CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Therapeutic Evaluation of Silver Nanoparticles Synthesized Using *Bryophyllum pinnatum* Plant Extract

by

Huma Noor

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

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

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Dedicated to Allah Almighty, Hazrat Muhammad (SAW) and my Parents whose utter support and sheer trust helps me to achieve this.

CERTIFICATE OF APPROVAL

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Abstract

Although the use of nanoparticles through the ages has lead to significant scientific discoveries recent breakthrough have opened a new era of revolutionary developments in the field of medicine, technology as well as environmental studies. Preparation of nanoparticles can be carried out by physical, chemical and biological methods but among these biological methods considered to be most preferred because they are ecofriendly, reproducable and produce stable nanoparticles. *Bryophyllum pinnatum* has long been used locally for wound healing and aseptic properties. İn this study we have used leaf extract with silver nitrate salt in a fixed ratio to produce silver nanoparticles. Characterization of synthesized nanoparticles was carried out by various physical techniques i.e UV visible spectrophotometer, FTIR, SEM, EDS and XRD. Antibacterial, antifungal, antioxidant and cytotoxic assays of synthesized particles were carried out to accertain their therapeutic significance. Absorption peak of synthesized silver nanoparticles was obtained between 400–450nm using UV vis spectrophotometer with average size 54±4 nm and having round shape analyzed through SEM. XRD studies confirmed the crystalline nature of particles with crystallite size 20nm. Presence of hydroxyl group and alkene as major functional groups were reported through FTIR and silver as major constituent was confirmed through EDS. Significant antifungal, antioxidant, antibacterial and cytotoxic potential was also confirmed and can be further explored in various applications.

Keywords: Antibacterial, Antifungal, Antioxidant, *Bryophyllum pinnatum*, Cytotoxic

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Abbreviations

µl	Micro Litre
$AgNO_3$	Silver Nitrate
AgNPs	Silver Nanoparticles
BBB	Blood Brain Barrier
CVC	Central Venous Catheters
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
UV-vis	Ultra Violet Visible Spectroscopy
XRD	X-ray Diffraction

Chapter 1

Introduction

1.1 Background

Nanotechnology is vast and innovative area which involves design, characterization, production and application of structures, devices and systems by regulating their shapes and size at nanoscale i.e 0.2 nm to 100 nm and nanobiotechnology has application in the field of biology. Because substances behave completely differently when they are at nanoscale compared to larger scale material just because of their large surface to volume ratio, increased reactive potential or stability in chemical processes and enhanced mechanical strength. Their use in variety of fields has been widely investigated. Nanobiotechnology is being used in new molecular imaging techniques for diagnosis of different diseases, as quantitative analytical tools to study the cell at molecular level and to find out better drug delivery systems for targeted and personalized therapy. The advancement in medical science and the discovery of new and old microorganisms with resistance to currently existing drug is making this field of vital importance for providing new arenas of research and development. Nanoparticles are being studied to make cancer a curable ailment. Silver nanoparticles have attracted greater interest recently in biomedical applications because of their increased electrical and thermal conductivity, surface enhanced resonance, chemical stability, catalytic activity and non linear optical

behavior [1]. They also have broad spectrum bactericidal and fungicidal activity [2]. They have also shown promising virucidal activity against HIV1, tacaribe virus, hepatitis B virus and influenza A/ HIN1 virus, herpes simplex and vaccinia virus [3]. They are also believed to have antitumor activity against number of cancerous cells like leukemic cells, MCF7 breast cancer cells etc. Currently they are used for wound dressing applications to avoid delayed diabetic wound healing [4] and to make CVC's (central venous catheters) with antibacterial properties [5, 6]. They also have got immense importance in the field of dentistry where they prevent black staining in teeth and prevent tooth decay due to their antimicrobial properties [7]. Their importance in field of orthopedic cannot be denied due to their efficient role in bone healing process by the differentiation of various bone cells and also promote the bone tissue mineralization process [8].

Nanoparticles synthesis can be carried out by top down or bottom up approaches. Various factors control their synthesis. Physical, chemical and biological methods can be employed. Out of all these, biological methods have considered as the best choice because they are simple, worthwhile, dependable and environmental friendly and also produce nanoparticles that are high yield and of defined size. Bacteria, fungi and plants can be used, while fungi produce larger amount of nanoparticles because of enzymes and proteins they secrete [9]. Plant extracts are one of the safest option and are quicker than microbes and other agents for synthesis of nanoparticles.

Medicinal plants use for the synthesis of nanoparticles is a form of green synthesis. Medicinal plants are those biological materials that have their usage either directly or after processing for treatment of diseases at either local or regional scales [10]. They have been used since ancient times in China, Greece, Egypt and India. Green synthesis is required because physical and chemical methods often lead to various toxic chemicals with adverse outcomes when used for medical applications [11]. Green synthesis donot require conditions that have economic burden like high pressure, energy, temperature and avoid production of toxic chemicals. Pakistan has approximately 6000 taxa of flowering plants. Studies show that round about 600 to 1000 plants possess medicinal properties but only 12% of them are used for

different pathological conditions [12]. However 350 to 400 species are locally used in herbal medicine [13].

Bryophyllum pinnatum belongs to genus *Bryophyllum* and family Crassulaceae. Plants of this genus have been used across the world for the treatment of various ailments like crused leaves for treatment of headache, pulmonary infection, rheumatoid arthiritis and gastric ulcers [14]. Extensive research have been done on the leaves of *B.pinnatum* but all parts have their use in various medical applications. Leaves are used for treatment of diabetes and heart diseases, decoctions for hypertension, use in combination with palm oil for abscesses and swelling and also used for treatment of jaundice. Leaves have antipyretic properties and have antimalarial effect. In traditional systems, leaves are extensivley used for their potential as antimicrobial, antifungal, anti ulcer, anti inflammatory and analgesic properties. They also have their potential against various worms and root infusion used for epilepsy. Phytochemical studies have extensively done for *B.pinnatum* that shows they possess flavonoids, tannins, coumarins, saponins, triterpenes, cardienolides etc [15].

1.2 Problem Statement

In presenet era, number of antibiotic and antiviral drugs are available in the market. The extent to which they are effective is still a question due to increasing resistance developed among the microbes. Many problems are associated with conventional drugs including potency, availability and solubility. To cope up with these issues, in present context we necessitate ourself to synthesize new agents that proves to be more effective and long lasting in their effect with no serious side effects. To achieve that purpose, we synthesized silver nanoparticles using green synthesis approach and then performed physical and biological evaluation of synthesized particles.

1.3 Aims

Nanotechnology is an exciting development with nanoparticle as its basic fundamental component. Nanoparticle synthesis have been considered as an important area of nanotechnology. Present study aims to synthesize silver nanoparticles by green chemistry approach and then to examine various features of silver nanoparticles including size, shape and morphology that affect their capacity of performing various biological activities. Present investigation expected to synthesize silver nanoparticles and to check out their potential as cytotoxic, antioxidant, antibacterial and anti fungal agents. Main motivation behind this investigational approach was to produce nanoparticles with maximum efficiency and least financial burden.

1.4 Objectives

1. Silver nanoparticles synthesis by green chemistry approach using *Bryophyllum pinnatum*.

2. Synthesized silver nanoparticles characterization through UV Vis spectrophotometer, Scanning electron microscopy(SEM), Energy dispersive X ray spectroscopy(EDX), Fourier transform infrared spectroscopy(FTIR) and X ray diffraction spectroscopy(XRD).

3. Biological assessment of synthesized silver nanoparticles by applying different bioassays including antibacterial, antifungal, antioxidant and cytotoxic assay.

1.5 Scope

Advancement in nanotechnology helps us to design and synthesize silver nanoparticles with distinct antimicrobial, optical and physical properties that allow their extensive use in medicine and many exclusive fields like bone healing, targeted drug

delivery, food preservation, wound healing and many more. Nanoparticles are designed with their potential as effective diagnostic tool and treating life threatening diseases like cancer with their outstanding property to cross blood brain barrier (BBB) ensuring maximum safety and efficacy.

Chapter 2

Literature Review

2.1 Nanobiotechnology

Nanobiotechnology is combination of two far off but existing fields of molecular biology and engineering which leads to development of advanced multipurpose systems and devices that have enhanced properties like sensitivity, specificity and higher rate of recognition and actually meant to improve the biological and chemical analysis [16]. These materials devices and systems are functionally organized in such a way that they possess at least one dimension in nanometer scale range. Due to this technology new therapeutic opportunities are opening up for many agents that cannot give better results using as conventional formulations due to poor functionality. Most commonly used nano objects in field of nanobiotechnology include nanotubes, nanochannels, nanoparticles, nanopores, and nanofibers [17]. Nobel laureate Richard P. Feynman presented nanotechnology while delivering his world renouned lecture " There is plenty of room at the bottom" [18], since then a lot of various revolutionary developments have been made in the field of nanotechnology. This is comparatively a new field and offers profound scope for various technical advancements in the field of human health proving to have a keen influence on disease prevention, diagnosis and treatment. Not only in the area of medicine but also in area of food and nutrition nanomaterials have proved

to enhance features like adding new tastes and flavors, manufacturing functional foods as well as hygienic processing and packaging of food items, detection of pathogenic organisms and their subsequent removal, smart light weight packaging of items and reduced agrochemicals, colors, artificial flavors and preservatives [19]. This technology has its use in enzyme immobilization, green synthesis of inorganic nanoparticles, preparation of nanoemulsions and their encapsulation, synthesis of nanosensors and packaging [20]. Nanomaterials are thought as solution to many technology related and environmental issues like in the field of solar energy conversion, catalysis, medicine, and water treatment. An increasing demand of nanomaterials can be accompanied by green synthesis approach with regards to global efforts to minimize the hazardous waste material. Bottom up approach helps to fabricate various building blocks that are in nanoscale range into functional structures and further into multifunctional assemblies and devices thus exploring new fields of research area.

2.2 Nanoparticles

Nanoparticles are fundamental components of nanotechnology. They serve as the primary components in the construction of nanostructures. These particles are chiefly composed of carbon, metals, metal oxides or organic matter and have a size ranges between 1 to 100nm [21]. There is significant difference between physical, chemical and biological properties of these particles at nanoscale as compared to their respective counterparts at macroscale. The reason for this difference is the relatively large surface to volume ratio, enhanced reactivity or stability during chemical processes as well as increased mechanical strength. Together these properties of the nanoparticles being allowed them to have applications in different fields. Nanoparticles are complex molecules that are made up of three different layers with the first layer called surface layer which can be made functionalized with a number of different small molecules, metal ions, surfactants and with use of polymers. Next layer is second layer and is called shell layer and that layer chemically differ from innermost layer. Core is innermost layer that is essentially

in the center of the nanoparticles and usually refers to the nanoparticles itself [22]. These characteristics make them of immense interest to the researchers in different disciplines. Metallic nanoparticles are another class of nanoparticles which differ from bigger metals in their physical and chemical characteristics like they have lower melting points, higher specific surface area, specified optical properties, mechanical strength including particular magnetization which set them apart from use in various industries. Nanoparticles can have different dimensions with zero dimensional particles being those whose length, breadth and height are fixed at a single point for example nanodots. One dimensional nanoparticles are those which can have only one aspect e.g graphene. Two dimensional particles have both length as well as breadth and include nanotubes whereas three dimensional particles include all aspects like length, breadth and height an example of which is gold nanoparticles.

2.3 History of Nanoparticles

Although it is commonly thought that nanoparticles are discovery of 20th century, various studies have revealed that as early as 9th century BC artisans in Mesopotamia were using finely divided material to produce sparkling effect on the surface of ceramic vessels. Specific silver and copper nanoparticles concentrated within the outermost layer called glaze imparted amazing optical properties to these decorative objects. Later on fine organic and inorganic dust particles which were very close to the size of nanoparticles were developed for use in different techniques of visuals art and painting [23]. One such example is of clay minerals which are only a few nanometers thick and hence are one of the finest representatives of natural nonmaterials in use since ancient times. It had been observed that clay had been used to bleach wools and garments even in 5000 BC in Cyprus [24]. In 1875 Michael Faraday published a paper in which he described the optical properties of nanometric metal particles scientifically thus becoming the first person to do so [25]. James clark Maxwell put forward the round of concepts of differentiation in nanotechnology ten years later i.e in 1876, but he did not use term nanotechnology

in order to define thin monomolecular layers [26]. Richard Adolf Zsigmondy used a technique known as dark field ultramicroscopy, later on this technique aided in the visualization of particles which were smaller in size when compared to the wavelength of monochromatic light. Thus this method enabled him to visualize 1/1000000 nm particles and applied the term nanoparticles to such particles for the very first time. In the year 1908, Mie explained why different metal colloids have different colors. In 1940's carbon black was substituted by SiO2 nanoparticles for rubber reinforcement [27]. Nowadays manufactured nanomaterials are being used to notably enhance the qualities of bulky materials like their strength, conduction ability and endurance as well as imparting useful self healing, self cleaning, antibacterial and anti freezing characteristics. A company named Samsung launched an antibacterial methodology in 2003 with the brand name Silver NanoTM in various appliances like air conditioners, refrigerators, washing machines, air purifiers and vacuum cleaners using ionic silver nanoparticles [27]. By the end of 2003, nanoparticles based clear coat was used by Mercedes Benz for production of both metallic and non metallic paint finishes. Such coats are more scratch resistant and glossy. Another advancement was made that includes an external iPad keyboard which was powered by light and was brought in the business by Logitech in the summer 2012, it represented the first significant market use of dye sensitized solar cells. By 2014, the number of commercially available nanotechnology based consumer products had already increased to 1814 and were available in over 20 countries [28].

2.4 Classification of Nanoparticles

Nanoparticles can be classified on the basis of various characteristics like structure, size or chemical characteristics. Some of the better recognized groups of nanoparticles depending on their physical and chemical properties are discussed below (Figure. 2.1).

Fullerenes and carbon nanotubes are two of the main groups of carbon based nanoparticles. Fullerenes consist of nanomaterial that is made of a spherical hollow cage like allotropic forms of the carbon. These are of notable commercial value because they have higher electrical conductivity, strength, unique structure, electron affinity and diversity [29]. Carbon nanotubes appear to be tubular in shape and have a diameter range between 1–2nm. Their peculiar physical, chemical and mechanical characteristics allow them to be used in original form as well as in the form of nanocomposites that have wide range usage in commercial applications including fillers [30, 31], and also have application in environmental remediation as efficient gas absorbents [32].

Metal precursors are the chief component of metal nanoparticles. These particles have distinctive optoelectrical properties because of their well known localized surface palsmon resonance characteristics. Metal nanoparticles can be synthesized by controlling their facet, size and shape in latest and advanced materials [32]. They are widely used in many research areas because of their advanced optical properties e.g. SEM technology uses gold nanoparticles for sampling in order to enhance the electronic stream to obtain high quality images.

Ceramic nanoparticles are inorganic nanometallic solids which are made via heat and subsequent cooling. They can be found in variety of structures for example as amorphous, polycrystalline, compact, porous or hollow structures [33] and have gained importance because of their utility in catalysis, photocatalysis, photodegradation of dyes and the process of imaging [34].

Semiconductor materials are a class of nanoparticles which have properties between those of metals and nonmetals due to which they are used in various applications [35] They have wide band gaps and hence their properties can be considerably changed by tuning their band gap. These qualities enable them to be use in photo catalysis, photo optics and electronic devices [36]. They are found to be extraordinarily effective in water splitting applications because their band gap and band gap positions are highly suitable for this use [37].

Among the organic based nanoparticles, polymeric nanoparticles are the important to be considered. Mostly they have a nanospherical or nanocapsular shape. The overall mass in nanospheres is mostly solid and outer molecules are adsorbed on the outer boundary of the surface of sphere. On the other hand, in nanocapsules, solid mass is completely enclosed inside the nanoparticles [38]. Lipid moieties are chief component of lipid nanoparticles which enable them for their use in several biomedical applications. Generally these are spherical in shape having a diameter from 10 to 1000nm. They have solid core that contains lipids and matrix which contains soluble lipophilic molecules. Surfactants or emulsifiers stabilize outer core of these nanoparticles [39]. Lipid nanotechnology is specialized field that deals with designing and creation of lipid nanoparticles used in several diverse fields for example as drug carriers and in drug delivery systems [40].

