CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Chemical Synthesis of Diversified Silver Nanoparticles along with Characterization and Biological Evaluation

by

Mehmoona Bibi

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

in the

Faculty of Health and Life Science
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Dedicated to Almighty ALLAH and the Holy Prophet Muhammad (P.B.U.H)
and My Loving Family.

CERTIFICATE OF APPROVAL

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Abstract

In present work, we have synthesized silver nanoparticles of different morphologies using different modifiers through chemical reduction method. Synthesized silver nanoparticles were characterized through UV-Vis spectrophotometer, scanning electron microscope (SEM), energy dispersive x-ray spectroscopy (EDX) and x-ray diffraction spectroscopy (XRD). Biological evaluation was done through antibacterial, antifungal, cytotoxic and antioxidant assay. UV-Vis spectroscopy revealed the formation of silver nanoparticles by exhibiting the surface-plasmon absorption maximum at 400-500 nm from UV-Vis spectrum. SEM results indicated plate-like round nanoparticles with normal size of 39 nm for maleic acid capped nanoparticles, rough surfaces flower shaped nanoparticles with size of 250 nm for citric acid capped nanoparticles and hexagonal shape with average size of 500 nm for uncapped silver nanoparticles. Their phase centered cubic crystalline nature was confirmed by XRD analysis. EDX analysis indicated presence of elemental silver. Synthesized silver nanoparticles were found more effective against the gram-negative bacterial strain as compared with the gram-positive. Similarly when tested for their antifungal activity, they were found reasonably effective. Moreover, these silver nanoparticles possess antioxidant activity in a concentration dependent way (percentage scavenging up to 74.4% for maleic acid capped nanoparticles and 56% for citric acid capped nanoparticles). These nanoparticles also have cytotoxic effects when tested against brine shrimp. Overall results showed that the maleic acid capped silver nanoparticles were more active due to their small size and smooth round surface as compared to citric acid capped and uncapped silver nanoparticles.

Keywords: Antifungal, Antimicrobial, Cytotoxic, Antioxidant, Modifiers, XRD, EDX, SEM.

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Abbreviations

UV-Vis	Ultra Violet Visible Spectroscopy
SEM	Scanning Electron Microscope
EDX	Energy-Dispersive x-ray Spectroscopy
XRD	X-ray Diffraction Spectroscopy
$AgNO_3$	Silver Nitrate
$FeSO_4.7H_2O$	Iron Sulfate Heptahydrate
AgNPs	Silver Nanoparticles
mV	Mili Volt
KeV	Kilo Electron Volt
μL	Micro Litre
nm	Nano Meter
Kv	Kilo Volt
μM	Micro Molar
mL	Mili Litre
ppm	Parts Per Million
rpm	Revolutions Per Minute

Chapter 1

Introduction

Our condition experiences a huge crumple, because of quick urbanization and industrialization, in addition to countless, gases or perilous and unnecessary materials are unconstrained, so now, we require to be familiar with the privileged awareness living in nature and it's normal products, that is coordinated towards the progressions required in the combination procedures of nanoparticles. For organic particles, nanotechnological applications are extremely fitting, because of their selected properties. In the combination procedure of metal nanoparticles, natural particles experience remarkably illicit assembly to make them reasonable, which have turned out to be solid and humble for the earth [1]. The strategy for the semiconductor and metallic nanoparticles union is a massive zone of research because of its approaching applications that are impelled to build up the novel and inventive advances [2]. One of the up and coming territories of research in the common field of material science is a field of nanotechnology. Nanoparticles show completely fresh out of the plastic new or upgraded properties, for example, measure measurement, appropriation, and particles morphology (size and shape), and so on. Novel and strange utilizations of nano materials and nanoparticles are quickly rising in various spaces [3].

Metallic nanoparticles have a great specific surface territory and a great division of surface molecules. In light of the one of a generous physicochemical potentials of

nanoparticles, together with reactant movement, photosensitive properties, electrical properties, antimicrobial properties, and attractive properties [4-7] they are attaining the interest of researcher for their innovative techniques for blend. In the passing few years, the blend of metallic nanoparticles is an imperative subject of research in present day material science. Nano-crystalline silver particles have been revealed massive uses in the fields of high affectability bio molecular discovery, antimicrobial (antibacterial and antifungal), diagnostics, catalysis, miniaturized scale hardware and therapeutics. Despite that, there is still requirement to design the economic industrially suitable as well as naturally uncontaminated combination course to orchestrate the silver nanoparticles. Silver is exceptional for having an inhibitory influence towards various bacterial strains and microorganisms normally present in restorative and mechanical processes [8]. In drugs, silver and silver nanoparticles have an adequate application including skin treatments and creams containing silver to avoid contamination of consumes and open injuries [9], therapeutic devices and inserts arranged with silver-impregnated polymers [10]. In material industry, silver-installed surfaces are presently used in draining apparatus [11].

