone was observed, maximum inhibition was measured at 100ppm against both strains gram-positive *(M. luteus, S.aureus, B. subitils)* and gram-negative *(A. tumefaciens, S. Setubal, E. aerogenes)*. Minimum zone of inhibition was observed against all the bacterium at 10 ppm [191]. It is reported that the antibacterial activity of ZnONPs is directly related to the concentration of nanoparticles and inversely related to the size of nanoparticles [192]. In another study, antibacterial activity was found to increase against both gram-negative and gram-positive bacterial strains with an increase in surface-to-volume ratio because of the decrease in particle size of ZnONPs [193]. ZnONPs have a more toxic effect against gram-positive bacteria as compared to the gram-negative bacteria [194, 195]. It has been reported that the higher sensitivity

of gram-positive bacteria is due to the difference in the cell wall, metabolism and cell physiology [196]. Another study demonstrated that ZnONPs prepared from the extract of *C. abyssinica* tuber having size 10.4 nm showed antibacterial activity against the gram-positive strains *B.coagulans, S. aureus* and gram-negative strains *S. dysenteriae, S. typhimurium and Sphingomonas paucimobilis.* It was observed that gram-negative show more resistance against ZnONPs as compared to gram-positive strains [197].

Nanoparticles utilize different procedures for attachment on the surface of both gram-positive and gram-negative bacteria due to the difference in transport inside the cell and difference in the structure of the membrane. As gram-positive bacteria cell wall contains thick layer of peptidoglycan and their cell consists of teichoic acid and lipoteichoic acid which facilitate the entry of ZnONPs inside the cell [198]. Cell wall of gram-negative composed of triple layer of peptidoglycan and also contain porins in the outer layer, these are ions channel that aid in passive diffusion of nanoparticles inside the cell [199].

Different mechanisms for antibacterial activity are proposed such as formation of the reactive oxygen species(ROS) [200, 201], interaction between ZnONPs and cell wall lead to loss of cellular integrity [20, 202], release of Zn^{2+}ions [22, 203] and internalization of ZnONPs [20]. Nanoparticles generate free radical which is the most common technique that is used to induced cell toxicity at the nano level. Reactive Oxygen Species (ROS) composed of hydroxyl ion OH^{-}, singlet oxygen 102, superoxide ion $O_2{}^{-}$ and peroxide H_2O_2 and these were produced under UV illumination [204, 205]. Due to the negative charge superoxide and hydroxyl ion enable to penetrate in the bacterial cell membrane, in contrast to these peroxide ions can penetrates in the bacterial cell membrane and cause the cell death [202,206]. Oxidative stress lead to the destruction of DNA, Lipid and Protein. Breakage of DNA into fragments such as breakage of single and double-strands, formation of adduct, cross-linkage of the DNA with proteins and the mostly damage of DNA due to the exposure of nanoparticle. First cell repair the DNA damaged but when cell is enable to reverse this situation it lead to cell death such as apoptosis or necrosis [207].

Table 4.2: Antibacterial activity of ZnONPs and plant leaf extract.

ZnONPs Con (ppm)	Zone of inhibition (cm)±S.E											
	Gram Positive Strains						Gram-Negative Strains					
	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	1.2 ±0.1	-	0.8 ±0.1	-	0.1 ±0.1	-	-	-	-	-	-	-
20	1.4 ±0.1	-	1 ±0.2	-	1.3 ±0.5	-	-	-	0.6 ±0.1	-	1 ±0.15	-
30	1.6 ±0.15	-	1.5 ±0.1	-	1.5 ±0.1	-	1.2 ±0.1	-	1.8 ±0.3	-	1.2 ±0.1	-
40	1.8 ±0.1	0.4 ±0.4	1.8 ±0.1	0.4 ±0.3	1.8 ±0.1	-	1.5 ±0.1	-	1 ±0.1	-	1.5 ±0.1	-
50	2 ±0.1	0.9 ±0.2	2 ±0.2	0.8 ±0.2	2.0 ±0.15	-	1.7 ±0.1	0.6 ±0.2	1.1 ±0.1	-	1.8 ±0.1	0.5 ±0.2
100	2.3 ±0.1	1.7 ±0.1	2.5 ±0.1	1.7 ±0.1	2.5 ±0.1	1.0 ±0.1	2 ±0.15	1.1 ±0.1	1.7 ±0.1	0.9 ±0.1	2 ±0.15	1.1 ±0.1
Negative control 100ppm	0	0	0	0	0	0	0	0	0	0	0	0
Positive control 100ppm	2	2	2.75	2.75	2.9	2.9	2	2	2	2	2.5	2.5