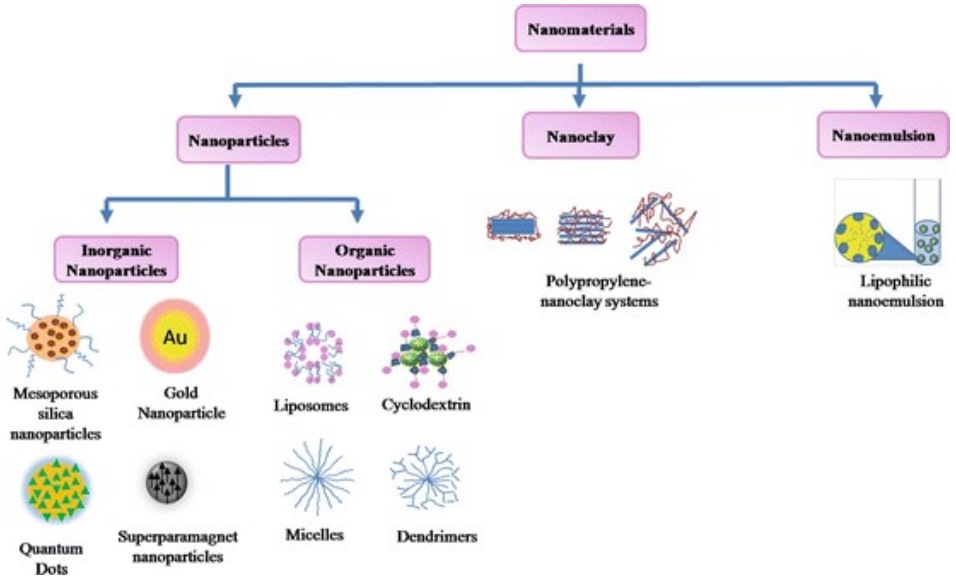

FIGURE 2.1: Classification of Nanomaterials [41].

2.5 Properties of Nanoparticles

Physical and chemical characteristics of nanoparticles are very important as they determine the different research areas where they can be utilized (Fig. 2.2). Physical properties include optical for example color of nanoparticles, its light penetration, ability to absorb and reflect light and UV rays in a solution or when coated

on any surface. Mechanical properties like elasticity, ductile, tensile strength and flexibility are also very important determinants of their applications. Many other physical characteristics like hydrophilicity, hydrophobicity, suspension, diffusion and settling properties are utilized for modern everyday purposes. Modern electronics and some other applications use the magnetic and electrical properties of these particles like conductivity, semiconductivity and resistivity. Size is one very important determinant of the characteristics of nanoparticles. Nanosizing is used to increase bioavailability, rate of diffusion, solubility and surface area at the same time decreasing the dose required.

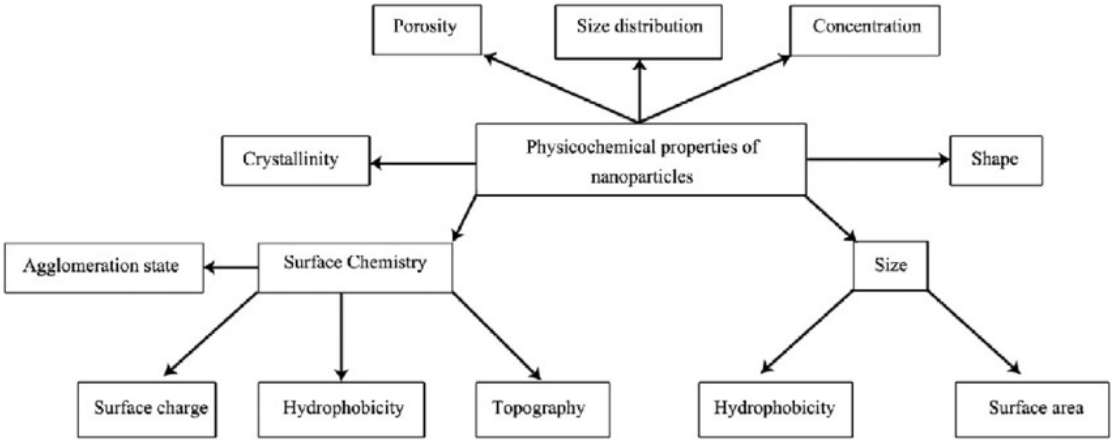

FIGURE 2.2: Various Physiochemical Properties of Nanoparticles [42].

Molecular and atomic assemblies like clusters, aggregates or filaments are defined by interaction of forces amongst the nanoparticles. Nanoparticles are functional bodies and are therefore linked to microbiological entities in a random manner through natural processes and they can be directed to living organisms and cell organelles. Chemical properties of nanoparticles are as important as physical e.g reactivity with the target, stability as well as sensitivity to different environmental conditions like moisture, atmosphere, heat and light. Their use is based on individual characteristic. Biomedical and environmental uses of nanoparticles depend upon their exceptional antibacterial, antifungal and disinfectant properties as well as their toxicity profile. Other important characteristics of nanoparticles determining their respective use are corrosive, anticorrosive, oxidation, reduction and flammability characteristics.

2.6 Applications of Nanoparticles

Nanoparticles have got their immense importance in various fields (Fig. 2.3). Drug delivery systems continuously face the challenge of releasing the drug agent at the right time safely to specific target site [43]. Conventionally pills and injections are used which don't provide precise control over drug release. The risk of destruction of drug agent during its passage through the intestine and poor absorption are also problematic. Nanotechnology and nanotechnological methods are now being used to develop futuristic tools for advance stage detection of disease as well as targeted delivery of drugs. Nanotechnology is now increasingly being employed for the diagnosis of disease. Nanoparticles of super paramagnetic iron oxide can be used for the imaging of brain tumors using MRI [44]. Tumor targeting using nanoparticles delivery systems is another tremendous use of nanotechnology in the field of health. Nanoparticles increase the penetrating ability and duration of retention of drug in tumor vicinity increasing the concentration of drug in desired area and at the same time decreasing the exposure of healthy tissue to drug by limiting the release of drug only to the targeted organ. Ligand attached or surface coated nanoparticles oppose the uptake by mononuclear phagocytic system and may be of more benefit. Various hydrophilic polymers are used to produce such coatings for conventional nanoparticles like polyethylene glycerol, poloxamines, poloxamers and polysaccharides, thus improving their therapeutic potential [45, 46].

Another important role of nanoparticles is as efficient gene delivery systems. Nanoparticles loaded with plasmid DNA can efficiently escape from degradation by endolysosomal systems and thus serve as excellent sustained release gene delivery system [47]. They are taken up inside the cell where they evade degradation by endolysosomal system and release DNA at a continuous rate therefore leading to enhanced gene expression [48].

The invention of new medicines for central nervous system is extremely difficult because of selective permeability of blood brain barrier (BBB). Nanoparticles can

interact with specific receptor mediated transport systems in the blood brain barrier and therefore are being employed for targeted therapy to the brain. Many nanoparticles were investigated in the past for biological applications, amongst these gold nanoparticles gained particular importance especially in the Ayurveda and Indian medical system in ancient times. Gold was commonly prescribed for the enhancement of memory. It was included in certain medical preparations to increase the mental fitness of baby. Passive adsorption of antibodies and proteins with colloidal gold is a long term practice. Antisense oligonucleotide conjugate GNP is an example of the use of these nanoparticles for DNA delivery inside the cell because not only it is effectively taken up by the cell but is also bring about expression of desired genes [49].

Another class of nanoparticles that has gained extraordinary attention is iron oxide nanoparticles, mainly due to their outstanding magnetic and optical characteristics. They are being extensively researched for use in biosensing, protein separation and purification, imaging, drug delivery and diagnostics, enhancement of MRI contrast, repair of damaged tissues, immunoassays, detoxification of biological tissues, hyperthermia, cell separation and drug delivery according to their surface chemistry. A high magnetic value is required for all of these applications and a size smaller than 100nm with narrow particle size distribution [50, 51].

Damaged tissue repair and reproduction can also be carried out using nanotechnology, replacing traditional treatments like artificial organ implants and organ transplants by technology like tissue engineering, for example carbon nanotubes scaffolds can be used for the growth of bones [52].

Nanoparticles have gained importance in almost all possible areas of medicine due to their capability to deliver drugs in the optimum dose range thereby increasing the therapeutic potential of drugs, decreased side effects and improved patient compliancy [53]. Hydrophilic nanoparticles have been recently developed to act as drug carriers. Amongst many distinct approaches, polyethylene oxide and polylactic acid appear to be promising system for intravenous drug administration [54]. Colloidal Au can also be used in combination to fluorescent dyes, enzymes

and radioactive compounds to label antibodies which can then be used to detect analytes in a tissue sample through antigen antibody interaction [55].

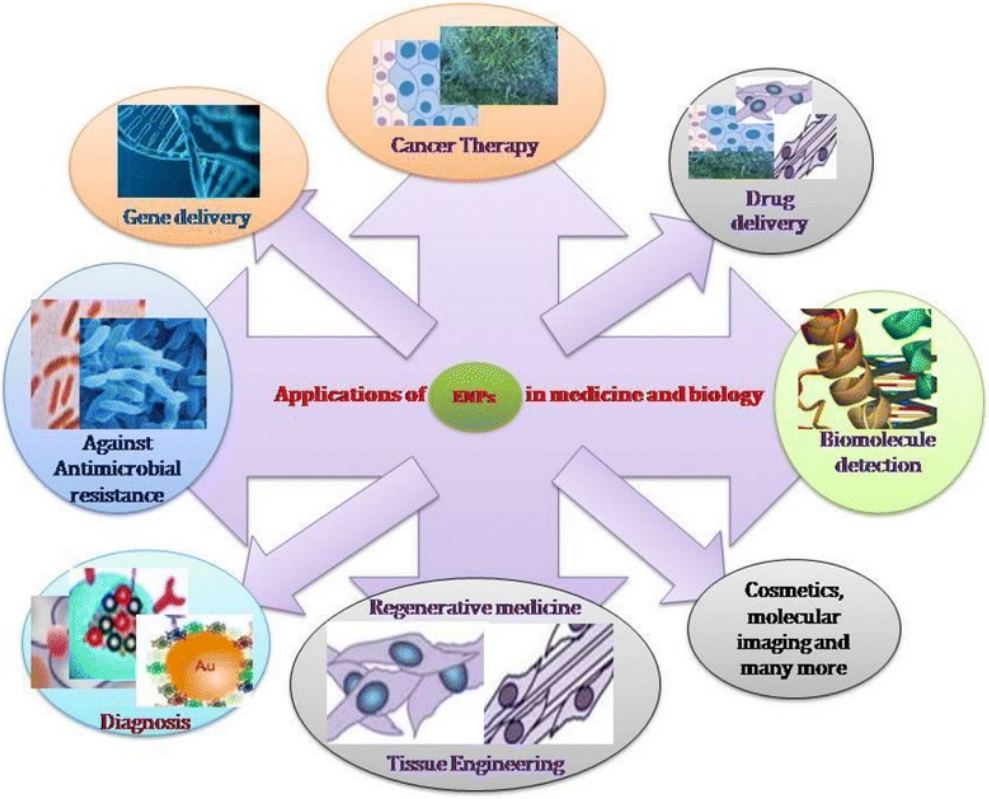

FIGURE 2.3: Applications of nanoparticles in various fields [62].

Numerous semiconductor and metallic nanoparticles with surface plasmon resonance, enhanced light scattering and absorption have immense potential in cancer diagnosis and therapy, for example gold nanoparticles can effectively produce localized heat by conversion of absorbed light which can further utilized for selective laser photothermal therapy of cancer [56]. Tumor targeting moieties like aptamers have been conjugated to gold nanoparticles for diagnostic applications because such aptamers can bind to target receptors in tumor neovasculature or surface of prostate cancer cells [57, 58].

Typical ultraviolet (UV) protection sunscreen lack stability in terms of long time range. Nanoparticles such as titanium dioxide are now being included in sunscreen to add numerous advantages. Titanium oxide and zinc oxide nanoparticles have Uv protection property because they have features like transparent to visible light

and efficiently absorb and reflect Uv rays. Iron oxide nanoparticles are also used as pigments in some lipsticks [59].

Nanoparticles are now being used increasingly in display technology such as computer monitors and televisions in order to achieve larger size and displays with high brightness. Light emitting diodes of modern displays uses the nanocrystalline material like lead telluride and many such particles [60]. Nanoparticles have also found much application in portable consumer electronics. Batteries that are made from nanocrystalline nickel and metal hydrides have advantage of acquiring less charging and ability to last longer due to exhibiting large surface area [61].

2.7 Silver Nanoparticles

Continuous rapid research is being done in the field of nanobiotechnology to produce nanoparticles and nanoproducts having novel and size related physiochemical properties those are considerably dissimilar from larger matter [63]. Properties thus attained can then be utilized in multiple ways in multiple fields. Silver (Ag); atomic number 47, is a shining very pliable and mouldable in different shapes and possess hardness greater than gold. Silver can exist chemically in four different oxidation states i.e Ag^0, Ag^{1+}, Ag^{2+}, and Ag^{3+} [64]. It is highly conductive metal but have limited use in electrical industry because of its costliness [65]. Metallic salts of silver are also being used to treat various diseases of human for example epilepsy, gonorrhea, and gastroenteritis. So far it is found to have no toxic and carcinogenic effects on human major body systems like nervous, immune, reproductive, or cardiovascular system [66]. That is why the demand of silver has been sky rocketed recently especially in fields such as medical, plastics, and textiles industries. Other distinctive properties of these nanoparticles are high electrical and thermal conductivity, surface enhanced resonance, chemical stability, catalytic activity and non linear optical behavior [1]. These properties are then utilized in materials like inks, microelectronics, and medical imaging systems [67]. Other properties important in determining their biological impact and interactions are

size, shape, surface charge and coating, agglomeration and dissolution rate. Shape is an important factor, the change in which can dramatically change the physical and chemical characteristics of silver nanoparticles. Most engineered nanoparticles possess the property of agglomeration. One example was displayed by agglomeration of these particles both in culture media as well as within cytoplasm and nuclei of HepG2 cells [68]. Ionic silver is produced by dissolution of silver nanoparticles due to surface oxidation. The rate of dissolution is determined by chemical and surface properties of the particle as well as its size and surrounding media also affect the dissolution [69]. Another extraordinary optical property of these nanostructures is localized surface plasmon resonance which is result of their unique interaction to light leading to collective coherent oscillation of their free conduction band electrons. Free electrons oscillate to result either in radioactive decay leading to strong visible scattering of light or in non radioactive decay leading to conversion of photon energy to thermal energy. Both of these decay mechanisms have been used in biodiagnostic and imaging as well as in therapeutic applications [70]. These particles also have broad spectrum bactericidal and fungicidal activity [2] and hence are used in a wide range of consumer products for example plastics, soaps, pastes, food and textiles which has increased their market value a lot [71, 72]. Use of silver nanoparticles as strong antimicrobial agents in surgically implanted catheters is well known and is very important to minimize the infections caused during various surgeries [73].

2.8 Therapeutic Uses of Silver Nanoparticles

Silver nanoparticles are considered to be potential antibacterial agents that can replace antibiotics in future and solve the age long problem of bacterial resistance against antibiotics. The large surface to volume ratios and crystallographic structure of these particles indicates that they might be better suited for use as antibacterial agents. Following are the three possible antibacterial mechanisms of silver nanoparticles (Figure. 2.4) [74]:

1. Silver nanoparticles adhere to the cell wall of bacteria and bring changes in the cell wall due to which cell wall can no longer protect the cell interior thus inhibiting bacterial growth and proliferation.

2. Silver nanoparticles proliferate the bacterial cell and damage its DNA thus changing the normal functioning of bacterial DNA which can lead to death of cell.

3. Silver ions(Ag+) interact with mostly sulfur containing proteins located in the cell wall of bacteria irreversibly and disrupt structure of the bacterial cell wall. This mechanism is considered to be the primary antibacterial mechanism during assessment of antimicrobial potential of silver nanoparticles [75].