Different approaches (physical, biological and chemical) can be used for synthesis of nanoparticles. Most common method is chemical method which requires very short period of time for large quantity of nanoparticles synthesis. This method have need of different modifiers or capping agents for stabilizing the size of nanoparticles (Citric acid, Maleic acid, Polyvinylpyrrolidone K-30, Sulfuric acid, N-methyl pyrrolidone and many others).

These silver nanoparticles are utilized in catalysis, hardware for circuit improvement and as self-collected fresh nanostructure resources, yet just couple of show diverse arrangements of uses with light association[12][13].

Nanoplasmonic explore is effectively picking up enthusiasm because of its different worth seeing applications, for example, super focal points, surface improved raman spectroscopy (SERS), single particle spectroscopy [14-18], plasmon-upgraded fluorescence, nanoscale lasing, upgrade of non-direct photosensitive signs, quantum

registering, plasmon helped photograph lithography, photocatalysis, light reaping, biochemical detecting and conceivably change of sun powered to substance vitality with plasmonic metal nanostructures [19-29]. Silver is fifty times less expensive than gold and turned into a decent hopeful in plasmonics because of its one of a kind and helpful physicochemical properties for the cutting edge plasmonic advances [30-32]. It is noticed that plasmonics limit to a pairing between electromagnetic excitation and metallic nanostructure for the age of surface plasmon and don't indicate plasmonic works without episode light. Nanoplasmonic is very special as various dimensions of nanostructures from 10 to 100 of nm can be utilized which go about as a connection among nanometer and micrometer levels. Accordingly, plasmonics is another branch of nanotechnology that has desire to comprehend the control of light with metallic nanostructure in creative methodology.

In biochemical applications, nanotechnology is an interdisciplinary approach and mainly focusing on nanoparticle synthesis which have enhanced antioxidant and antimicrobial properties against the progressive diseases and cancer [33]. At this time, research trends (in natural and synthetic antioxidant) led the identification and screening of novel antioxidant. Synthesized antioxidants is reported to have several properties for example anticarcinogenicity, antimutagenicity, antiallergic and antiaging [34]. Antioxidant activity play significant role in reducing singlet and triplet oxygen, decaying peroxides or neutralizing the free radicals. So, it is supposed that nanoparticle antioxidant activity is because of the better absorption of the antioxidant material. Toxicity of nanoparticles is determine by different parameters like size of particles, their surface area and surface reactivity. Cytotoxicity of the silver nanoparticles on cancer cell line was reported by Jacob et al. [35]. Metallic nanoparticles especially AgNPs have grew more consideration because of their microbial resistant [36] and play significant role as antimicrobial agent [37]. So among all of the other metallic nanoparticles, silver nanoparticles are broadly used in commercial products. In this manner, we synthesize silver nanoparticles of different morphologies using different modifiers through chemical

reduction method and then characterize these confined sizes and shape nanoparticles through UV-Vis spectrophotometer, SEM, EDX and XRD. Biological evaluation is also being done through different assay which includes antibacterial assay, antifungal assay, cytotoxic assay and antioxidant assay.

1.1 Problem Statement

In the market there are enormous drugs available, but how much they are effective and to which extent, is still a question. As time is passing, bacteria, viruses and other microbes are getting resistant to these drugs. Silver nanoparticles have been successfully used as antimicrobial agent with no side effects. So in that context, we necessitate ourselves to prepare new agents that are much more effective and long-lasting. For that purpose, we are going to synthesize silver nanoparticles using chemical reduction method and characterize through UV-Vis spectrophotometer, SEM, XRD and EDX and their biological evaluation through different assays.

1.2 Aims and Objectives

Nanotechnology can possibly be utilized in huge number of procedures and products. The nanoparticles synthesis is one of the most important areas of present day nanotechnology. The present investigation is expected to categorize the synthesis, characterization, cancer preventive agent, cytotoxic, antioxidant, and antimicrobial activities of silver nanoparticles that are synthesized by wet chemical method using different capping agents or modifiers. The principle motivation behind this investigation is to produce balanced out nanoparticles with substantially less exertion and financial esteem. This investigation aims to find out the shape, size and structure of silver nanoparticles and their capacities of performing various biological activities.

1.2.1 Objectives

This study requires the following objectives:

- Silver nanoparticles synthesis by chemical reduction method using different modifiers.
- Synthesized silver nanoparticles characterization through UV-Vis spectrophotometer, scanning electron microscope (SEM), energy dispersive x-ray spectroscopy (EDX) and x-ray diffraction spectroscopy (XRD).
- Biological evaluation of synthesized silver nanoparticles by using different bioassays including antibacterial, antifungal, antioxidant and cytotoxic assays.

1.3 Scope

Improvements in silver nanotechnology assist us with designing and synthesizing silver nanoparticles. Their special antimicrobial (antibacterial and antifungal), optical and physical properties would lead AgNPs across the board practices in medicinal and distinctive divisions like wound healing, food sanitation, targeted drug delivery and so on. Examinations' with respect to the preparation of AgNPs goes for designing of effective drug delivery agent, diagnosing and treating fatal diseases as well ensuring higher safety and efficacy.