*M.luteus ;Micrococcus luteus; S.aureus Staphylococcus aureus; B.subtilis Bacillus subtilis; A.tumefaciens Agrobacterium tumafaciens ; S.Typhi Salmonella typhi; E.aerogenes Enterobacter aerogene*s ZnONPs=zinc oxide nanoparticles, p.extract = plant extract

In a study another study of the antibacterial activity of ZnONPs was reported in this mechanisms release of Zn^{2+} ions cause the inhibition of bacterial growth. In solution partial dissolution of ZnONPs lead to the release of Zn^{2+} ions, which possess antibacterial activity. Antibacterial activity due to the dissolution of ZnO [203,208] by reducing the metabolism of amino acid and lead to enzymatic system [209].

Antibacterial mechanisms of ZnONPs related to the relationship between ZnO particle and the cell wall, lead to the loss of bacterial cell integrity. The toxicity of ZnO against the bacteria cells reported that damaged bacterial cell cause the membrane disorganization which lead to the increase in membrane permeability and resulting the ZnONPs accumulation in the bacterial cell membrane as well as the NPs internalization [20, 203]. In a study it was reported that, *S. aureus* interaction with ZnONPs results in formation of holes in the cells membrane [210]. ZnO NPs toxicity is not always due to the internalization in the bacterial cell membrane. ZnO NPs can alert the environment that is close to the bacteria, generating the ROS or enhanced the ZnONPs solubility, result in cellular damage [211, 212].

4.3.2 Antifungal Assay

Fungus exhibited high resistance against many traditional fungicides such as dicarboximides and benzimidazoles, so it is very difficult to control fungal growth. To reduce or minimize this resistance, it is important to use some strategy that can replace these conventional antifungal agents [213]. Nowadays nanoparticles have gained much attention because of their novel physical and chemical properties [214]. Present study was conducted to evaluate the antifungal activity of synthesized ZnONPs against different fungal strains which are responsible for several fungal diseases. The leaf extract of *B. pinnatum* mediated synthesis of ZnONPs were tested against five different fungus strains. It was observed that ZnONPs showed a maximum inhibitory effect against the *A.flavis* 60%.While inhibition observed against the Mucor species was 43%. ZnONPs also showed inhibition

against the other three strains *A.Niger, A.Fumigatus*, *F. Solani* the such as 45%, 40%, and 50% respectively.

The results are mentioned in (Table 4.3). For comparison, the extracts of *B. pinnatum* leaves, positive and negative control were used. Plant extract of *B. pinnatum* leaves only exhibited a significant effect against three strains of fungi. In the case of leaf extract, the maximum antifungal activity was observed against *Fumigatus* 25%, *Solani* 20%, *Flavis* 15%, *Mucor* 10% and *Niger* 5% (Table 4.3).

TABLE 4.3: Precentage inhibition of ZnONPs and plant leaf extract (*B.pinnatium*) aganist diferent fungal species

S.No	Samples	Percentage inhibition against Fungal Species (%)				
		Mucor.sp	*F.Solani*	*A.fumigatus*	*A.Flavis*	*A.Niger*
1	ZnONPs	43	50	40	60	45
2	Plant extract	10	20	25	15	5
3	Distilled water (-ve Control)	-	-	-	-	-
4	Positive control (terbinafine)	100	100	100	100	100