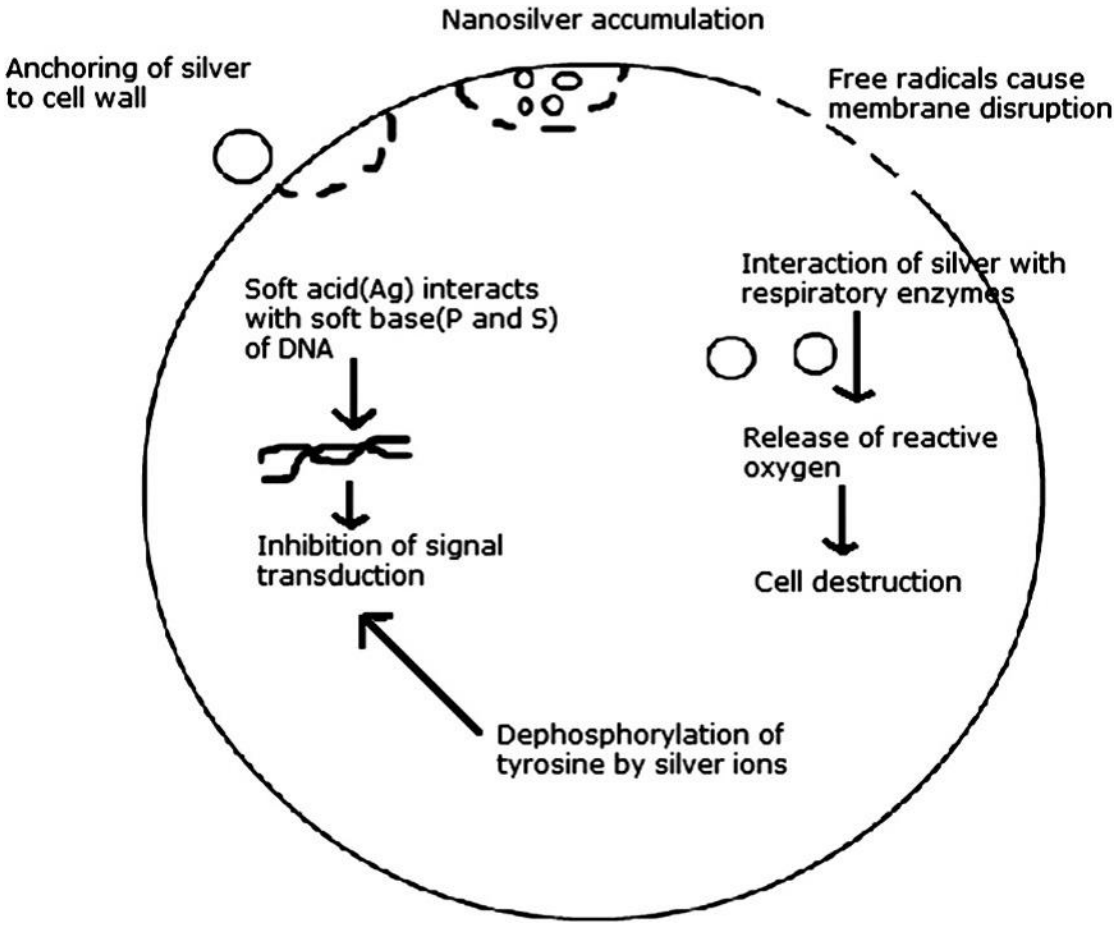

FIGURE 2.4: Various Means of Action of Silver Nanoparticles on Bacteria [76].

The antimicrobial properties depend on various parameters like size, shape and surface charge of the particles. In this regard silver nanoparticles have better potential antibacterial properties because they can easily penetrate the bacterial cell wall and alter its nuclear content, inactivating DNA and enzymes especially in gram negative bacteria leading to cell death [77]. Morphology of silver nanoparticles is also an important determinant of their antibacterial properties. Silver nanoparticles that have same surface area but different shapes show very different antibacterial activities because of their different effective surface areas and different number of active facets. One example is of truncated triangular silver nanoparticles which displayed the maximum antibacterial activity speculated because of large surface area to volume ratio and crystallographic surface structure. Electrostatic attraction between positive charged nanoparticles and negative charged bacterial cell is another very important factor that contributes towards their antibacterial properties.

Another promising antimicrobial use of silver nanoparticles is that they behave as antiviral agents in addition to antibacterial agents. A new micrometer sized magnetic hybrid colloid decorated with variously sized silver nanoparticles has been evaluated as an antiviral agent with lesser chances of being released into the environment [78]. Not only in treatment, but these nanoparticles have also been found to have beneficial effects in prevention of influenza virus infection invitro as well as invivo [79]. One study [80] concluded that silver nanoparticles showed better virucidal activity against herpes simplex virus and human influenza virus(80– 90% inhibition) at the same time being less cytotoxic to vero cells and can also have inhibitory effect against replication of vaccinia virus [3].

Silver nanoparticles had got their immense role as photosensitizers and radiosensitizers. LSPR of nanoparticles enables the use of silver nanoparticles in nonionizing radiation and ionizing radiation. Recent studies have shown that aptamer- Ag- Au shell core nanostructures have a high ability to absorb NIR irradiation and have potential to be used in photothermal therapy of the A549 cells at a low irradiation power density without making any damage to healthy cells and the surrounding normal tissues [81]. In addition, it was reported that hollow Au– Ag nanoshells

that make stable when PEGylated under the laser illumination and thus having great potential for photothermal therapy [82].

Cancer is a modern world menace and the promising antitumor effect of silver nanoparticles can be life saving for millions (Fig. 2.5). Although toxicological data concerning nanoparticles makes us doubtful as regards to their use but the same toxicity is highly useful in cancer treatment where damage to cancer cells is our main target and many positive outcome have been recorded. They have also been found to actively mediate molecular process to regulate cell functions and positively interact with non tumor healthy cells. As angiogenesis play pivotal role in many disease process including tumor growth and proliferation. The antiangiogenic properties make them a potential agent of choice. Silver nanoparticles have also been found to inhibit the formation of new blood vessels in vivo. In another study these particles were also seen exhibiting cytotoxic effects against Dalton's lymphoma ascites cell invitro as well as invivo and also enhance the survival time in tumor mouse model significantly (approximately 50% as compared with tumor controls) [83]. Leukemia is categorized as group of cancers that begins in the bone marrow and results in large number of abnormal white blood cells in the circulation and silver nanoparticles have been reported to have cytotoxic effects against these cells as well as against THP1, jurkat and K562 cells. Silver nanoparticles also have considered effect on breast cancer cells. They have been reported to exhibit dose dependent cytotoxic effects in MCF7 breast cancer by inducing apoptosis in cancer cells. These particles have also displayed cytotoxic effects in lung cancer cells. Studies have revealed dose dependent reduction in mitochondrial function of human alveolar cell line A549 cells [84]. They are easily taken up by the cells and cause ROS production leading to apoptosis and necrosis. Research has shown the effectiveness of silver nanoparticles as anticancer agents against HT144 cell lines [85].

The pharmacokinetics and pharmacodynamics properties of drugs are as important as their intrinsic therapeutic effects in field of medicine [87]. The outstanding biocompatibility and viability of silver nanoparticles for the nanoscale derived therapeutic settings makes them successful choice for drug delivery systems with

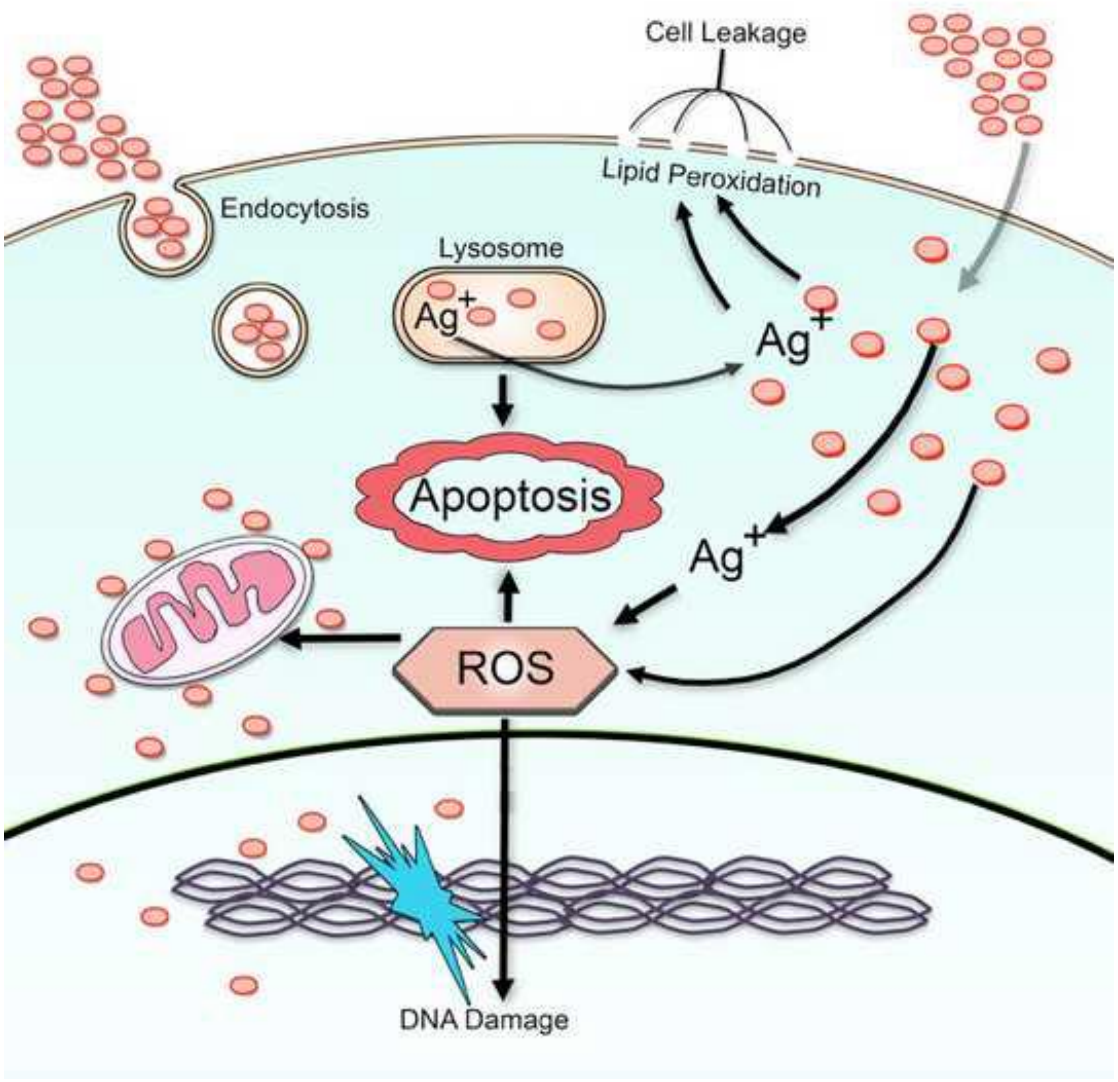

FIGURE 2.5: Mechanism of Action of Ag^+ Nanoparticles on Cancer Cells [86].

enhanced performance and such systems are responsive to thermal, optical, or pH modulators in order to target inflammatory infections and malignant diseases. These nanosystems are being investigated as appropriate carriers of different therapeutic molecule like anti inflammatory, antioxidant, antimicrobial and anticancer biosubstances [88]. Silver nanoparticles also perform well as active or passive nanocarriers for anticancer drugs hence making them of special use in tumor drug delivery systems [89]. A lot of effort has been put lately toward the development of silver nanoparticles based drug delivery systems including research to collect scientific knowledge as well as financial support in order to use intrinsic features of nanosilver like its ability to bind with wide range of organic molecules, its tunable

and strong absorption properties and low toxicity profile [90].

Central venous catheters (CVC's) are devices that have application in providing intravenous access for fluid administration, hemodynamic monitoring, drug delivery and nutritional support in a wide range of clinical conditions like renal disease and cancer especially in the critically ill [5, 91]. These devices when contaminated by microbial colonization have a high risk of becoming a source of hospital acquired infections [92, 93]. Various strains of *Staphylococcus aureus* were found to be the culprits in many of these catheter related infections and out of these 82% were methicillin resistant having many genes related to biofilm development and bacterial dispersion process [94]. Silver nanoparticles were extensively investigated to impart antibacterial effects to such clinically relevant materials and equipments. They were used for the modification of one dimensional and two dimensional surfaces such as cotton fabrics, natural and artificial fibers, thin polymer films and wound pads [95]. Although silver can undergo rapid oxidation process, large surface to volume ratio of silver nanoparticles allows them to provide sustainable local supply of silver ions Ag+ at the coating or tissue interface [6]. Central venous catheters coated with such particles showed significant inhibitory effect against both the gram positive and gram negative bacterial film development [5, 96]. These catheters with antimicrobial properties can provide an easy way of avoiding microbial contamination along with other aseptic techniques thus decreasing this risk of colonization and life threatening sepsis [93].

Among the most widespread oral cavity related disease across the globe is dental caries which possess a huge economic burden as well [97]. Nanotechnology derived dental related techniques decrease and even eliminates the clinical impact of caries by elevating the remineralization process as well as controlling the biofilm development. For the control of pathogens that are responsible for dental related biofilm formation leading to implant failure various metal coated implants were evaluated [98]. Silver nanoparticles have their use in other fields of dentistry like dental prostheses, restorative and endodontic dentistry and implantology [99]. Silver based nanostructures are embedded in general use dental materials worldwide by dentists to provide additional contact bactericidal effects [100]. The surface

area of silver nanoparticles can be significantly increased by decreasing their size and nanosilver use thus prevent the black staining in teeth which occurs after application of nanosilver diamine fluoride [7].

Wound infections, another important clinical challenge contribute to patient morbidity and mortality as well as posing immense economic burden. Prevention of wound dehiscence and surgical site infections is critical to good clinical practice but a challenge difficult to cope with [101]. Permanent disability or even death may result from the disturbance of cutaneous layer of skin and its functional integrity as a result of physically or chemically induced cutaneous wound depending upon how severe the injury is [102]. The intrinsic physiochemical and biological features of nanosilver impart them with effective biocide activities against a large variety of anaerobic, aerobic, gram negative and gram positive bacterial strains. Metallic or elemental silver is poorly absorbed by bacterial and mammalian cells because of chemical inactivation, so ionization of silver is required before it can be used to produce particular antibacterial effect under physiological conditions. Silver ions penetrate cell and interact with enzymatic as well as structural proteins [103]. They also have application in absorbent wound dressings where they interact with and destroy the bacteria found in exudates [104]. Incorporation of silver nanoparticles in novel and naturally derived biomaterial has also shown promising result in enhancing wound healing management for example modified cotton fibers, bacterial cellulose, chitosan and sodium alginate [105]. Diabetic wound are usually infected by numerous bacteria and use of silver nanoparticles and silver ions can provide efficient healing process thus helping in early wound healing stages and decreasing life threatening complications [4].

Because bone is an active tissue frequently undergoing regenerative and restoration process through its inherent and complex bone remodeling process, orthopedic and bone implant related infections are associated with highly inflammatory process leading to implant failure accompanied by bone destruction phenomena [106]. According to some studies, silver nanoparticles were found to improve the differentiation process of MC3T3–1 pre osteoblast cells and subsequent bone like tissue mineralization when compared with other nanoparticles [8]. These particles are

used as doping materials for synthetic and bio inspired bone scaffolds in bone replacement procedures with considerably relevant results [107]. They can promote osteogenesis and proliferation of mesenchymal stem cells thereby enhancing the healing process of fractured bone [108]. Studies of correlation between uptake and growth in clathrin dependent endocytosis revealed positive results in case of mesenchymal stem cells and osteoblasts, which indicates that this may be the principal route by which silver nanoparticles are internalized on a cellular level [109].

2.9 Methods of Synthesis of Nanoparticles

There are two common approaches to synthesize nanoparticles, the top down approach and bottom up approach (Figure. 2.6).

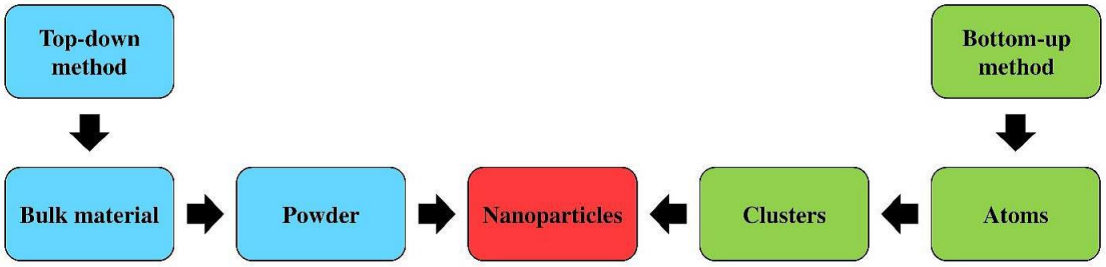

FIGURE 2.6: Synthesis Process of Nanoparticles [110].