Chapter 2

Literature Review

Nanotechnology is a modern investigation zone that mainly concerns by way of synthesis, design and structure handling of particles ranges from 1 to 100 nm (ultrafine) at least in one dimension. In recent years, there is a significant growth seen in this field that has unlocked new fundamental and applied frontiers, which includes nanoparticles synthesis and their applications in exotic physiochemical and optoelectronic properties. Nanotechnology has much more importance in different fields of life such as environmental health, cosmetics, chemical industries, space technology, drug delivery, gene delivery, photo electrochemical applications, single electron transistors, energy science, non-linear optical devices, light emitters, biomedical science, electronics, mechanics, optics, catalysis, food and feed, health care, targeted drug delivery and gene delivery [38, 39].

Nano scale materials are now considered as ultimate solution to many health, environmental and technological problems (catalysis, medicine, water treatment and solar energy conversion). Nanotechnology is necessarily altering the approach where nanoparticles are manufactured and devices are fabricated. Bottom-up approach is used to achieve the assimilation of nanoscale building blocks into functional assemblies and further into multifunctional strategies. Nanomaterials are of great concern due to their exclusive properties such as mechanical, magnetic and optoelectronics as compared to bulk material of that compound [40].

2.1 Nanoparticles

The word "nanoparticles" is broadly used to define a particle with size in the range of 1-100 nm (ultrafine), at least in one of the three possible dimensions. In this size range, the physical, chemical and biological properties of the nanoparticles changes in fundamental ways from the properties of both individual atoms/molecules and of the corresponding bulk materials. Nanoparticles can be made of materials of various chemical nature, the most common being organics, silicates, metal, metal oxides, polymers, non-oxide ceramics, carbon and biomolecules. Nanoparticles occur in several morphologies for example cylinders, tubes, spheres, platelets and many more. Mostly the nanoparticles are designed with surface modifications tailored to meet the needs of specific applications they are going to be used for. The huge diversity of the nanoparticles arising from their extensive chemical nature, shape and morphologies, the dispersion state of the particles, the mediocre in which the particles are present and most prominantly, the various possible surface improvements that makes nanoparticles a dynamic field of science [41].

2.2 Silver Nanoparticles

Silver nanoparticles has great significance because of their unique shape and size depending electromagnetic and optical properties which can be merged into electronic components, combined fibers, cosmetic products, biosensor tools, cryogenic superconducting materials and antimicrobial application. Different approaches (e.g. physical, chemical and biological) have been adopted for stabilizing and synthesizing silver nanoparticles [42, 43].

One of the most common approach is chemical approach that includes chemical reduction (by using different inorganic and organic reducing agents), radiolysis, photochemical reduction and photochemical reactions are broadly used for the synthesis of AgNPs.

2.3 Synthesis of Silver Nanoparticles

Nanoparticles have been made physically and artificially for a long time, (Figure 2.1) anyway late changes exhibit the fundamental occupation of microorganisms and natural systems in progress of metal nanoparticles.