In a study antifungal property of prepared ZnONPs was reported against *B. cinerea and P. expansum.* After application of ZnONPs alteration in morphology and cellular composition of hyphae was observed by the help of Scanning electron microscopy (SEM) and Raman spectroscopy. ZnONPs inhibited growth of both strains but was found more toxic against *P. expansum.* The hyphae malformation was observed in case of *B. cinerea*, referred to inhibition that impact on cellular function and cause enhanced nucleic acid obtained from the hyphae due to oxidative stress. In case of *P. expansum* ZnONPs inhibited the growth of conidia and conidiophores. These susceptibility difference was due to genetic tolerance and morphological growth difference [22]. In another antifungal activity of ZnONPs is reported against *Candida albicans* due to the ROS. Oxygen radicals removed by histidine and added into *Candida albicans* culture to observed the inhibitory effect

on toxicity generated by ZnONPs. It showed that 5nM of histidine cause the inhibition of the antifungal activity of ZnONPs. Researcher reported that generation of ROS in aqueous media cause cell death. It was also reported that when ZnONPs contact with visible light leading to increased cell death rate [215]. Mechanisms of action of the antifungal activity of ZnONPs was reported according to it by the process of diffusion and endocytosis ZnONPs penetrate into the fungal cell, when these particles reached into the cytoplasm of the cell, these can also take part in mitochondrial function, result in generation of ROS and Zn^{2+}. These generated ions enter into nuclear membrane and penetrate into the DNA, leading to nuclear destruction such as irreversible chromosome damage which cause death of the cell [216].

4.3.3 Antioxidant Assay

Antioxidants can cause the inhibition of the production of reactive oxygen species (ROS), changing intracellular redox state and scavenging of free radicals [217]. Antioxidant activity of synthesized ZnO NPs was measured by the DPPH (2,2 dipheny l-1-1 picryl hydrazyl) assay. In antioxidant property the synthesized ZnONPs donate an electron of oxygen toward the hydrogen. Synthesized nanoparticles donate electron and DPPH accept these electron, DPPH is one of the stable free radicals and become stable diamagnetic molecules [218]. The synthesized ZnONPs and plant leaves extract both exhibited significant free radical scavenging activity. In present study the antioxidant activity of synthesized ZnONPs evaluated along with the plant leaves extract *B. pinnatum*. The synthesized ZnONPs showed greater free radical scavenging activity as compared to plant leaves extract. At maximum concentration 300ppm ZnONPs showed scavenging activity up to 88.5%, while at 300ppm concentration the leaves extract of *B. pinnatum* plant showed 56.3% free radical scavenging activity. At the 200ppm concentration the ZnONPs exhibited the 74.7% free radical scavenging activity and extract of *B. pinnatum* leaves showed 42.5% free radical scavenging activity at 200ppm concentration. At lowest concentration 100ppm ZnO NPs exhibited 47.1% free radical

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

Source of variation	Df	Sum of squares	Mean square	F-Value	P-value	Significant
Interaction	4	1392	348.0	39.81	0.0001	Yes
ZnONPs	2	13830	6917	791.4	0.0001	Yes
Concentration	2	2588	1294	148.0	0.0001	Yes
Residual	18	157.3	8.741	_	_	_

scavenging activity and plant extracts of *B. pinnatum* leaves showed 26.4% free radicals scavenging activity as shown (Table 4.4). IC_{50} of ZnONPs were 136ppm, plant extracts have IC_{50} 227ppm and IC_{50} of standard (Ascorbic acid) is 55.89ppm.

TABLE 4.4: %age scavenging and IC_{50} of zinc oxide nanoparticles, leaves extracts *B.pinnatum* and ascorbic acid against DPPH

Samples	Percentage scavenging			
	100ppm	200ppm	300ppm	IC_{50}(ppm)
ZnO NPs	47.1	74.7	88.5	136
Plant extract	26.4	42.5	56.3	227
Ascorbic Acid	100	100	100	55.8

As results showed that plant leaves extract have greater value of IC_{50} which indicate that ZnONPs have more effective antioxidant property as compared to plant extract (Table 4.4).

It was also noted that the free radical scavenging activity of ZnONPs increased with increase in ZnONPs concentration. It was reported in a previous study that tuber extract of *Coccinia abyssinica* based ZnO nanoparticles possessed significant antioxidant activity having IC_{50} Value of 127.74 μg/ ml, antioxidant activity increased with increase in concentration of ZnONP [197]. The obtained results were statistically significant($P<0.0001$) (Table 4.5).