In the former mechanical methods are used to decrease size of the bulk materials and convert them into nanosized particles. This can be done by specialized ablation methods for example lithography, thermal decomposition, laser ablation, mechanically milling the raw material or by etching and sputtering (Figure. 2.7) [111]. On the other hand,bottom up approach uses principle of arrangement of atoms and molecules to form structures that come in nanoscale range [112]. Homogenous systems are used which involves use of catalyst e.g reducing agent and enzymes that help to synthesize nanostructures. Various conditions and factors control and affect this mechanism for example catalyst properties, reduction media, solvent stabilizers as well as a temperature. The most common mechanism to synthesize metal nanoparticles is chemical reduction [113].

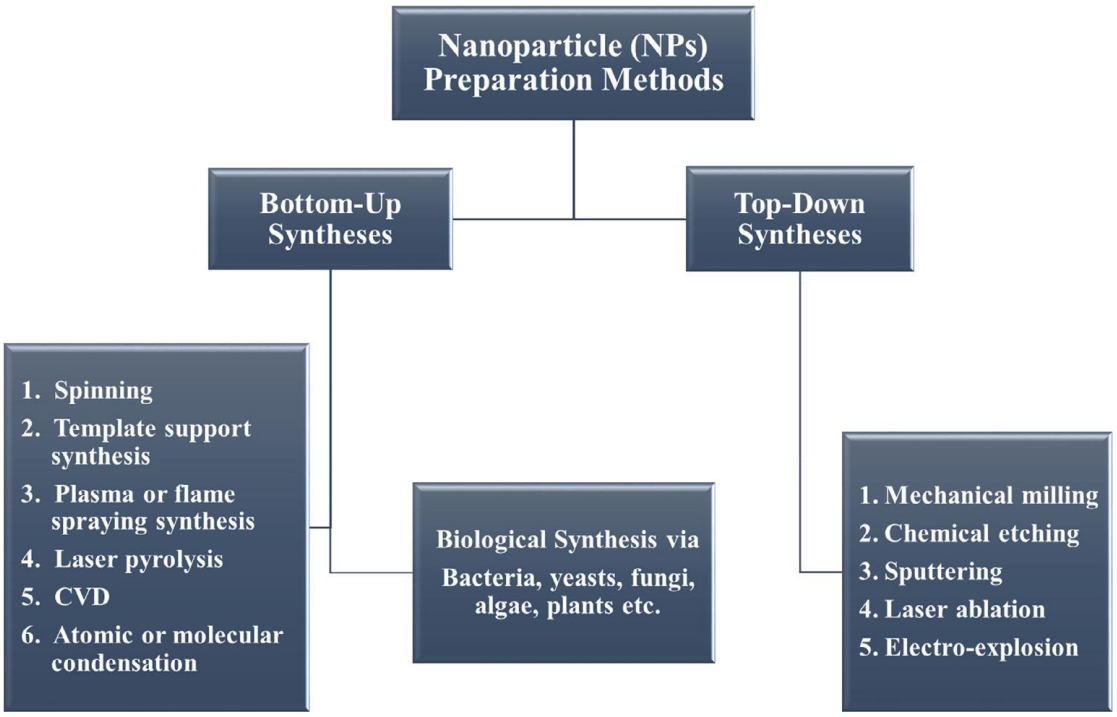

FIGURE 2.7: Classification of "Top Down" and "Bottom Up" Approaches [114].

2.9.1 Physical Methods

To physically synthesize nanoparticles evaporation condensation method is usually used in which tube furnace is used at atmospheric pressure. But this method possess various limitations like large space, huge energy consumption which raises the temperature of environment in surrounding of source material along with extra time requirement to attain thermal stability.

We can also synthesize silver nanoparticles with small ceramic heater which heats the local area. This creates steeper temperature gradient as compared to tube furnace because of which vapors that get evaporated cooled down at suitably rapid rate and hence small nanoparticles can be made in high concentration [115].

Another method to synthesize silver nanoparticles is through laser ablation of the metallic bulk material in solution form. Many factors like laser wavelength impinging metallic substrate, duration of the laser pulses, fluency of laser and the total time for ablation as well as the effective liquid medium as well as presence or absence of surfactant determine the properties of metal particles formed and the efficiency of ablation [116]. The most important parameter out of all of these

is laser fluence. A least power or fluence is required for the metal particles to be ejected from target. Increasing laser frequency increases mean size of nanoparticles, and smallest size is seen for fluencies which are not much larger than the laser breakdown threshold. Number of laser shots also determines concentration along with structure of metal particles released in medium like liquid. Metal particle concentration is expected to increase when exposed to the laser beam for longer duration of time but it gets saturate because light gets adsorbed in the colloidal material that is highly concentrated in the metal particles. When nanoparticles are synthesized in solution that have high concentration of surfactant, they have got small size as compared to those synthesized in solution with low surfactant concentration [117].

2.9.2 Chemical Methods

In this method metal ions are reduced in solution using chemicals to make nanoparticles. It is the most commonly deployed method to synthesize silver nanoparticles that act as the stable colloidal dispersions in water as well as in organic solvents. Usually borohydrite, citrate, ascorbate and elemental hydrogen are utilized as reductants in this process. Previously it was observed in various studies that use of strong reductants for example borohydrite created smaller particles which were monodispersed but creation of the larger particles was found to be difficult to control. On other hand when weak reductants like citrate were used, a slower reduction rate was achieved but size distribution assumed to be not narrow at all [118]. Stabilizing agent like dodecanethiol can be used which bind to the surface of nanoparticles hence preventing aggregation of particles and thus making them soluble in some solvents. Hence it may be stated that smaller changes in the synthetic factors can help to attain extraordinary huge modifications in the structure of nanoparticles, their average size, size distribution, width, stability and self assembly pattern. Three main component used in this process are metal precursor, reducing agent along with stabilizing or capping agent. Colloidal solutions formed allow the reduction of silver salts. The process has two stages of the nucleation

followed by growth. Both the initial nucleation process and subsequent growth of the initial nuclei thus formed can be manipulated through controlling different parameters of reduction like temperature, PH, precursors, reducing agents and stabilizing agents [119]. One disadvantage of using this method is that it is very expensive. The materials which are used in this process like citrate, borohydrite, thioglycerol and 2–mercaptoethanol are potentially toxic and can have hazardous consequences [120]. Along with these drawbacks, this method generates particles which are not of expected purity and have surfaces which are sedimented with chemicals. Many toxic and dangerous by products are formed and released during this process.

2.9.3 Biological Methods

Because of the many disadvantages of chemical methods, the biological methods have been developed to synthesize nanoparticles. In contrast with chemical methods, they are simple method, cost effective, dependable and environmental friendly. The nanoparticles synthesized by using multiple biological systems like bacteria, fungi, plant extracts (Table 2.1) and small biomolecules like vitamins and amino acids are high yield and of defined size. The better defined size and structure of the particles produced by the method gives this process a lead over many other physiochemical methods [121]. Three main factors determine the synthesis of nanoparticles using biological systems. These are solvent, reducing agent and non toxic material. The presence of amino acids, proteins and secondary metabolites in synthesis process provides a major advantage and no further step is needed to prevent the aggregation of synthesized particles. These biological molecules are ecofriendly and do not increase pollution. Nanoparticles of controlled size and shape of particle are produced using this method which can then be utilized in number of biomedical applications [122]. Bacterial proteins and plant extract are used as reducing agents to control features like shape, size and monodispersity of nanoparticles [123].

2.9.3.1 Synthesis of Silver Nanoparticles Using Bacteria

Bacterial mode of silver nanoparticles synthesis was first time documented and established by using *Pseudomonas stutzeri AG259* strain which was isolated from a silver mine [124]. There are certain micro organisms which show possible survival and growth in some metal ion concentrations because they show resistant behaviour to those metals. Many mechanisms are employed for resistance by these microorganisms including efflux systems, changing their solubility patterns and toxicity profile by reduction or oxidation, biosorption, bioaccumulation, extracellular complex formation, metal precipitation and absence of specific metal transport systems [125]. It is widely accepted that main mechanism involved in silver biosynthesis is because of presence of nitrate reductase enzyme. This enzyme shows its involvement by converting nitrates into nitrites. When nanoparticles are synthesized invitro using bacterial system, alpha nicotinamide adenine dinucleotide phosphate which has been reduced by NADPH dependent nitrate reductase and use of this enzyme just avoid the downstreaming processing step which may be essential in other cases [126].

2.9.3.2 Synthesis of Silver Nanoparticles Using Fungi

Fungi secrete large amount of proteins and hence produce larger amount of nanoparticles as compared to bacteria, leading to higher productivity of nanoparticles [9]. Following steps are required in this process: Ag+ ions get trapped at surface of fungal cells and are further reduced by the enzymes that are present in fungal systems [127]. This reduction seems to be facilitated by the extracellular enzymes like naphthoquinones and anthraquinones. For example in Foxysporum, and proposedly other fungi, NADPH dependent nitrate reductase enzyme and a shuttle quinine extracellular process is involved in the formation of nanoparticles [128]. Although we don't know the exact mechanism by which silver nanoparticles are synthesized by fungi, but it may be believed that mechanism given above mainly responsible for synthesis. One main disadvantage of using microbes for the formation of silver nanoparticles is that process carried out by microbes is slow when

compared to plants extract. Thus the use of plant extracts is more attractive option.

2.9.3.3 Synthesis of Silver Nanoparticles by Plants

Plant extracts are most often safe non toxic and most easily available substances and have a great number of metabolites which can facilitate the process of reducing silver ions. Their faster speed compared to microbes in the synthesis process also makes them more advantageous for use. Plant assisted reduction because of phytochemicals is thought to be the main mechanism by which plant extracts synthesize nanoparticles. Terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids are main phytochemicals that are involved. Flavones, organic acids and quinine are water soluble and reduce the ions immediately. Xerophytes have been shown to have emodin which is an anthraquinone that have the ability to undergo tautomerization, that can lead to synthesis of silver nanoparticles. On the hand mesophytes have three types of benzoquinones that are cyperoquinine, dietchequinone and remirin and it is considered that these phytochemicals are directly involved in the reduction of ions and synthesis of nanoparticles [129].

Table 2.1: Nanoparticles Synthesis With Use of Different Biological Sources And Possible Mechanism

S.NO	Biomass	Possible mechanism of nanoparticles synthesis	References
1	Plants (leaves, stem, roots, shoots, flowers, bark, seed)	Secondary metabolites i.e alkaloids,flavonoids, saponins, steroids, tannins etc acts as reducing and stabilizing agents	[130]

Table 2.1 continued from previous page

S.NO	Biomass	Possible mechanism of nanoparticles synthesis	References
2	Fungi	Produce reducing enzyme intracellularly or extracellularly also membrane bound and cytosolic oxido reductase and quinines	[131, 132]
3	Bacteria	Microbial cell reduces metal ions using specific enzyme like NADH dependent reductase or nitrate dependent reductase	[133]

2.9.4 Medicinal Plants

Nature is a best example for the coexistence phenomenon. Human diseases and ailments are treated with natural products from either source including plants, animals or minerals [134]. According to WHO definition of medicinal plants, these are considered as those natural materials that are either used directly or after processing for treating various diseases of living beings either at local or regional level [10]. In countries like China, Greece, Egypt and India medicinal plants are being transformed into one of the oldest sciences. Apart from many other uses, these materials are used as drugs, disinfectant and as aromatic agent in ancient Persia [135]. Use of plants by humans is not a new area infact it dates back to history of human life, when they are trying to find out a suitable tool from the environment to treat diseases and plants serve as the only choice for them. Reason for using medicinal plants for treating diseases is that they are chief source of many compounds that are used as reference for the synthesis of drugs at industrial scale [136]. Seeds, roots, leaves, flower and even whole plant

sometime used for treating diseases. Therapeutic or medicinal effect of these active compounds may be direct or indirect and thus are considered as medicinal agents.

2.9.5 Medicinal Plants in Various Cultures

Greek civilization was true reflection of science and philosophy. In pharmaceutical sciences notable contributions have been made by them. For the treatment of various pathological conditions almost 500 crude drugs were discovered by Aristotle [137]. Hippocrates was considered as father of allopathic medicine. One of Aristotle student named Theophrastus also wrote about 500 crude drugs in his own book. By using various extraction techniques named galenicals, Claudius was able to produce vegetable drugs and he also introduced idea to formulate stable and effective drugs using the concept of pharmaceutical formulations [138].

Among the oldest system of treatment, TCM was important to mention. Pioneer of this system was Fu His. Those exogenous factors are considered in this system that are involved in pathology [139]. Most inclusive pharmacopoeia named Ben Ca Gang Mu, which was published in 1596 was written by Li Shizen. It contains 1894 prescriptions which were used in China and other countries as a reference and for research guide. This system includes knowledge that passes from generation to generation, but it was formatted into academic educational training in 1950s.

Oldest health care system on earth is Traditional Indian medicine or Ayurveda. Ancient literatures like Rig Veda and Atharva Veda provide the description of this system about 5000 years BC [140]. Ayurveda is Sanskrit language word that means knowledge of life. According to this system, seven basic tissues makes the human matrix and for normal functioning these components work in harmony, while any imbalance among them results in a disease condition [141].

After the fall of Roman Empire, Arabs made enormous progress in the field of medicine and science. These scholars translated many books from Greece and Rome. Concept of diet control and exercise along with medication was introduced

by Arab physicians [142]. In the basic pharmacy practices these scholars are considered as pioneers. Jabber Bin Hayan made an extraordinary contribution in field of chemistry and provide with concept of extraction and isolation of many chemicals like alcohol, nitric acid and sulfuric acid etc. Toxic aspects of several plants were also reported by Arabs apart from healing and therapeutic characteristics. Comprehensive book on plant poisons and antidotes was also written by one of Arab scientist [143].

Important factors like climatic and edaphic gives the Pakistan a very incredible position in world in terms of biodiversity. Biodiversity is not localized instead spread throughout country. There are almost 6000 taxa of flowering plants reported in Pakistan. Studies suggest that 600 to 1000 plants exhibit medicinal properties and among them only 12% are used for treating pathological conditions. [12]. Almost 350 to 400 species of plants are available in local drug markets and traded for various herbal preparations [13].

2.9.6 Need for Green Synthesis

Oxidation / reduction reaction is the main process that involves in the synthesis of nanoparticles through bottom up approach. We will look for green chemistry because conventional methods like physical and chemical were of economic burden. Main problem that is associated with chemical method is the presence of toxic chemicals byproducts that get adsorbed on the surface of nanoparticles and in return exhibit such adverse effects when use in various biomedical applications [11]. In order to find out the most cost effective way for synthesis of nanoparticles, scientist look for microbial enzymes and plant extract. These materials allow the reduction of metal compound into respective nanoparticles because they possess specific antioxidant or reducing properties. Green approach is more preferred over other methods just because it serve as least economic burden, ecofriendly, easily scaled up for large scale production of nanoparticles and also reduces the demand for conditions like high pressure, temperature, energy and harmful chemicals [144].

By adopting biological methods it is more convenient to control the size of crystal as well as get more stable product.

2.9.7 Genus Bryophyllum

Term *Bryophyllum* get derived from two Greek words 'bryo' mean 'sprout' and 'phylon' mean 'leaf'. This genus comes under Crassulaceae family. This genus includes almost 40 species that are originally native of South Africa, Madagascar and Asia. In present time, plants of this genus are cultivated in almost all parts of tropics and reason for their cultivation is their attractiveness and interesting mean of reproduction. Plantlets are produced in large quantity on the edges of leaves and when they get detached and fall will result in production of whole plant i.e. vegetative mean of reproduction. In most collections, they are considered as weeds.

In Brazil juice of *Bryophyllum* can used to treat many periodontal ailments like chelitis, cracking of lips, wounds, bruises and boils etc [145]. In Nigeria plants of genus are used to treat insect bites, ear infections, tissue injuries and skin infections [146, 147]. Practice of rubbing or get tied the crushed leaves on head result in relief from headache in Africa. Plants of genus also proved to be effective against number of diseases including respiratory infections, rheumatoid arthritis and gastric ulcers [14].