2.3.1 Physical Approach

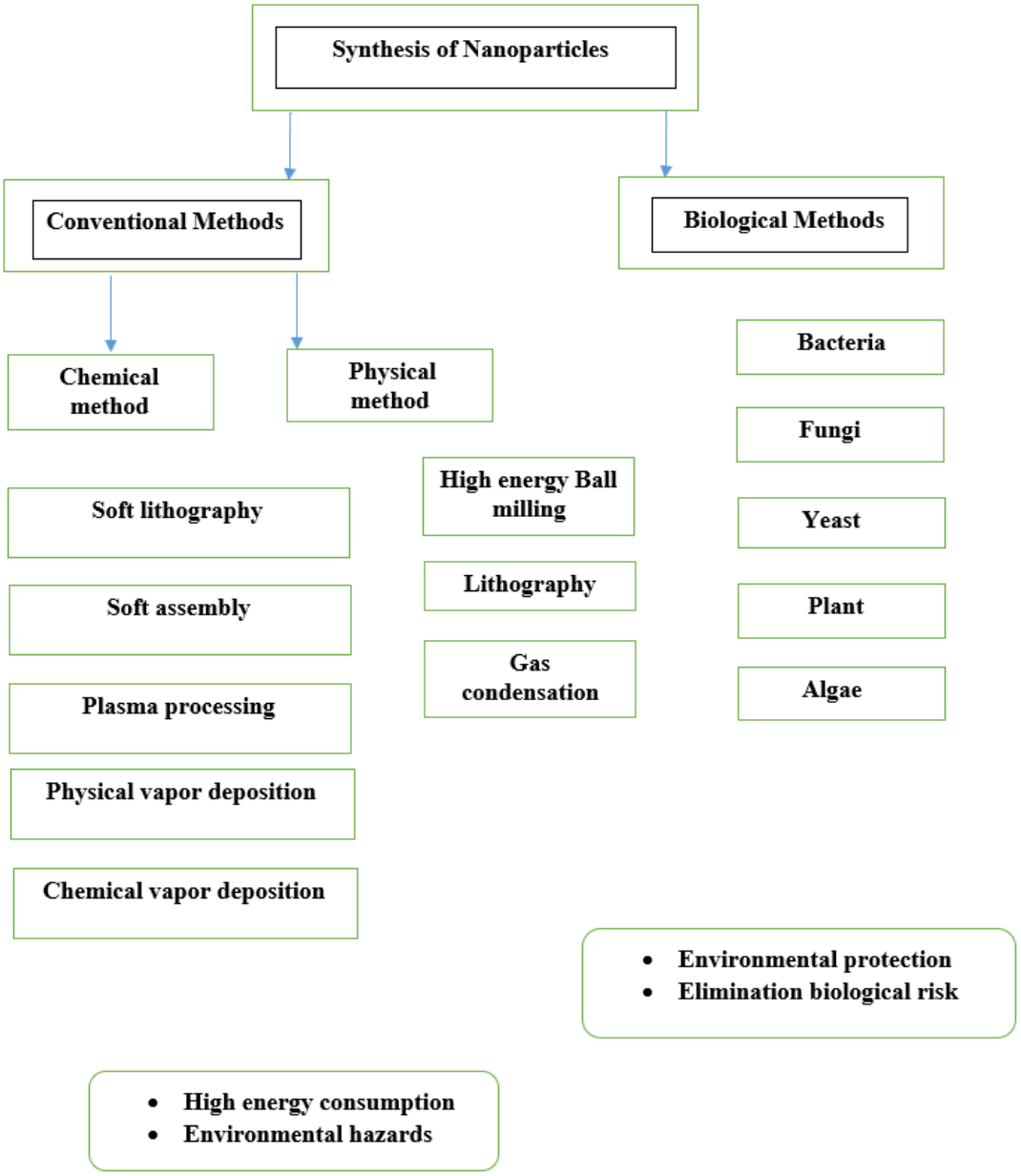


FIGURE 2.1: Overview of Different Methods for Nanoparticle Synthesis

The maximum important physical procedures includes degeneracy accumulation and laser removal. Different types of metallic nanoparticles, for example, gold, cadmium sulfide, fullerene, silver and lead sulfide all have earlier been incorporated by using the degeneracy collection strategy.

The absence of dissolvable pollution in the organized skinny cinemas and the symmetry of nanostructures rotation are the paybacks of physical approach in association with multiple procedures. [44, 45]. It was proved that nanoparticles of silver might be synthesized through a small porcelain furnace with a natural central heating source [46].

At a reasonable fast rate the dissipated vapor can cool down on grounds that the temperature slope in the section of the heater surface is abrupt extremely in analysis with that of a tube heater. This makes believable progress of minute nanoparticles in great attention.

This physical method can be valued as a nanoparticle procedure for long pull tests for inner breath toxic quality examinations, and as an alignment tool for nanoparticle assessment hardware [47-52]. AgNPs can be incorporated by laser removal of metal mass materials in organization [52-58].

2.3.2 Biological Approaches

Later, the development of nominal green science methods utilizing characteristic are diminishing, coating, and matching out operators to get ready AgNPs with preferred shape and size have turned into a significant focal point of scientists. Biological methods can be employed to compose AgNPs deprived of the use of some hard, lethal and costly artificial substances [59, 60].

The bio reduction of metallic particles by mixtures of biomolecules set up in the concentrates of particular life forms (e.g vitamins, amino acids, compounds or proteins and polysaccharides) is earth meager, yet synthetically mind boggling. Many inspections have declared the effective mixture of silver nanoparticle using life forms (microorganisms and organic frameworks) [61, 62].

2.3.3 Chemical Approach

Generally, it is very difficult to control the morphology and structure of material at nanometer scale because it has huge influence on the properties of that particular material.

A lot of effort has been dedicated to the silver nanoparticles synthesis to attain specific shapes and size like nanocubes [66, 69], nanodisks [70-73], nanoprisms [74-76], nanoplates [70-72], nanorods [73], and nanowires [77-85]. Chemical approach is the most common and less time consuming approach among the others. Chemical reduction occur by using organic or inorganic reducing agents and a strong stabilizing agent.

Different reducing agents are used for this purpose for example, elemental hydrogen, polyol process, Tollens reagent, N, N-dimethylformamide, sodium citrate, ascorbate, sodium borohydride (NaBH4), all are utilized as reducing agents for silver ions in the non-aqueous and aqueous solutions.

Here is the detail of photochemical and chemical methods:

2.3.3.1 Light Mediated Synthesis

Here, light radiation is used for the synthesis of nanoparticles. So, laser light or laser ablation on the aqueous solution of the metallic salt in the existence of a stabilizing agent to produce specific size, shape and structure of nanoparticle where light source acting like a reducing agent [86] [87].

At a reasonable fast rate the dissipated vapor can cool down on grounds that the temperature slope in the section of the heater surface is abrupt extremely in analysis with that of a tube heater.

Modification in the morphologies of nanoparticles is being done by using laser light such as simply melting the silver nanospheres to silver nanoplates [88-93]. Extremely desirable and well controlled metallic nanoparticles are synthesized by light irradiation or light mediated process.