Figure 4.6 showed percentage scavenging of Zinc oxide nanoparticles and plant leaves extract against DPPH. It was studied that metal ion (Zn) is used by enzymes as a cofactor, scavenge H_2O_2 free radicals, and the nanoparticles having Zn ion

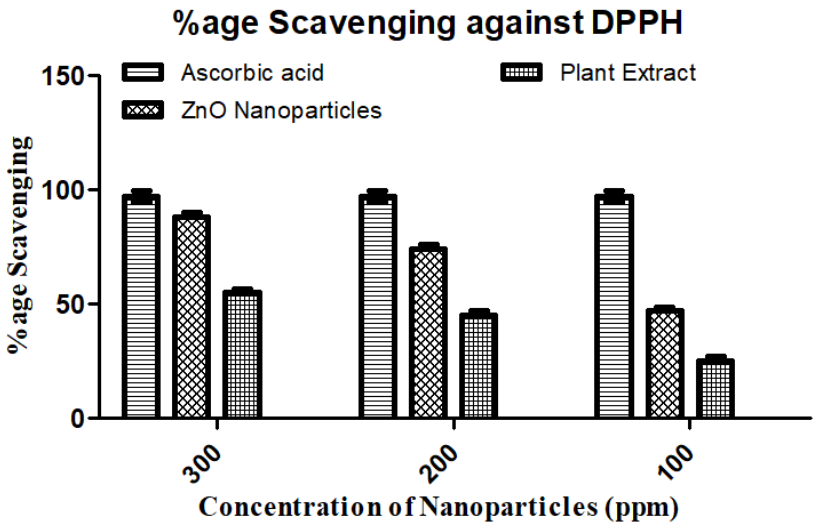

FIGURE 4.6: %age scavenging Of Zinc Oxide Nanoparticles and plant leaves extract against DPPH

may increase scavenging activity against H_2O_2 free radical as compared to the plant [219]. It is reported that plant extracts have phenolic compounds which have high antioxidant activity and they also are involved in the green synthesis of nanoparticles [220].

It was reported that the antioxidant activity may be due the electrostatic attraction between positively charged nanoparticles ($ZnO = Zn^{2+} + O_2{}^-$) and negatively charged bioactive compounds (COO^-, O^-) of plant. Bioactivity of ZnONPs have increase synergistically when they bound to the phytochemicals [221, 222].

4.3.4 Cytotoxic Assay

Toxicity of ZnONPs was evaluated by brine shrimp cytotoxic assay. Different concentrations of ZnO nanoparticles and plant extract were used such as 50ppm, 100ppm and 250 ppm and showed significant toxic effect. At the highest concentration of 250ppm ZnONPs showed the 70% mortality of brine shrimp and *B. pinnatum* leaves extract showed 33.33%. At 100ppm concentration, ZnONPs exhibited the 56.6% mortality and plant extract showed the 26.6% mortality. At the lowest concentration of 50ppm ZnONPs exhibited 40% mortality and plant leaves showed 3.33% mortality. Hence, ZnONPs exhibited more mortality rate at 250ppm

TABLE 4.6: Analysis of Variance for Factor Affecting the Mortality of Brine shrimps

Source of variation	Df	Sum of squares	Mean square	F-Value	P-Value	Significant
Interaction	4	957.0	239.3	52.52	0.0001	Yes
ZnO Nano particle	2	14060	7031	1543	0.0001	Yes
Concentration	2	1807	903.3	198.3	0.0001	Yes
Residual	18	82.00	4.556	_	_	_

TABLE 4.7: %age Mortality and IC_{50} of ZnO nanoparticles and plant extract

Samples	Percentage Scavenging			
	50ppm	100ppm	250ppm	IC_{50} (ppm)
ZnONP	40	56.66	70	56.63
Plant extract	3.33	26.66	33.33	298.69

70% as compared to plant extract 3% as shown in Table 4.7. It was observed that results were significant statistically ($P<0.0001$) (Table 4.6) and IC_{50} for ZnONPs were 439.8 ppm and plant extract have IC50 540.2. As plant extracts have greater IC_{50} value which indicated that ZnONPs showed more effective results than plant extract and so, in future it can be used on cancer cell lines (Table 4.7).