2.9.7.1 *Bryophyllum pinnatum*

Bryophyllum pinnatum is a perennial herb of genus *Bryophyllum* belonging to family Crassulaceae (Figure. 2.8). It is erect and grows about 1.5 m height. The leaves and stem are fleshy and succulent. Leaves are alternate, opposite, simple and ex stipulate. Flowers are usually cymose. It reproduces from seeds and also vegetatively from leaf bubbils [148].

2.9.7.2 Habitat of *Bryophyllum pinnatum*

It is found naturally in hot and humid tropical regions of the world. It is a native herb of Madagascar.

2.9.7.3 Taxonomy of *Bryophyllum pinnatum*

Kingdom:	Plantae
Class:	Dicotyledon
Order:	Saxifragales
Family:	Crassulaceae
Genus:	Bryophyllum
Specie:	*B. pinnatum*

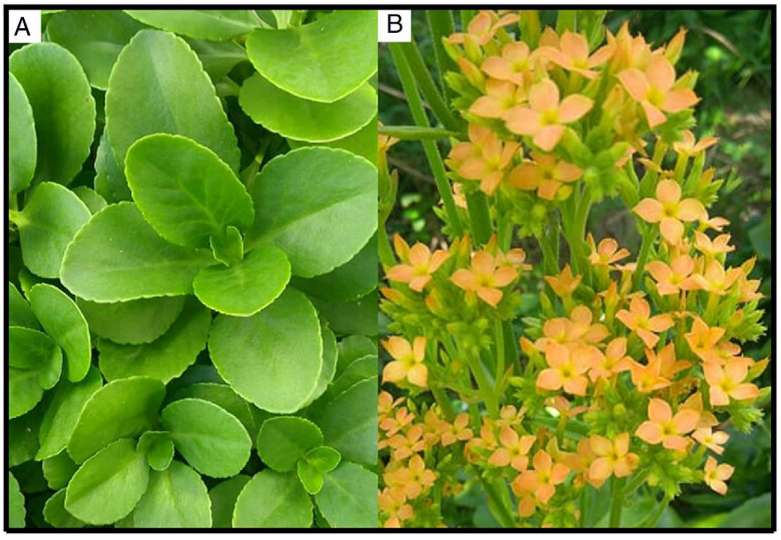

FIGURE 2.8: Leaves and Inflorescence of *Bryophyllum pinnatum* [149].

2.9.7.4 Parts Used

Almost all parts of the herb have been utilized in the folklore system for the treatment of various ailments. However, the extensive research has been made on leaves.

2.9.7.5 Ethnobotanical Uses

It has been used in treatment of various diseases including diabetes and heart diseases. Leaves of the plant have much higher therapeutic applications than other parts of the plant. Decoctions of leaf are used for the treatment of hypertension. It has been used with palm oil and shear butter in abscesses and swelling. In India, the leaves are used as hepatoprotective herb to treat jaundice. In Africa, and Asia leaves are used as an antipyretic and for treatment of malaria. In traditional medicine, the leaves of this plant have been reported to possess antimicrobial activity, antifungal, anti ulcer, anti inflammatory and analgesic activity. It also possesses anti hypertensive activities. Aerial parts of plant have been used against guinea worm. Lightly roasted leaves are applied to boils and skin ulcers. Leaves and stem are soaked in cold water overnight and then drink this water for heartburn, urethritis, and fevers and for all sort of respiratory conditions. Root infusion has used in epilepsy [150].

2.9.7.6 Phytochemical Studies of *Bryophyllum pinnatum*

Phytochemical studies have shown that plant contains, flavonoids, tannins, anthocyanins, glucosides, alkaloids, phenols, bufadienolides, saponins, coumarins, carotenoids, sitosterols, quinines, tocopherol and lectins [151]. It also contains steroids, lipids, triterpenes, and cardienolides [15]. Lipids , carbohydrates, proteins, vitamins and minerals have also been present. The vitamins reported in the plant include ascorbic acid, riboflavin, and niacin. The leaves are reported to contain bufadienolides such as bryotoxin A, B, C which are very similar in structure and activity like cardiac glycosides, digoxin and digitoxin [152]. Aerial parts of the plant contain varying amount of phenol acids such as caffeic acid, synergic acid, hydroxycinnamic acid, ferulic acid, para coumaric acid, 4- hydroxy- 3- methoxycinnamic acid, protocatechuic acid, 4-hydroxybenzoic acid [15]. Flavonoids found in the plant include friedelin, astragalin, epigallocatechin-3-o-syringate, luteolin, kaempferol, rutin etc. The cardienolide and steroidal contents in the aerial parts of plant includes, β- sitosterol, bryophyllol, bryophynol, bryophyllin B, bryophyllin

A, bryophyllin C and bersaldegenin-3-acetate, codisterol, clerosterol, and peposterol etc. Plant also proved to be the source of triterpenes that includes α-amyrinacetate, β- amyrinacetate, bryophollenone, bryophollone, taraxerol, pseudo taraxasterol, friedelin etc [152]. The plant contains amino acids such as thiamine, pyridoxine, glycine, ascorbic acid and cysteine and casein hydrolysate nicotinamide in its dry and fresh leaves. Minerals present in plant leaves include sodium, calcium, phosphorus, copper, potassium, magnesium, zinc and ferrous. Fatty acids such as palmitic acid, steric acid, behenic acid, malic acid, oxalic acid, citric acid, succinic acid, hydrocyanic acid are found in plant. Sugar found in plant includes raffinose, lactose, sucrose, glucose, galactose, fructose, maltose and arabinose which justify its uses in diarrhea [153].

2.10 Biological Evaluation

2.10.1 Bioassay

Bioassay is a term that is generally used to find out the effect of certain substance on specific type of living parts [154]. Any living system is generally exposed to varied amount of particular stimulus. The extent to which biological system respond to such stimulus depend on amount of dose. Biological assays are mostly comparative in nature. The potential of any substance to cause specific effect is found by making comparison with certain standard.

2.10.2 Types of Biological Assays

Quantitative biological assays are classified into two types i.e. direct and indirect assays. Direct assay aims to measure standard and test preparations doses that result in production of specific response. Direct assay has applied in limited areas because it requires exact amount of dose that is required to produce certain effect. Response in return can be recognized easily and dose is given in manner such that exact amount that is producing specific response will recorded [155].

While in indirect assay, varied amount of doses are provided to different biological units. In return, response of biological unit to each dose is recorded separately. Thus it helps to determine relative potency of test sample compared to standard by using statistical analysis of dose response relation [155].

2.10.3 Significance of Bioassay

By plotting the concentrate response curve it will enable us to find out sensitivity of tissue. Bioassay not only helps to find out concentration that is effective for producing a response but also find out potency of sample. It is used for standardization of drugs, vaccines, toxins or poisons, disinfectants, antiseptics etc [156] as biological system is exposed to any of them in some or other form. Specificity of a compound to be used will also determine using such assays. When we are performing structure activity relationship studies during drug invention process where our aim is to compare the relative potency and effect of certain compound on intact tissue system, principles of bioassay will serve as the better option [157].

Chapter 3

Materials and Methods

The current research work was carried out in wet lab of Department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad. The materials used and methodology adopted is as follows (Figure 3.1).

3.1 Materials

Material utilized for the research work that is mentioned below :

Table 3.1: Materials Utilized For Research Work

Chemical	Compnay Name
Silver nitrate salt	Sigma Aldrich
Nutrient agar	Sigma Aldrich
Luria broth	Sigma Aldrich
Sabourad dextrose agar	Sigma Aldrich
Brine shrimp eggs	Sigma Aldrich
Sea salt	Sigma Aldrich
DPPH (2,2-diphenyl-1-picrylhydrazyl)	Sigma Aldrich
Ascorbic acid	Sigma Aldrich

Table 3.1 continued from previous page

Chemical	Compnay Name
Streptomycin	Sigma Aldrich
Distilled water	Sigma Aldrich
Terbinafine	Sigma Aldrich
Glass vials	Sigma Aldrich
Fungal strains	Sigma Aldrich
Bacterial strains	Sigma Aldrich
Petri plates	Sigma Aldrich
Test tubes	Sigma Aldrich
Pestle and mortar	Sigma Aldrich
Micropipette	Sigma Aldrich
Micropipette tips	Sigma Aldrich
Cotton swabs	Sigma Aldrich
Aluminium foil	Sigma Aldrich
Falcon tubes 50ml	Sigma Aldrich
Eppendorf tubes	Sigma Aldrich
Beakers	Sigma Aldrich
Test tube racks	Sigma Aldrich
Para film or masking tape	Sigma Aldrich
Discs	Sigma Aldrich
Ethanol	Sigma Aldrich
Forceps	Sigma Aldrich

3.2 Microogranisms Used

Fungal strains *(Mucor species, A.flavis, A.fumigatus, A.niger, Fusarium solani)*
Bacterial strains *(Gram positive: M.luteus, S.aureus, B.subtilis) (Gram negative: A.tumefaciens, S.setubal, E.aerogenes)*

3.3 Methods

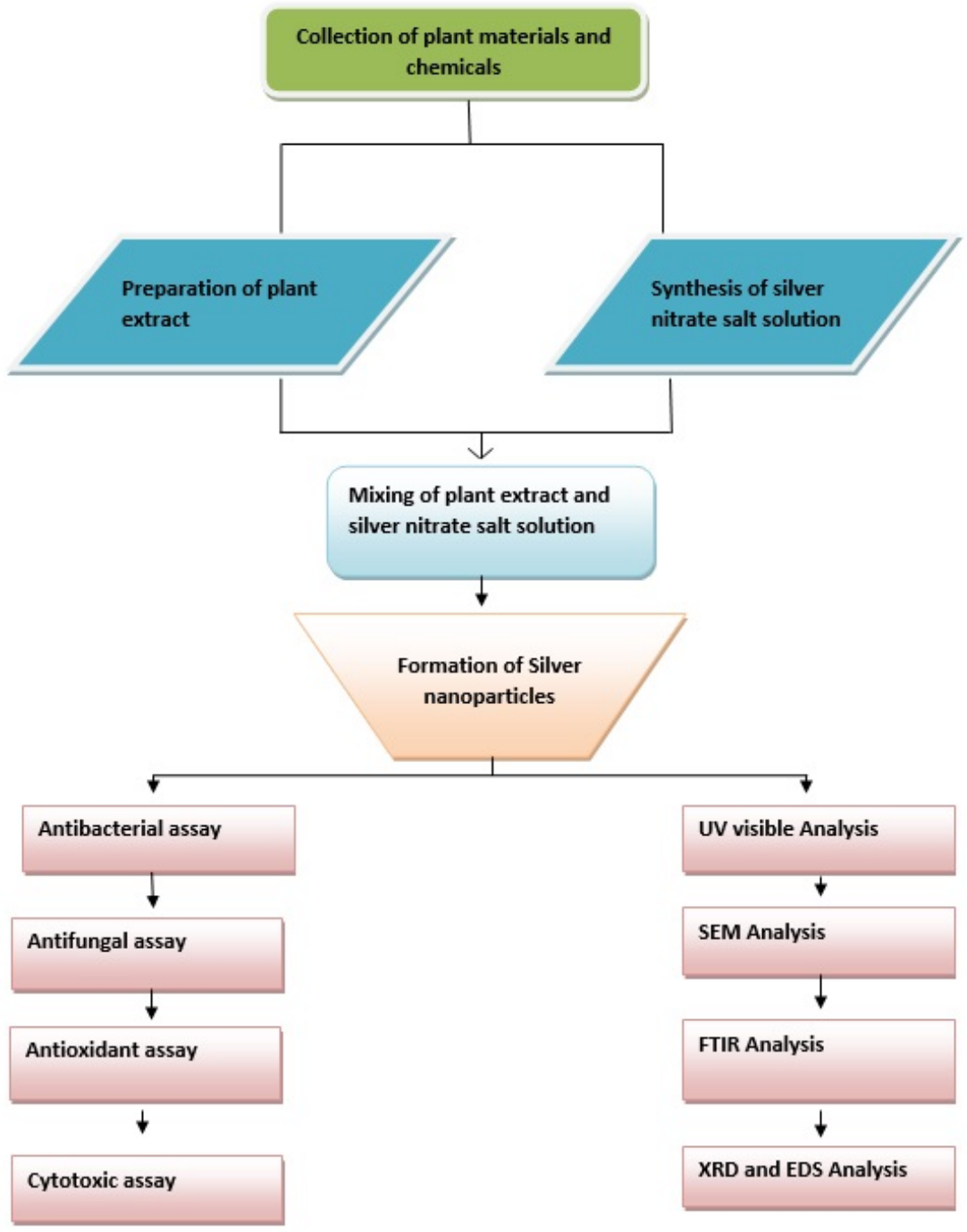

FIGURE 3.1: Overview of Methodology

3.4 Silver Nanoparticles Synthesis

3.4.1 Preparation of Plant Extract

100 g of fresh leaves of *Bryophyllum pinnatum* were taken and washed with distilled water. Leaves were then cut down into pieces and chopped in pestle and mortar. Chopped leaves were then boiled in 100ml of distilled water for 15 to 20 minutes to get maximum extract (1g/ml). After boiling, all the contents were then cooled and filtered using Whatmann No.1 filter paper. Filtrate thus obtained was stored at 4℃ for its further use as reducing and stabilizing agent [158].

3.4.2 Synthesis of Silver Nitrate Solution

0.05M silver nitrate solution was prepared by dissolving 4.25g of silver nitrate salt in 500ml of distilled water. Magnetic stirring was carried out at room temperature for 25 minutes. Prepared salt solution was stored in a flask that was covered with aluminium foil to prevent reduction and kept in refrigerator for further use [159].

3.4.3 Synthesis of Silver Nanoparticles

45ml of silver nitrate salt solution was taken in falcon tube and 5ml of plant extract was added in solution so that to make final volume of 50ml. Solution was then kept in sunlight for about 30 minutes for reaction to proceed. Appropriate color change confirmed the completion of reaction. The suspension was then preceded for centrifugation with condition of 6000 rpm for 30 minutes so that excess plant extract and unreduced silver was removed. Supernatant was discarded and pellet thus obtained were washed with distilled water to remove any impurities and other substances that get adsorbed on surface of nanoparticles. This process was repeated three times so that to get pure pellets. Pellets of silver nanoparticles were then dried in incubator at 60℃ for 24 hours. Dried pellets were then crushed to made fine powder and stored in eppendorf tubes [158].

3.5 Characterization of Silver Nanoparticles

For the purpose of physical characterization of silver nanoparticles following analysis were carried out:

3.5.1 UV- Vis Analysis

3.5.1.1 Sample Preparation

After centrifugation, supernatant was discarded and pellets were collected for sample preparation. Sample was prepared by dissolving 20ml of water in a vial containing pellets so that homogenous mixture was prepared. This mixture was later used for UV Vis analysis.

3.5.1.2 Experimental Procedure

To determine optical properties of silver nanoparticles, UV Vis analysis was done with the help of UV Vis spectrophotometer. About 5ml of sample was taken from stock and subjected to analysis with water as blank reference. This method was carried out to confirm the synthesis of silver nanoparticles. UV Vis spectras were observed in the range of 200-800nm [158].

3.5.2 Scanning Electron Microscope (SEM)

To determine the morphological features of prepared silver nanoparticles Scanning electron microscope (SEM) was used. Slide of sample was prepared and subjected to analysis at voltage of 5Kv with the help of Leo 440i, Cambridge, UK. Sample preparation involves well coating with conductive material. Sample was analyzed at different resolutions to get the best image for determining size and shape of particles [158].

3.5.3 X-ray Diffraction Spectroscopy

X-ray diffraction technique is most commonly used technique to determine the phase identification of crystallite material. Main principle of this technique is to bombard X rays on sample that were deflected at different angles thus spectra obtained provides the information about unit cell dimensions and structural finger prints of the unknown sample material. X ray diffraction of biologically synthesized silver nanoparticles was done by casting solution of nanoparticles on glass slide and then scanning was done in region from 20° to 80° [158].

3.5.4 FTIR Analysis

For identification of different components of sample FTIR analysis was carried out. FTIR analysis was done using Thermo Scientific, Nicolet iS50; Germany on liquid sample and infrared spectrum was obtained in range of 4000 to 500 cm^{-1}. Spectrum was then analysed for identification of different chemical groups present in sample and comparison based study was carried out [158].