2.3.3.2 Electrolysis and Pyrolysis

Very small number of research papers are available that by some means describe the usage of electrochemical method for silver nanoparticles synthesis. Such as, sphere-shaped silver nanoparticles is being synthesized by reducing Ag^{+} ions in the presence of PVP (Polyvinyl pyrrolidone) using this method.

AgNPs that are amalgamated by this process are of average size of 10 nm. Moreover, thin platinum plate as anode and titanium electrode as cathode are also part of this process.

Another method of spray pyrolysis is also used for the silver nanoparticles synthesis, in which the average size of particles is 100 nm. As, no harmful and toxic synthesizing agents (reducing and stabilizing agents) are used in pyrolysis and electrolysis processes so that's why they are considered as environment friendly [94] [95].

2.3.3.3 Citrate Reduction

Citrate reduction of silver ions is one of the most prevalent method to synthesize the nanoparticles. This method does not needs any extraordinary laboratory skills. First time, Lee and Meisel presented this process in 1982 [99].

Commonly, silver nanoparticles are designed when a specific quantity of sodium citrate is mixed in an aqueous solution of silver nitrate at boiling for 1 hours.

One major drawback of this process is that it does not produce controlled size nanoparticles. Size range of synthesized nanoparticles is from 20 nm to 600 nm. One of the important factor is pH control.

Different morphologies of nanoparticles are synthesized by adjusting of pH level for instant, triangular shape at pH = 5.7 and spherical shape at pH = 11.1 [100, 101].

2.3.3.4 Polyol Synthesis

Silver salt precursor and stabilizing agent are bring together into polyols for the production of and development towards nanoparticles synthesis. Different reducing agents like 1,2-propylene glycol, 1,5-pentanediole or propylene glycol are the reducing agents which reduce silver ions present in aqueous solution [107].

At a reasonable fast rate the dissipated vapor can cool down on grounds that the temperature slope in the section of the heater surface is abrupt extremely in analysis with that of a tube heater. This makes believable progress of minute nanoparticles in great attention.

Polyol synthesis is one of the most important method for synthesis of wide range of sizes and shapes. Temperature and concentration shows significant part in controlling the morphologies of AgNPs.

2.3.3.5 Seed Mediated Synthesis

In seed mediated synthesis, nanocrystals act as seed for additional growth. End product size and shape is being highly controlled by this process [108-111]. Such as, Xia et al. synthesized silver nanocubes by using globular shaped single crystal seed for specific size 30-200 nm.

2.3.3.6 Silver Mirror Reaction

In 1835, silver mirror reaction was discovered by Justus von Liebig [112] and became widespread for put down silver nanoparticle on targets. Tollens reagent is reduced by any aldehyde containing compound like sugar for synthesizing silver.

$$2[Ag(NH_3)_2]^+ + RCHO + 2OH \longrightarrow 2Ag + RCOOH + 4NH_3 + H_2O$$

A shiny layer is formed on the internal layer of the container in which the reaction was carried out. This layer shows the success of reaction.

2.4 Application of Silver Nanoparticles

The medicinal properties of AgNPs acknowledged for many decades. Since the 19th century, silver-based composites have been utilized in many antibacterial and antifungal applications.

Nanoparticles have been used for many biological, pharmaceutical, physical applications, and antimicrobial agents. It is a fact that silver and silver based composites are extremely lethal to bacterial and many fungal strains.

Because of their very small size and extremely large surface to volume ratio, nanoparticles are of great interest which leads to the differences of physiochemical properties as related to bulk of the same chemical composition. AgNPs have been utilized broadly as antibacterial specialists in human health, food storage and packaging, fabric industry and several environmental applications.