ZnONPs synthesized using aqueous extract of *Deverra tortuosa* have shown remarkable cytotoxic potential against two cancer cell lines such as human colon adenocarcinoma "Caco-2" and human lung adenocarcinoma "A549" and the study also revealed that cytotoxic activity was due to synergetic action of both nanoparticles and the phytocompounds that were attached on the surface of nanoparticles [223]. The cytotoxic mechanism of ZnONPs were not fully understood yet, however it was study that from the ZnONPs generation of superoxide anion (O^{2-}), hydroxyl radicals and perhydroxyl radicals these were main components of cytotoxic effect. When nanoparticles penetrate into cells, cellular immune system or protection mechanisms were stimulated to minimize harm. When the production of highly active free radicals lead to the antioxidative defensive ability of the cell, which cause oxidative damage to the biomolecules and lead to the cell death [224, 225]. It was observed that increased concentration is directly associated with increased mortality rate (Figure 4.7).

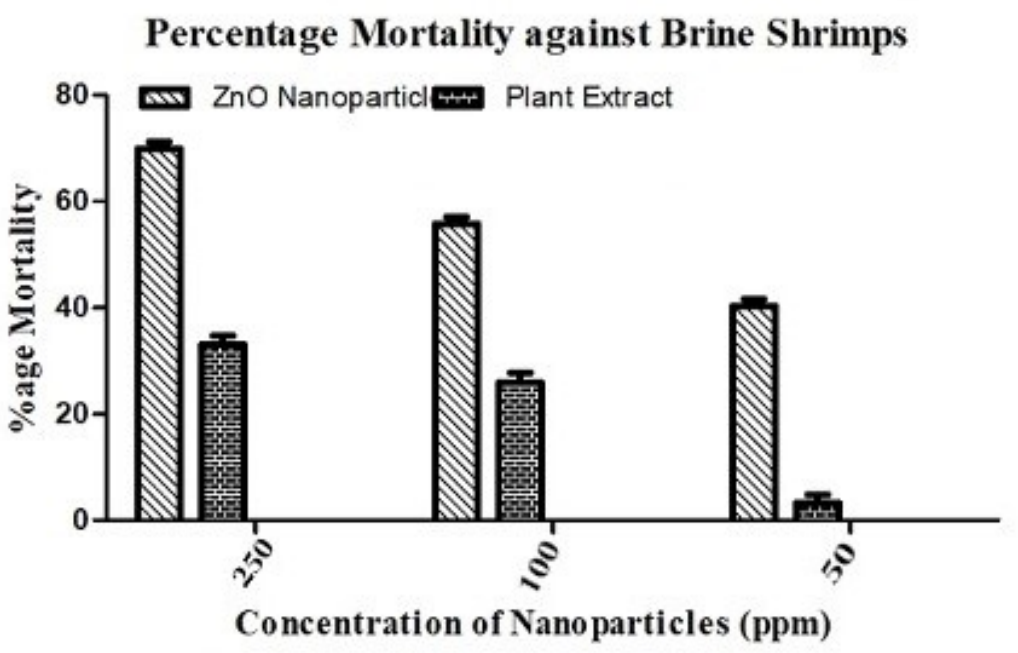

FIGURE 4.7: %age mortality Of ZnONPs and seed extract against Brine Shrimps

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Green synthesis approach was used in the present research to synthesize zinc nanoparticles from the *B. pinnatum* extract due to their oxidizing and reducing abilities. It was noticed that synthesized zinc nanoparticle, showed absorption peak at 370nm confirmed by Uv-Vis spectroscopy, an average size of 80nm and triangular in shape as confirmed by SEM analysis. The XRD analysis confirmed crystalline nature of the zinc nanoparticles. The synthesized zinc nano particles exhibited significant antimicrobial, antifungal, antioxidant as well as cytotoxic activities with lesser IC_{50} and MIC values as compared to plant extract.

5.2 Recommendations

Synthesized ZnO NPs have exhibited significant antimicrobial, antifungal, antioxidant and cytotoxic activity, current research can be explored further for development of better antifungal and antibacterial, anti-cancerous drugs with enhanced efficacy can be explored in future as medical. Moreover, the smooth morphological

features can also be used as drug carriers in controlled and targeted drug delivery systems in future.

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