3.5.5 Energy Dispersive X-ray Spectroscopy

Elemental composition of sample was obtained using EDX analysis. This technique aims to provide both qualitative and quantitative information about sample. Qualitative analysis involves the identification of respective line in spectrum while quantitative analysis results in measuring line intensities of each element thus helps in correct identification of desired element. EDS analysis of silver nanoparticles was done at energy level of 6.0475Kv for correct identification [158].

3.6 Biological Evaluation of Silver Nanoparticles

For the purpose of biological evaluation of synthesized silver nanoparticles, various biological assays were carried out. These assays are following.

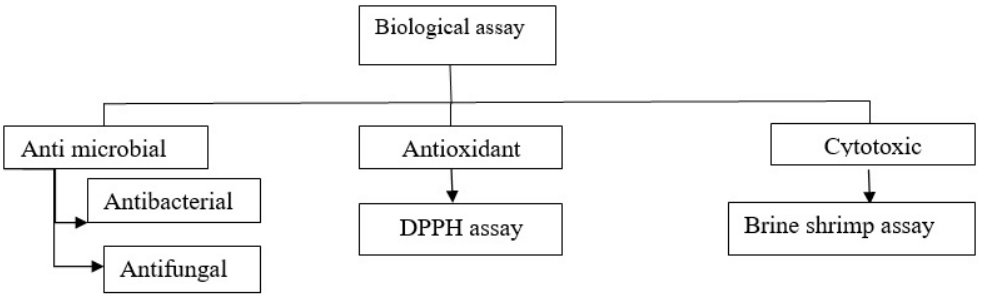

FIGURE 3.2: Biological Assays

3.6.1 Anti-microbial Assay

Two types of antimicrobial assays were carried out in order to determine the biological activity of prepared silver nanoparticles and these are as follows:

- Antibacterial assay
- Antifungal assay

3.6.2 Antibacterial Assay

Five pathogenic strains of bacteria were used for determination of bactericidal activity of silver nanoparticles using disc diffusion method as described by Ruparelia et al [160].

3.6.2.1 Bacterial Strains

Bacterial strains both gram positive and gram negative, that were used to determine bactericidal activity of silver nanoparticles are as follows:

- **Gram Positive Strains**
  1. *Bacillus subtilus*
  2. *Micrococcus luteus*

3. *Staphylococcus aureus*

- **Gram Negative Strains**

1. *Agrobacterium tumifaciens*
2. *Salmonella setubal*
3. *Enterobacter aerogenes*

3.6.2.2 Culture Refresh

For culture refreshing, twelve test tubes with caps were taken, two for each bacterial strain. 50ml of Luria broth was prepared by dissolving 1.25g/50ml of distilled water. Media along with test tubes and tips were autoclaved in order to maintain sterile conditions. After autoclaving, all the material was placed in laminar flow which was spared with 70% ethanol before placing material in it for sterilized conditions. Test tubes were marked in triplet for each strain. 4ml of media was poured in each test tube. 4µl of strain was added in each tube using yellow tips. Tips were changed for each strain. After inoculation of strains, tubes were incubated for 24hrs at 36℃. At medium turbidity further experimental procedure was carried out.

3.6.2.3 Sample Preparation

Pellets (50mg) were taken that were collected after centrifugation. About 50ml of distilled water was added in order to get homogenous mixture of stock. That mixture was used at different concentrations i.e. 10ppm, 20ppm, 30ppm, 40ppm, 50ppm, and 100ppm in order to determine antibacterial property of silver nanoparticles.

3.6.2.4 Media Preparation For Bacterial Growth

For bacterial growth in petri plates, media was prepared. 6.25g of Luria broth and 3.75g of agar was dissolved in 250 ml of distilled water for media preparation. Flask was sealed with cotton plug.

3.6.2.5 Experimental Procedure

Media, petri plates, tips, filter paper discs, cotton swab, forecep and distilled water were autoclaved at 121℃ for 20 minutes. After autoclaving, all autoclaved material was placed in Laminar flow. Plates were labeled with strains in triplet. After that media was poured in each plate in equal quantity and let it to solidify. After solidification, strains were streaked with help of cotton swab in each plate. Then discs were placed in each plate, six for different concentration of sample and one for positive control that was streptomycin (100ppm) and one for negative control that was distilled water. Petri plates were then sealed and placed in incubator at 37℃ for 24 hours. After 24 hrs zone of inhibition was calculated with help of ruler.

3.6.3 Antifungal Assay

Antifungal activity of silver nanoparticles was determined by using tube dilution method [161].

3.6.3.1 Fungal Strains

Five fungal strains were used which are as follows:

- *Aspergillus flavus*
- *Aspergillus fumigatus*
- *Aspergillus niger*

- *Mucor*
- *Fusarium solani*

3.6.3.2 Sample Preparation

Pellets (50mg) were taken that were collected after centrifugation. About 50ml of distilled water was added in order to get homogenous mixture of stock (1000ppm). That mixture was used at different concentration i.e. 50ppm, 100ppm, 150ppm in order to determine antifungal activity of silver nanoparticles.

3.6.3.3 Media Preparation

3.25g of Sabouraud dextrose agar was added in 50ml of distilled water to prepare media used for the growth of fungal strains.

3.6.3.4 Experimental Procedure

Test tubes, media, cotton plug, tips, loop and distilled water were autoclaved at 121℃ for 20 minutes. After that all the material was placed in Laminar flow. Test tubes were marked to 10cm. 4ml of media was poured in each test tube and sample was added at different concentrations and cotton plug was placed in each test tube. Tubes were placed horizontally so that slant was made up to mark. Ten test tubes were taken for each fungal strain and three were labeled as positive control i.e. Terbinafine, three for negative control i.e. water and three for different concentration of sample. Procedure was done in triplicate for each strain. After that, test tubes were inoculated with fungal strain, positive and negative controls and cotton plugged. Tubes were incubated at 28℃ in dark for two days. Fungal growth in slanting region was recorded. Following formula was used in order to calculate percentage inhibition.

$$\%I = \left[\frac{(Linear\ growth\ in\ -ive\ control) - (Linear\ growth\ in\ samples)}{Linear\ growth\ in\ -ive\ control}\right] \times 100$$

3.6.4 Antioxidant Assay

To determine antioxidant potential of silver nanoparticles DPPH method was used as reported by Gyamfi et al. [162].

3.6.4.1 Sample Preparation

Stock solution was prepared by diluting pellets with distilled water such that homogenous mixture was obtained (1000ppm). That solution of silver nanoparticles was used in three different concentrations for determination of antioxidant potential. Concentrations were 25ppm, 50ppm and 100ppm.

3.6.4.2 Preparation of DPPH Solution

12mg of DPPH was dissolved in 100ml of ethanol and magnetic stirring was done for one hour. Flask containing solution was covered with foil.

3.6.4.3 Experimental Procedure

For antioxidant assay to precede eighteen glass vials of volume 10ml were used and 2.8ml of DPPH solution was added in all vials. Out of eighteen vials, nine vials were used for positive and negative control i.e. in 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 remaining nine vials sample of 25ppm, 50ppm and 100ppm concentration was added such that there were three vials for each concentration. After that vials were placed in dark for 30–40 minutes at room temperature. Then readings were recorded at 517nm with ethanol was

used as blank reference. Following formula was used to calculate percentage of free radical scavenging.

$$\%Scavenging = \left[\frac{Control\ absorbance – Nanoparticle\ sample\ absorbance}{Control\ absorbance}\right] \times 100$$

3.6.5 Cytotoxic Assay

Cytotoxic activity of silver nanoparticles was determined using Brine lethality assay by using method followed by Bibi et al, [163].

3.6.5.1 Sample preparation

Pellets of silver nanoparticles were diluted and this stock (1000ppm) was used in three different concentrations for determination of their cytotoxic potential. Concentrations were 25ppm, 50ppm and 100ppm.

3.6.5.2 Sea salt preparation

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

3.6.5.3 Hatching of Brine Shrimp Eggs

Brine shrimp eggs were added in sea salt for hatching. Box having eggs and sea salt was placed for 24 hours. After 24 hrs shrimps were collected for checking cytotoxic potential of nanoparticles.

3.6.5.4 Experimental Procedure

Twelve glass vials were used out of which three were labeled with negative control i.e. 5ml of sea salt was added. In remaining nine vials sample of three different concentrations i.e. 25ppm, 50ppm and 100ppm was added such that there were three vials against each concentration of sample. Then sea salt solution was added to make final volume 5ml. After 24 hrs brine shrimp eggs were hatched and shrimps were seeing floating on surface. About 15 shrimps were added with the help of pasture pipette in each vial. Vials were kept in light for 24hrs at 24℃temperature. After 24hrs alive brine shrimps were counted with help if pasture pipette. Following formula was used to calculate percentage viability.

$$\%age\ Mortality = \left[\frac{(control) - (test)}{control}\right] \times 100$$

Chapter 4

Results and Discussion

This chapter covers all the phases that were carried out during synthesis of silver nanoparticles and their characterization by using UV vis spectrophotometer analysis, SEM (scanning electron microscope), XRD (X ray diffarction spectroscopy), EDS (Energy dispersive x ray spectroscopy) and FTIR (Fourier transform infrared spectroscopy). Results of various biological assays were also found and included in this chapter. Results of analysis are as under:

4.1 Synthesis of Silver Nanoparticles

Color change of solution confirms the formation of silver nanoparticles. This color change was observed within 30 minutes after addition of plant extract in the solution of silver nitrate salt. Color of pure silver nitrate salt solution remained unchanged during entire period of incubation. When plant extract was added, reduction of silver ions takes place that results in formation of dark black color silver nanoparticles (Figure 4.1). In a similar study, *Bryophyllum pinnatum* plant extract was used for synthesis of silver nanoparticles and appearance of dark brown color confirms the synthesis of silver nanoparticles [158]. In one of other study conducted by group of researchers, it was found that color change was observed

when *Azadirachta indica* leaf extract was used for the synthesis of silver nanoparticles and color change from colorless to brown indicated the formation of silver nanoparticles [164]. Results of these studies correlated with results of current research thus validated the present results.

FIGURE 4.1: Synthesis of Silver Nanoparticles

4.2 Characterization of Silver Nanoparticles

Following are the results of various physical characterization techniques.

4.2.1 Analysis of Silver Nanoparticles Through UV Vis Spectrophotometer

Uv vis spectrophotometer confirms the synthesis of silver nanoparticles and also used to determine the optical properties of synthesized particles. Silver nanoparticles showed the absorption peak around 400nm (Figure 4.2).

Surface palsmon resonance is the major phenomemon exhibited by metallic nanoparticles due to absorption in visible range. This was due to collective oscillation of conductive electrons after exposure of particles to visible light. Silver nanoparticles

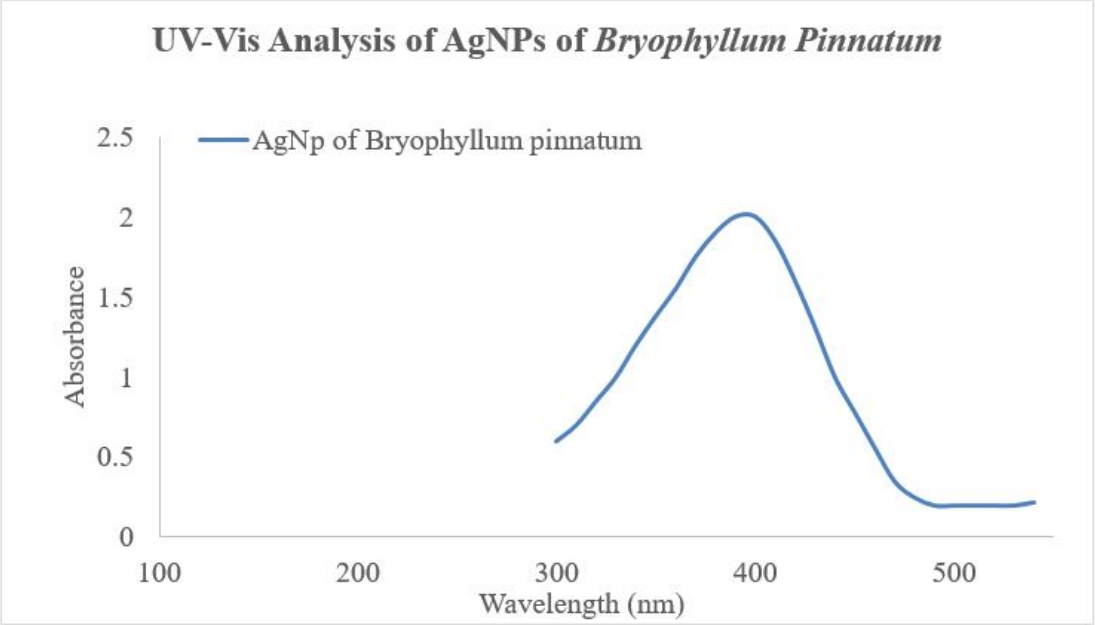

FIGURE 4.2: UV visible spectra of silver nanoparticles of *Bryophyllum pinnatum*

exhibit interesting optical properties that were directly related to surface palsmon resonance which inturn depend on the morphology of particles [158]. In a previous study, silver nanoparticles synthesized using *Bryophyllum pinnatum* plant extract exhibited characteristic brown color in aqueous solution with absoption ranges close to 418 nm [158], the results which correlates with present study. In another study performed by group of researchers, it was found that silver nanoparticles synthesized using same palnt have shown the absorption at 421nm [159] which further supports the present study results.

4.2.2 Analysis of Silver Nanoparticles Through SEM

SEM analysis was carried out in order to find out the morphological features and size of silver nanoparticles. Size of particles in present study was found to be 54±4 nm calculated using SEM with spherical round shape (Figure 4.3). It is a test process that involves the sample scanning with electron beam to produce the highly magnified image for further analysis. Under high magnifications, this technique generates images that allows precise measurement of very small objects and

features. Signals that were generated during SEM analysis results in production of two dimensional image that reveals the information about sample including external morphology and orientation of material and also provides information regarding chemical composition when used in conjunction with EDS [165].

Maginification of SEM ranges between 5X to 30,000X with resolution ranges between 50 to 100nm. Use of SEM in various applications proves to be advantageous due to its ability to gather data from sample surface and then spatial differences between samples can be studied by generating two dimensional image [165].

In one of the previous study, same plant specie was used for the synthesis of silver nanoparticles and size of particles was reported to be 84.85nm [159]. Another group of researchers performed the synthesis of silver nanoparticles and they had confirmed the synthesis of spherical shape nanoparticles with size ranges between 70–90nm [158], results which correlates with the present study.