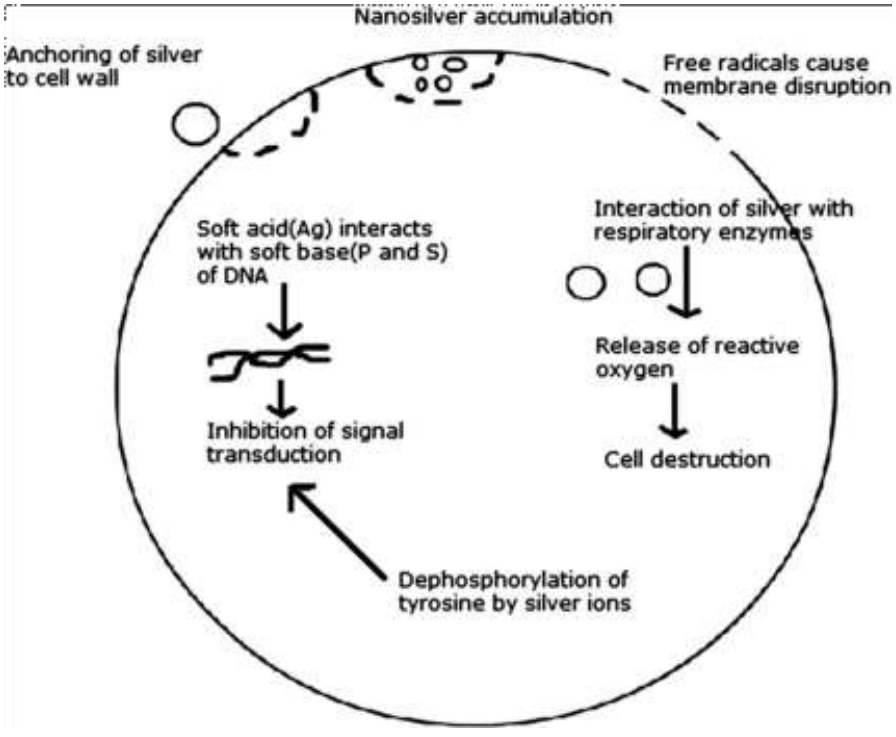


FIGURE 2.2: Several Modes of Actions of AgNPs on Bacteria

Antimicrobial (antibacterial and antifungal) properties of AgNPs triggered the utilization of these nano-metals in various medicinal fields, various industries,

bundling and packaging, animal farming, cosmetic industry, safety and military. [113, 114].

When all is said in done, therapeutic influences of silver nanoparticles (in suspension form) rely upon important properties , together with size of particles (surface region in addition to energy), shape of particles (reactant activity), concentration of particles and charge of particles (oligodynamic quality) [115].

The mechanism of antibacterial effects of silver nanoparticles is still a mystery but a number of studies shown that particles of silver may attach to gram-negative bacterial cell wall and break the cell wall, which eventually leads to the protein denaturation and death of the cell (Figure 2.2).

2.4.1 Mechanism of Action

To examine the antibacterial properties of AgNPs, fluorescent bacteria are used. Silver based compounds attached to the S-proteins (sulfur containing proteins) of bacteria which eventually cause death.

In addition, fluorescent capacities of cell-free supernatants exposed the AgNPs effects on recombination of bacteria. Nanoparticles of silver attached to the cell wall surface which results the additional growth of envelope protein precursors that leads to the immediate degeneracy of proton motive force [116]. Catalytic activity of silver based compounds, nanoparticle and their destruction towards the cell by connection with sulfur and phosphorous containing compounds, for example DNA have been examined [117]. In addition, AgNPs showed interruption of external membrane and break down the plasma membrane, in this way causing consumption of ATP [118]. An additional mechanism includes the silver nanoparticles association with the oxygen and its reaction with sulfhydryl groups on the cell wall, where R-S-S-R bond formation take place, that result the blocking of respiration and causing cell death [119]. Nanoparticles have different application in different fields of life. Some application are given below in the figure 2.3.