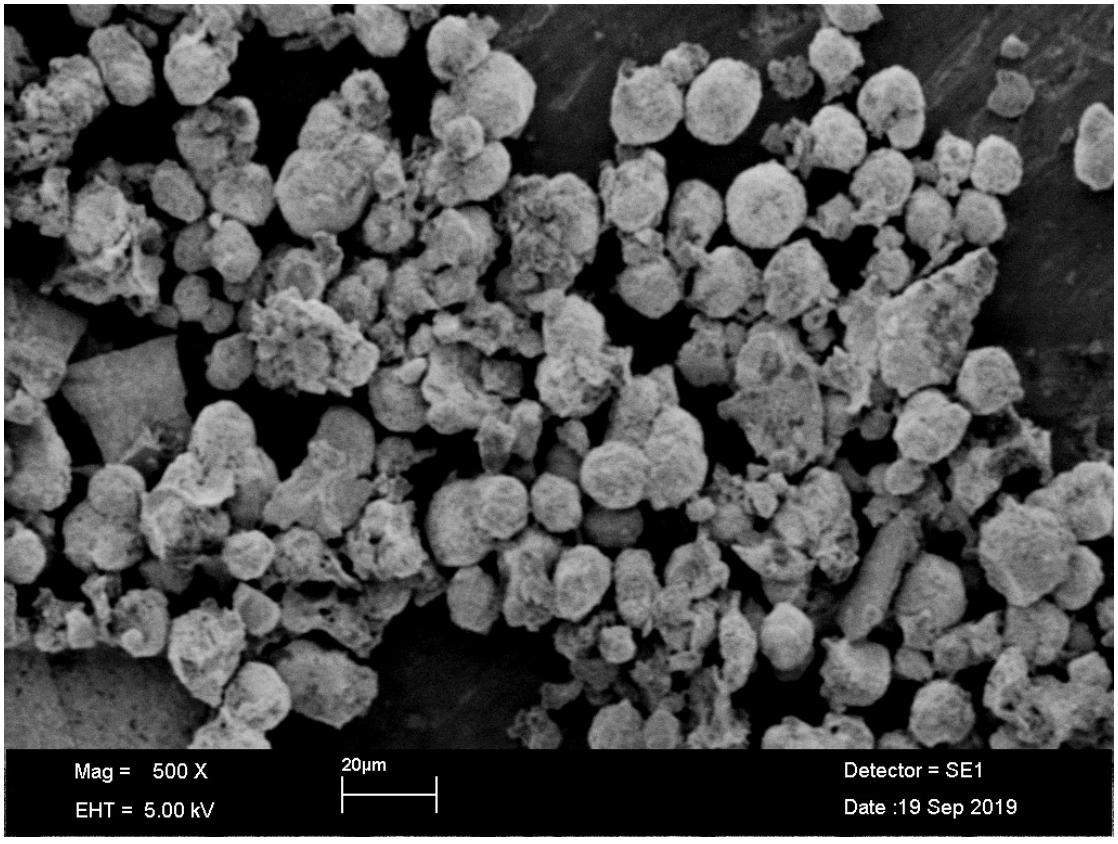

FIGURE 4.3: SEM Analysis of Synthesized Silver Nanoparticles

4.2.3 Analysis of Silver Nanoparticles Through EDS

The chemical composition of synthesized silver nanoparticles can be determined using Energy dispersive X ray spectroscopy equipped with SEM. It was confirmed from EDS spectrum that silver is the major constituent present in the sample (Figure 4.4). Corresponding peaks except silver shows element that may be utilized for conducting SEM analysis. In previous study, synthesis of silver nanoparticles using *Bryophyllum pinnatum* showed EDS spectra that showed a strong signal at 3kev [159].

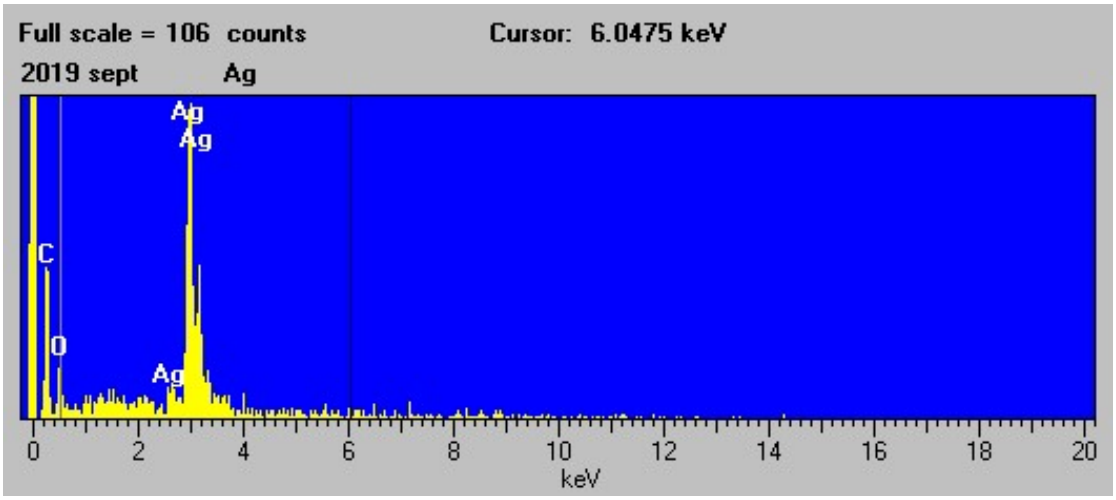

FIGURE 4.4: EDS Spectra of Synthesized Silver Nanoparticles

4.2.4 Analysis of Silver Nanoparticles Through XRD

Study of crystal structure either the amorphous or crystalline can be confirmed using X ray diffraction spectroscopy analysis, because knowledge of cryatallininty is highly relevant as the cryatalline form is most preferred in development. In contrast to amorphous material, crystalline material have well developed properties including melting point, solubility etc. The result of XRD analysis is a diffractogram that shows the intensity as a function of difraction angles. Characteristic X ray diffraction pattern generated in an analysis provides unique fingerprint of crystal present in sample. Proper interpretation and comparison with standard

reference pattern and measurements, fingerprint allows the identification of crystalline form[166].

Different diffraction intensities were recorded from 20° to 80° in 2θ angles. Strong peak values of 2θ with in angle of 38°, 44.3°, 64.6° and 77.0° have been observed (Figure 4.5). Similar peaks for silver nanoparticles were found when they were synthesized using leaf extract of ocimum [167]. Diffraction intensities were compared and analyzed which confirms the face centered cubic crystalline structure of synthesized silver nanoparticles. Crystallite size was also recorded using diffraction data and it was found that cryatallite size is 20nm. In another study peaks for silver nanoparticles synthesized using *Bryophyllum pinnatum* extract was recorded within angle of 38° to 80°, thus results correlate with present study [158].

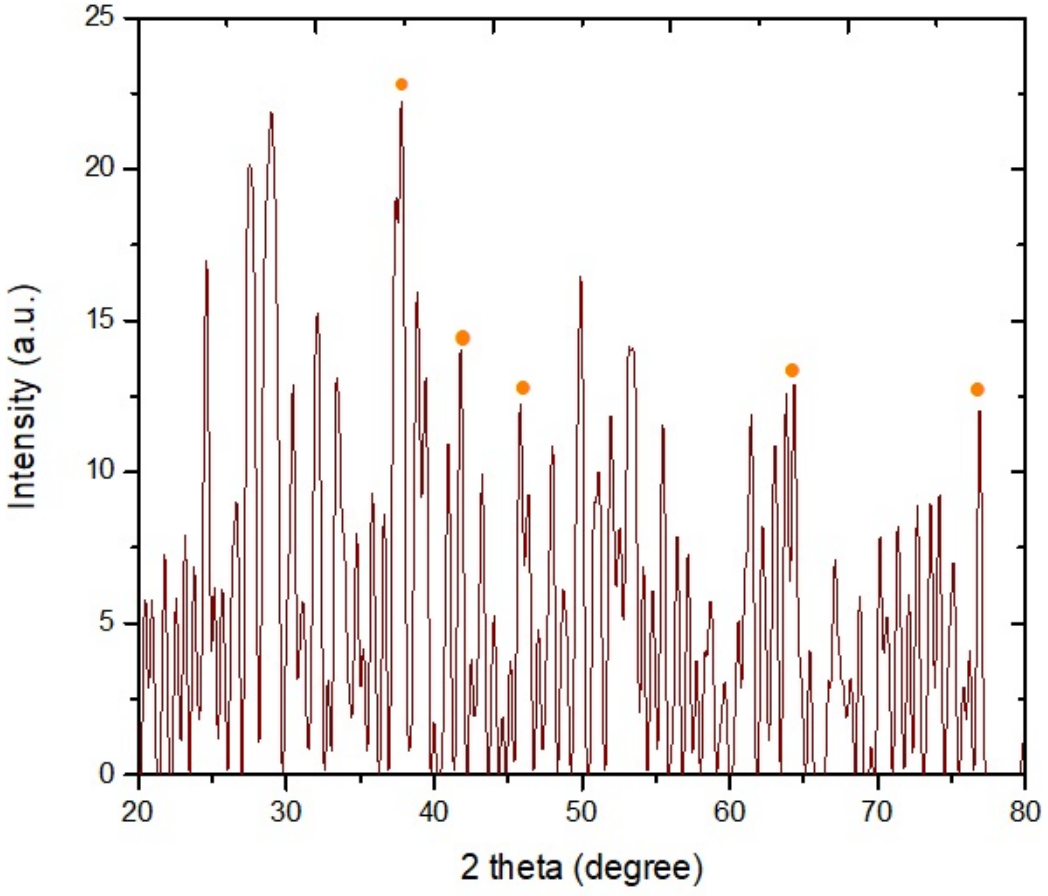

FIGURE 4.5: XRD Peak Diffractogram of Silver Nanoparticles

4.2.5 Analysis of Silver Nanoparticles Through FTIR

For determination of various biological molecules present in *Bryophyllum pinnatum* plant extract that were involved in synthesis of silver nanoparticles FTIR analysis was carried out using FTIR spectrophotometer. The peaks represent the FT-IR spectrum of the AgNPs (Fig 4.6). It displays several small bands between 460-500 cm^{-1}. The observed peaks bands may be assigned to silver stretching, O=C=O is stretching vibration (1481 cm^{-1}), O-H stretching (3500-3354 cm^{-1}), alkanes (2353 cm^{-1}) and O-H bending vibration (1680 cm^{-1}). The C=O bonds are stretched on 1620-1610. The lowest stretching can be observed on 730-665 which indicates strong C=C alkene. The observed vibration bands at low frequency regions suggest the formation of AgNPs. It was reported that O–H stretching vibration which indicates the presence of hydroxyl group responsible for reduction of silver ions into silver nanoparticles [159]. Presence of C=O bonds may be assigned to carbonyl group of proteins which clearly indicates the presence of proteins as capping agents for silver nanoparticles. Proteins have strong affinity to bind silver nanoparticles thus impart stability to these metal nanoparticles [168].

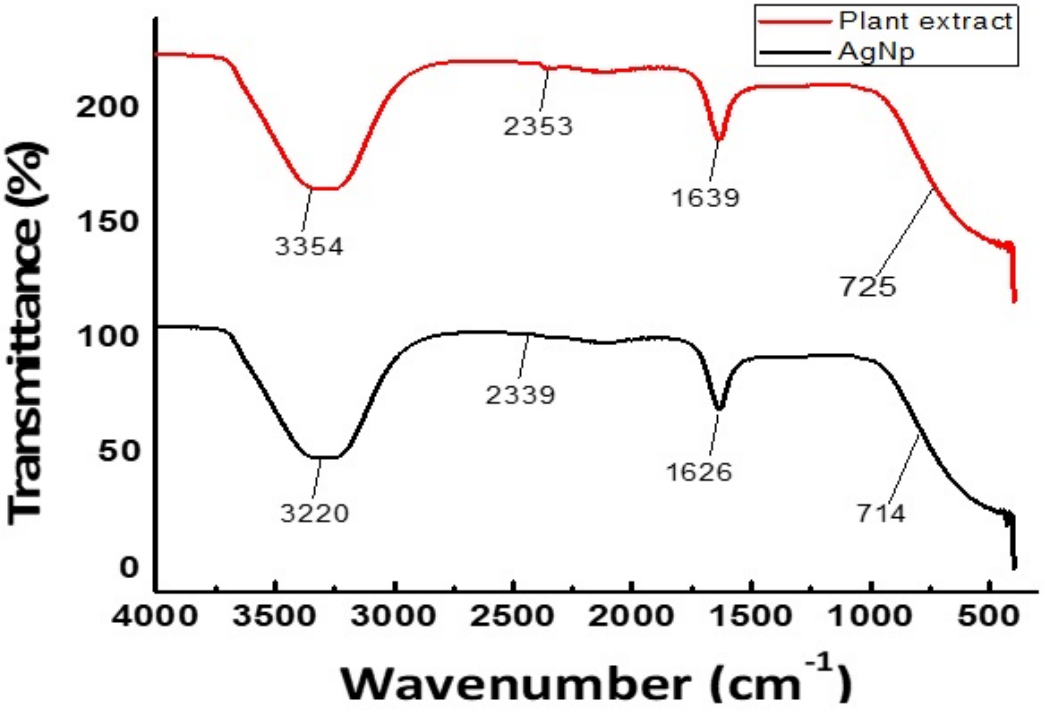

FIGURE 4.6: FTIR Spectrum of Silver Nanoparticles and Plant Extract

4.2.6 Antibacterial Assay

Antibacterial activity of synthesized silver nanoparticles was determined using disc diffusion method. Six bacterial strains including both gram positive and gram negative were used in this assay. Gram positive strains were *M. Luteus, S. aureus, B. subtilis* and gram negative strains that were used include *A. tumefaciens, S. Setubal, E. aerogenes*. Results are shown in table 4.1. Antibacterial potential of silver nanoparticles was determined by making comparison with control treatments. Minimum inhibitory concentration was determined against different concentrations of silver nanoparticles including 10ppm, 20ppm, 30ppm, 40ppm, 50ppm and 100ppm. Minimum inhibitory concentration (MIC) is the lowest concentration of any chemical or drug that results in retardation of bacterial growth. After an incubation period of 24 hours clear zone of inhibition was observed. Growth of all bacterial strains was inhibited at 100ppm. MIC value in case of gram positive bacteria was 30ppm while it was 10ppm for gram negative bacteria. From MIC value it was clearly shown that silver nanoparticles have shown greater antibacterial potential against gram negative bacterial strains as compared to gram positive strains. Another previous study confirms that silver nanoparticles synthesized using Azadirachta indica leaf extract showed prominent inhibitory effect against gram negative bacteria as compared to gram positive bacteria [169]. It was related with the fact that cell wall of gram negative bacteria is mainly composed of peptidoglycan layer with outer membrane composed of lipopolysaccharide. While in case of gram positive bacteria cell wall is composed of thick layer of peptidoglycan with various cross linkages between linear polysaccharides and peptides thus giving the membrane a rigid structure. Thus this rigid structure minimized the accessibility of silver nanoparticles to penetrate cell wall of gram positive bacteria thus showed minimum inhibitory effect [170].

Inhibitory effect of silver nanoparticles was due to interaction of nanoparticles with the sulfur containing proteins of bacterial cell like thiol group and phosphorous containing proteins like present in DNA of cell. These both were the most likely binding sites available for silver nanoparticles. Damage to bacterial cells caused

when silver nanoparticles bind with phosphate group of bacterial DNA due to altering protein structure. While when interacting with thiol group i.e sulfur containing group which is most important functional group of enzymes, silver nanoparticles can act by inactivating transcription and translation enzymes. Silver nanoparticles can also inhibit the bacterial growth by modulating the phosphotyrosine of bacterial proteins [171].

Another mechanism that was associated with inhibition of bacterial growth by silver nanoparticles involved the electrostatic interactions between the bacterial cell and silver nanoparticles that leads to the leakage of cell. Various chemical moieties including hydrogen peroxide, superoxide radicals, hydroxyl radicals and singlet oxygen including ROS, all of them were responsible for inducing oxidative stress to bacterial cells thus leading to damage to bacterial proteins and DNA [172].

TABLE 4.1: Antibacterial Activity of Synthesized AgNPs Using Disc Diffusion Method

AgNPs	**Zone of inhibition (cm)±S.E**											
Conc (ppm)	**Gram Positive Strains**						**Gram Negative Strains**					
	M. luteus		***S.aureus***		***B. subtilis***		***A. tumefaciens***		***S. Setubal***		***E. aerogenes***	
	AgNPs	***P.extract***	***AgNPs***	***P.extract***	***AgNPs***	***P.extract***	***AgNPs***	***P.extract***	***AgNPs***	***P.extract***	***AgNPs***	***P.extract***
10	-	-	-	-	-	-	0.5±0.01	0.36 ±0.02	-	-	1±0.15	0.5±1.2
20	-	-	-	-	-	-	0.8±0.15	0.48±0.03	-	-	1.5±0.14	0.66±1.1
30	0.5±0.05	0.66±0.02	1±0.1	-	0.9±0.1	-	1.5±0.1	0.64±0.02	0.8±0.08	-	2±0.15	0.76±0.56
40	0.8±0.2	0.73±0.03	1.3±0.15	o.42±0.01	1.3±0.1	-	1.7±0.2	0.72±0.01	1±0.11	0.92 ±0.01	2.2±0.15	0.8±1.1
50	1±0.3	0.83±0.02	1.5±0.1	0.51±0.02	1.7±0.11	0.34±0.02	1.9±0.1	0.83±0.02	1.6±0.30	1±0.06	2.4±0.2	1±1,1
100	1.3±0.05	1±0.05	1.8±0.05	0.6±0.01	1.9±0.05	0.71±0.01	2±0.15	1±0.10	1.8±0.12	1.4±0.2	2.7±0.1	1.7±0.3
Streptomycin +ive control	1.5		3		2.5		2.5		2.5		3	
–ive control	0		0		0		0		0		0	

P. extract: Plant extract, AgNPs: Silver Nanoparticles, *M. luteus: Micococcus. luteus, S. aureus: Staphylococcus. aureus, B. subtilis: Bacillus. subtilis, A. tumefaciens: Agrobacterium. tumefaciens, S. Setubal: Salmonella. setubal, E. aerogenes: Enterobacter. aerogenes*

4.2.7 Antifungal Assay

Fungal strains gain major attention because of their great potential to cause serious diseases as well as development of resistance among drugs with time. To cope up with such issues, in this study silver nanoparticles were tested against various fungal strains that are responsible for various diseases. Significant inhibition of fungal growth had been observed against four treatments i.e three concentrations of silver nanoparticles includes 50ppm, 100ppm and 150ppm along with 150ppm of plant extract. Maximum inhibition was observed in case of positive control. As far as different concentrations of silver nanoparticles are concerned maximum inhibition was shown in case of Solani strain (70% growth inhibition). In contrast, minimum growth inhibition was observed in case of Mucor strain (3% growth inhibition). Nanoparticles also showed considerable activity against different fungal strains including Fumigatus 60%, Flavis 43%, Mucor 38% and in case of Niger 60% growth inhibition was observed (Table 4.2). Comparison based study was done in which comparison was made between antifungal activity of silver nanoparticles and plant extract. In case of plant extract, maximum inhibitory activity was observed in case of Niger i.e 45% and lowest activity was observed in case of Flavis 20% (Table 4.2). Overall it was observed that silver nanoparticles have exhibited greater antifungal activity as compared to plant extract.

Antifungal activity of silver nanoparticles was due to interaction of silver nanoparticles with the fungal spores thus causing death of fungal spores by destructing the membrane integrity of those fungal spores [173]. In a recent study conducted by group of researchers in which silver nanoparticles were synthesized using A.indica leaf extract and synthesized particles were tested against various phytopathogenic fungal strains. The study clearly showed the inhibitory activity of these particles against pathogenic strains including Alternaria, Sclerotinia and Curvularia specie [132]. In another study, *Euphorbia hirta* extract was used for synthesis of silver nanoparticles and these particles were tested against various fungal species that includes *Candida albicans*, niger, flavus and fumigatus. All of these strains showed

growth inhibition against the highest concentration of silver nanoparticles that was used in study [174].

TABLE 4.2: Antifungal Activity of Silver Nanoparticles And Plant Extract

Anti Fungal Assay						
Sr No	Nanoparticles	Fungal strains	Growth (cm)			
	AgNPs		Sample	+ive	-ive	% inhibition
1	50ppm	Fumigatus	9	0	10	10
	100ppm	Fumigatus	6	0	10	40
	150ppm	Fumigatus	4	0	10	60
	Plant extract 150ppm		6.3	0	10	37
2	50ppm	Solani	9.5	0	10	5
	100ppm	Solani	6.5	0	10	35
	150ppm	Solani	3	0	10	70
	Plant extract 150ppm		6.8	0	10	32
3	50ppm	Flavis	7.7	0	10	23
	100ppm	Flavis	7.5	0	10	25
	150ppm	Flavis	5.7	0	10	43
	Plant extract 150ppm		8	0	10	20
4	50ppm	Mucur	9.7	0	10	3
	100ppm	Mucur	7.7	0	10	23
	150ppm	Mucur	6.2	0	10	38

Table 4.2 continued from previous page

Anti Fungal Assay						
	Plant extract 150ppm		6.8	0	10	32
5	50ppm	Niger	9.5	0	10	5
	100ppm	Niger	7.2	0	10	28
	150ppm	Niger	4	0	10	60
	Plant extract 150ppm	Niger	5.5	0	10	45

4.2.8 Antioxidant Assay

Antioxidant potential of silver nanoparticles was evaluated by DPPH assay. Free radical scavenging activity was exhibited by both silver nanoparticles and plant extract. In present study silver nanoparticles exhibited greater antioxidant activity as compared to plant extract. Silver nanoparticles showed maximum free radical scavenging activity at higher concentration (100ppm) and it was recorded to be 95.5% whereas at same concentration plant extract showed 52.6% scavenging activity (Fig 4.7). At 50ppm silver nanoparticles showed 80.2% antioxidant activity while plant extract at same concentration showed 33.3% free radical scavenging activity. At lowest concentration of both plant extract and silver nanoparticles i.e. 25ppm minimum activity was shown and it was 64% in case of silver nanoparticles and 20.7% for plant extract. Results are also found significant by comparing EC_{50} values of both silver nanoparticles and plant extract. EC_{50} value was found 23.3ppm for silver nanoparticles while it was 163.3ppm for plant extract (Table 4.3). Lower EC_{50} value of silver nanoparticles as compared to plant extract showed their great antioxidant potential. Free radical scavenging activity was increased in dose dependent manner and as the concentration decreases there is significant decrease in antioxidant potential.

DPPH is nitrogen centered free radical which shows a clear color change from violet to yellow upon reaction with antioxidants due to reduction. DPPH is a stable free radical that reduces upon accepting electron or hydrogen from silver nanoparticles. It was confirmed from previous studies that plant extracts possessed various groups that adds to the antioxidant potential of silver nanoparticles. *Bryophyllum pinnatum* plant extract has methanol and ethyl acetate content that gives characteristic antioxidant property [175]. It was reported in a previous study that *Chenopodium morale* plant extract based silver nanoparticles possessed significant antioxidant activity due to presence of phenolic compounds that act as reducing agents, hydrogen donors and free singlet oxygen quenchers [130]. In one study it was shown that apple contain polyphenols that were responsible for its antioxidant activity and thus silver nanoparticles synthesized using apple extract showed excellent antioxidant potential [176].

Table 4.3: Antioxidant Activity of AgNPs and Plant Extract

Antioxidant Activity of AgNps				
Sr No	Sample and Conc	Abs	%Scavenging	EC_{50}(ppm)
1	Positive control	0.001	99.97	
2	Negative control	0.002		
3	AgNps 25ppm	1.3	64.1	
4	AgNps 50ppm	0.51	80.2	23.3
5	AgNps 100ppm	0.02	95.5	
6	Plant extract 25ppm	3.1	20.7	
7	Plant extract 50ppm	2.9	33.3	163.3
8	Plant extract 100ppm	1.8	52.6	

Impact of silver nanoparticles and plant extract as antioxidant agents was found significant statistically as $p<0.0001$ (Table 4.4).

TABLE 4.4: Analysis of Variance For Factors Affecting The Free Radical Scavenging Activity of Silver Nanoparticles

Source of variation	Df	Sum of squares	Mean square	F-Value	P-Value	Significant
Interaction	4.0	867.0	216.7	23.54	<0.0001	Yes
Silver Nano particle	2.0	18440	9218	1001	<0.0001	Yes
Concentration	2.0	1417	708.4	76.93	<0.0001	Yes
Residual	18	165.7	9.207			

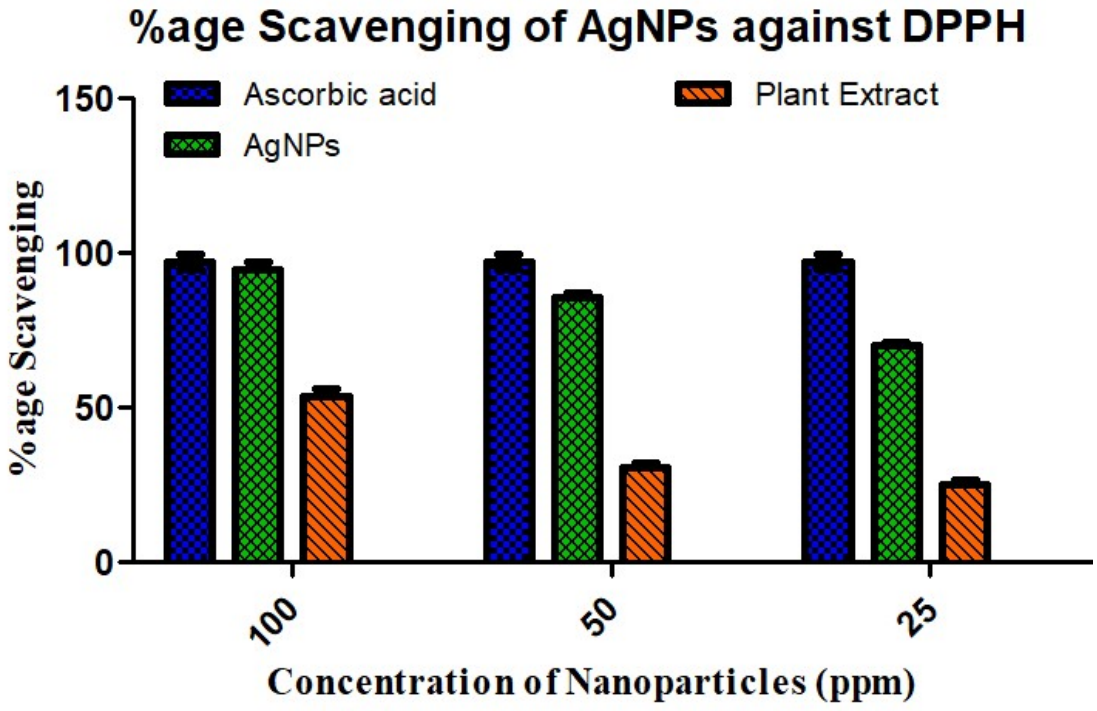

FIGURE 4.7: % Scavenging of Silver Nanoparticles Against DPPH

4.2.9 Cytotoxic Assay

Toxicity profile of silver nanoparticles was evaluated using brine shrimp lethality assay. Three different concentrations of both plant extract and silver nanoparticles

i.e 25ppm, 50ppm and 100ppm were used and all of them showed significant cytotoxic effect. At highest concentration i.e 100ppm silver nanoparticles showed 80% mortality while plant extract showed 33.3% mortality at same concentration. At 50ppm silver nanoparticles showed 66.6% mortality while at same concentration plant extract showed 26.6% mortality. At lowest concentration i.e 25ppm silver nanoparticles showed 46.6% mortality while plant extract showed 13.3% mortality. Results are validated by comparing IC_{50} values of both plant extract and silver nanoparticles. It was found that for silver nanoparticles the value is 117.6 while it was much higher for plant extract i.e. 298. Lower IC_{50} value of silver nanoparticles thus showed their greater cytotoxic potential against brine shrimps (Table 4.5). It was observed that higher concentration can be associated with higher mortality rate (Fig 4.8) and these results are found significant statistically as $p<0.0001$(Table 4.6). Thus it can be concluded from these results that silver nanoparticles has cytotoxicity thus they can be tested against various cancer cell lines. In earlier studies it was reported that cytotoxic activity of silver nanoparticles depends on several factors which are dose, time and size of nanoparticles. All of these factors have a combined effect on the cytotoxic potential of silver nanoparticles. Cytotoxic potential of silver nanoparticles have been confirmed by various studies. In one report, silver nanoparticles synthesized using cannonball leaf extract have shown anticancer effect against MCF– 7 cells and the activity was observed to be increased in a dose dependent manner [176]. In another study *Piper longum* extract based synthesized silver nanoparticles have shown efficient cytotoxic effect against HeP–2 cell lines. Cytotoxic effect was significantly increased as the concentration of silver nanoparticles was increased. Mechanism that was reported for cytotoxic effect includes the production of ROS which can cause damage to lipids, DNA and protein component of the cell thus eventually causing cell death [177]. Silver nanoparticles can efficiently reduce the ATP content of cell thus causing ultimate damage to the mitochondria of cell and thus leading to production of ROS in a dose dependent manner [178]. Silver nanoparticles synthesized using *Erythrina suberosa* have shown remarkable cytotoxic potential against A–431 osteosarcoma cell line 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 [178].

TABLE 4.5: Cytotoxic Effect Of AgNPs And Plant Extract Against Brine Shrimps

Cytotoxic Assay						
Brine shrimp lethality checked by AgNPs						
Sr No	Treatments	Concentration	Total no. of Shrimps	Alive	% Toxicity	IC_{50}
1	AgNPs	25ppm	15	8	46.66666667	117.6
2	AgNPs	50ppm	15	5	66.66666667	
3	AgNPs	100ppm	15	3	80	
4	Plant extract	25ppm	15	13	13.33333333	298
5	Plant extract	50ppm	15	11	26.66666667	
6	Plant extract	100ppm	15	10	33.33333333	
7	Sea salt (-ive control)		15	15	0	

TABLE 4.6: Cytotoxicity of Silver Nanoparticles Against Brine Shrimps Analysis of Variance For Factors Affecting The Viability of Brine Shrimps

Source of variation	Df	Sum of squares	Mean square	F-Value	P-Value	Significant
Interaction	4.0	884.3	221.1	19.83	<0.0001	Yes
Silver Nano particle	2.0	24140	12070	1083	<0.0001	Yes
Concentration	2.0	1488	744.2	66.76	<0.0001	Yes
Residual	18	200.7	11.15			

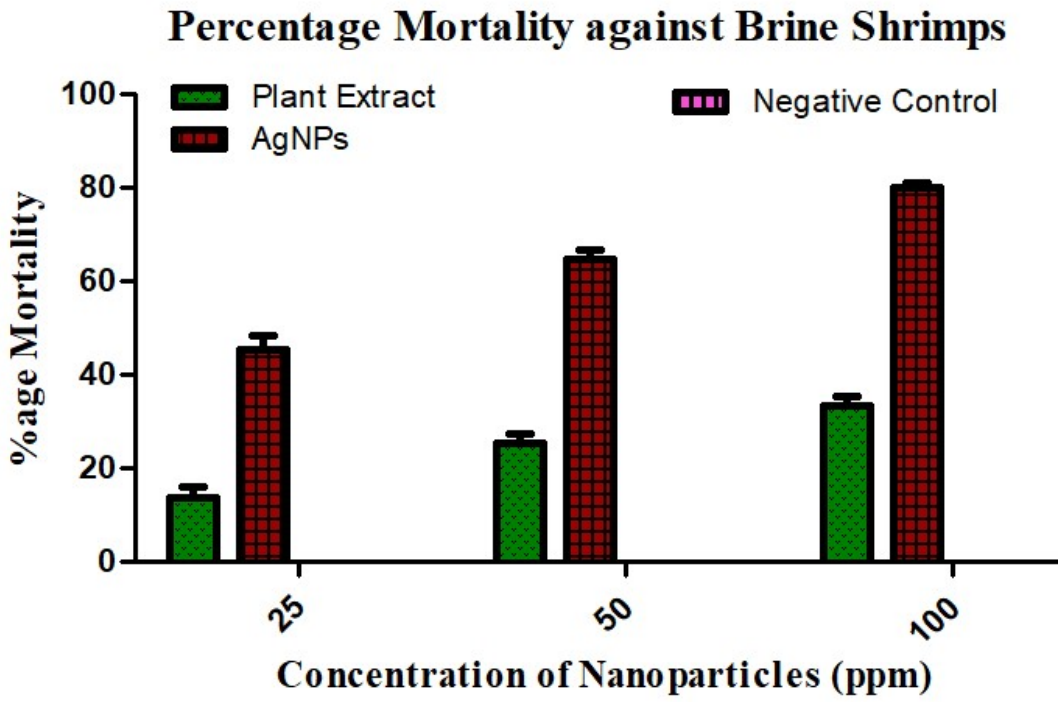

FIGURE 4.8: Percentage Mortality of Brine Shrimps Against Plant Extract and Silver Nanoparticles

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

In the present research green approach using *Bryophyllum pinnatum* plant extract was utilized which lead to the successful development of silver nanoparticles due to presence of reducing and stabilizing agents in the plant extract. It was observed that synthesized silver nanoparticles showed absorption spectra at 400nm, with size ranges from 54±4 nm, standard peaks for various chemical moities including alkanes, hydroxyl group, alkene and carbonyl group of proteins observed between 500 – 4000 cm-1 through FTIR analysis and face centered cubic crystalline nature of particles with crystallite size 20nm was confirmed from XRD analysis.

Synthesized particles have also exhibited significant antifungal, antibacterial, antioxidant and cytotoxic properties with lesser IC_{50}, EC_{50} and MIC values as compared to plant extract.

5.2 Recommendations

Owing to greater potential against number of fungal and bacterial strains, current research can be explored further for development of better antifungal and antibacterial drugs with enhanced efficacy. Surface and morphological features of nanoparticles can be modified in such a way so that they can be explored as efficient drug delivery tools in future.

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