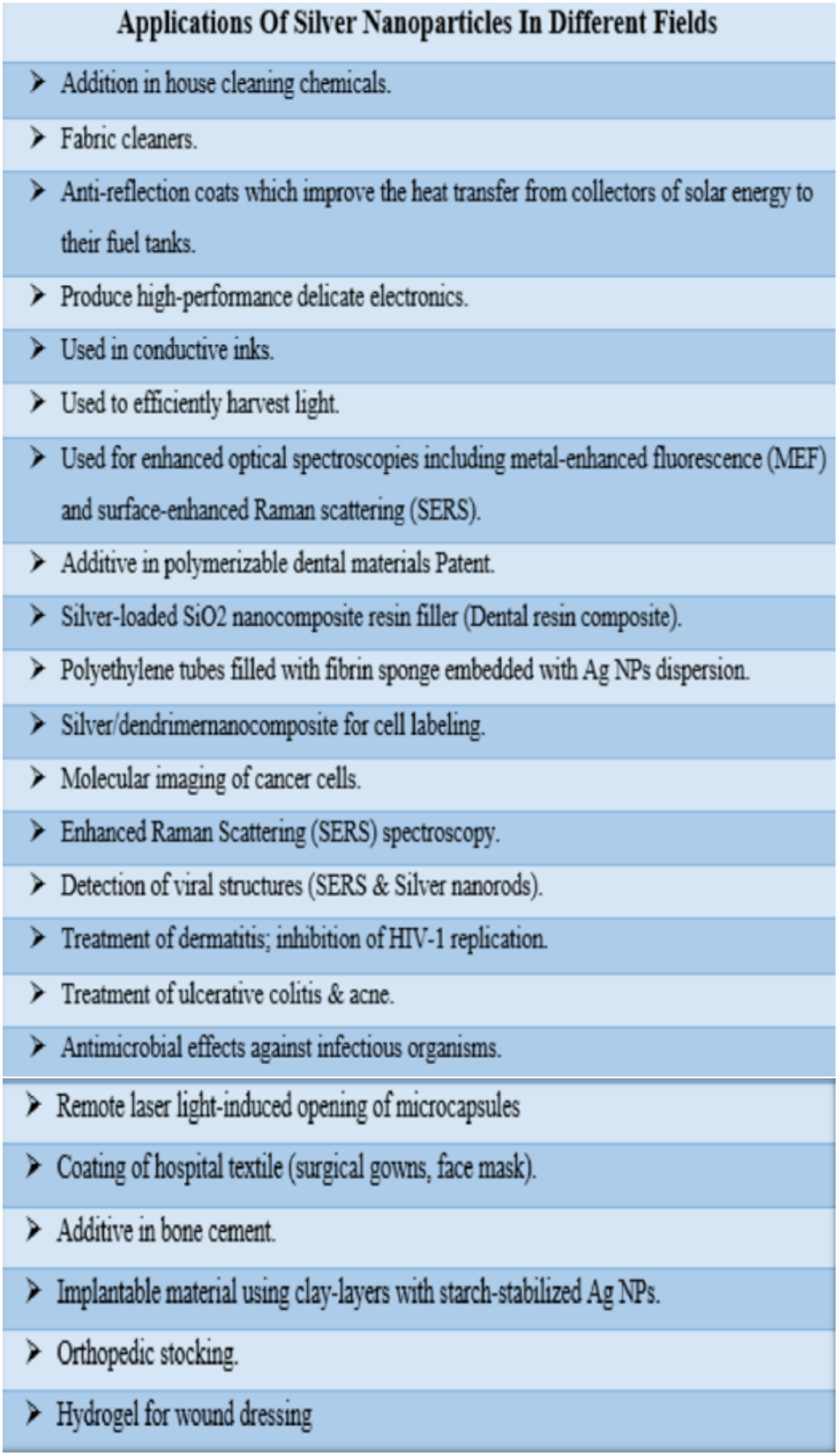


FIGURE 2.3: Silver Nanoparticle's Applications [120, 121]

2.5 Characterization and Biological Evaluation of Silver Nanoparticle

2.5.1 UV-Vis Spectrophotometer

The optical properties of AgNPs was determined with the help of UV-Vis spectrophotometer (UV-1602) (Figure 2.4). After mixing of $AgNO_3$ solution and $FeSO_4.H_2O$ solution along with different modifiers, AgNPs solution was formed. Then by setting distilled water as a blank reference, about 4 mL AgNPs solution were subjected to spectrophotometer [122].

FIGURE 2.4: UV-Vis Spectrophotometer

2.5.2 Scanning Electron Microscope

The morphological (size and shape) features of AgNPs that was prepared by using the chemical method were analysed by SEM (Scanning Electron Microscope) (JEOL-JSM-6490 LATM), effective at the voltage of 20 Kv with the frequency of 2838 cps (max). Magnified micrographs were taken up to the resolution of 5 μm in scale bar. Scanning electron microscope slips were arranged by making a solution smear on the slips. Platinum thin layer coat was made. This will help the samples to become more conductive [123].

2.5.3 X-ray Diffraction Spectroscopy

The phase identification and structural analysis of AgNPs was analyzed by the help of XRD (X-ray diffraction spectroscopy) [124].

2.5.4 Energy Dispersive X-ray Spectroscopy

Elemental composition and chemical characterization of AgNPs was analyzed by EDX (Energy-dispersive X-ray spectroscopy). The signal for silver nanoparticles will be achieved at energy level of 3 keV which indicates the silver nanoparticles have been correctly recognized [125].

2.5.5 Bioassay

The method which are used for the biological evaluations, concentration and purity of substances by calculating its impact on cell, tissue, organism and enzyme or receptor preparation associated to a standard ground work are known as bioassay. In developing novel drugs and investigation of pollutants in the environment, bioassays plays vital role.

Types of Bioassays

Bioassays can be categorized into two types.

Quantitative Bioassays

This type of assays are generally examined by using the bio statistical methods. Quantitative assays are also involved in assessment of the meditation or effectiveness of a substance by calculating its biological reaction that a specific substance produces.

Qualitative Bioassays

This type of assays are generally used to analyze the physical impact of a specific substance that may not be quantified.

Why we use Bioassays?

- To determine the pharmacological activity of substance.
- To determine the level of drug toxicity.
- To measure the unknown substance concentration.
- To investigate the role of endogenous mediators.
- To assess the amount of pollutants that are being released by a specific source [126].

2.5.5.1 Antibacterial Assay

Pathogenic microbes like bacteria, fungi and algae are causing hundreds of different diseases in plants, animals and human. In higher organisms, bacterial and fungal infections are the major cause of death. The antibiotic penicillin discovery is considered one of the most significant discoveries in all over the world. Different new antibiotics were isolated from the natural sources and organic products and after that synthesized artificially and used in medical practices.

Humans efforts against the pathogenic bacteria and fungi is unfortunately far from over because of various reasons. One of the reason is that with the passage of time, new species of pathogens are discovered and their incredible abilities to gain resistance against antibiotics. This is an ongoing process (development and discovery of new antimicrobial agents) [127]. The reason of choosing bacterial strain in this study were mainly based on their significance as latent human pathogens.

Bacillus subtilis is a gram-positive strain, rod shape and spore forming broadly spread all over the environment, mainly in air, soil and decaying plant residues. This strain produces numerous toxins and causing the food poisoning. It is not human natural biota but may isolated from human infections. Pneumonia, endocarditis and septicemia are the main diseases caused by *B. subtilis* [128].

Micrococcus luteus is a gram-positive bacterial strain. It may cause different infections which includes meningitis, arthritis, pneumonia in immunological compromised patients and endocarditis [129].

Enterobacter aerogenes is a gram-negative bacterial strain and mainly active in food degradation. It causes the respiratory and urinary tract infection and pathogenic infections in immunosuppressed hosts [130].

Salmonella setubal is a gram-negative bacteria that is in charge for triggering the severe food poisoning and typhoid [131].

Staphylococcus aureus is a gram-positive and round fashioned bacteria and generally responsible for causing skin infections, food poisoning and respiratory infections [132].

Agrobacterium tumefaciens is a gram-negative and rod shaped soil bacterial strain and mainly involved in Crown gall disease [133].

2.5.5.2 Antifungal Assay

In immune-compromised patients, fungal infections are one of the most important adaptable infections. Due to the intensive therapy being managed with cancer therapy, HIV infections (human immunodeficiency virus) and organ transplant, the immuno-compromised patients are increasing day by day. Fungal pathogen control and designing new drugs for antifungal control are crucial requirements [134]. The less number of drugs availability in market and multi drug resistant fungal strains makes it compulsory to design fresh modules of antifungal drugs and other composites that prevent these mechanisms of resistance [135].

Fusarium solani is a common soil fungus and colonist of plant materials. *Solani* is implicated in plant diseases as well as human diseases notably infection of the cornea of the eye and many other including skin infection, disseminated disease, fungemia, osteomyelitis and endophthalmitis [136].

Mucor species is generally present in digestive systems, rotten vegetables materials, soil, on plant surfaces and residues of iron oxide in biosorption procedure. Many mucor species are not capable to infect endothermic animals and humans because of their lack of ability to grow in warm environments. Heat tolerant species of mucor like *Mucor indicus* may cause opportunistic and most frequently spreading necrotizing recognized as zygomycosis [137].

Aspergillus fumigatus is very common fungal specie normally present in soil and dead decaying organic matter where it plays important role in carbon recycling. In immuno-compromised persons (such as AIDS, organ transplant recipients or leukemia) fungus become more pathogenic and triggering a variety of diseases usually known as aspergillosis. Numerous virulence factors have been suggested to explain this opportunistic behavior [138].

Aspergillus flavus is recognized for its legumes, tree nuts and cereal grains colonization. Flavus infections can occur even though hosts are still in the ground (preharvest), but symptoms are not appear until postharvest (transport). Along with the preharvest and postharvest infectious, many species produce toxic compounds (mycotoxins) that are toxic to living things when consumed. Flavus cause aspergillosis and consider opportunistic pathogen in immuno-compromised persons [139].

Aspergillus niger is very common fungal species. On certain vegetables like onion and fruits like peanuts, grapes and apricot, it may cause a disease called "black mold" and also a mutual contaminant of food. It is present everywhere but most commonly in soil and indoor environment, where it may confused with the *Stachybotrys* ("black mold") because both species have characteristic of making black colonies [140].

2.5.5.3 Cytotoxic Assay (Brine Shrimp Assay)

Cytotoxic assay is performed by using brine shrimps, as reported by Ismail et al. (2015). This assay was generally used to assess the nanoparticles toxicity. Cell

viability may be defined as total number of healthy cells in a sample and cell propagation is a vital sign for understanding the mechanism of action of specific proteins genes and pathways involved in survival of cell and death after revealing the toxic agents. Mostly, the methods are same for both the cell propagation detection and viability determination. Cytotoxic assays are used for drug screening to identify weather particles have some effects on cell propagation or exhibit direct toxic effects. On the basis of cell functions like cell adherence, enzymatic activity and permeability of cell membrane, co-enzyme production, ATP production and nucleotide uptake activity, multiple assay techniques are used.

FIGURE 2.5: Brine Shrimp Eggs

2.5.5.4 Antioxidant Assay

To predict the antioxidant activities, DPPH (2, 2 diphenyl-1-picrylhydrazyl) was used. This is a mechanism in which lipid oxidation inhibits, so scavenging of DPPH radical and consequently determinate free radical scavenging capacity. The assay measures the reducing ability of antioxidants toward the DPPH radical [141]. Methanol and ethanol are most utilized solvents for determining the radical scavenging activity by DPPH. Miller et al. used methanol as a solvent for this assay.

Chapter 3

Material and Method

Following research work was carried out in biological laboratory of Department of Bioinformatics and Biosciences, Faculty of Health and Life Sciences, Capital University of Science and Technology, Islamabad.

3.1 Materials

Material utilized for the research work is given below:

Table 3.1: Material Utilized for Research Work

Chemicals	**Company Name**
Iron sulfate heptahydrate	Sigma-Aldrich
Citric acid	———-
Maleic acid	———-
Sulfuric acid	———-
Polyvinylpyrrolidone (PVP k30)	———-
Nutrient agar	———-
Luria broth	———-
Sabouraud dextrose agar (SDA)	———-
Brine shrimps egg	———-

Chemicals	**Company Name**
Sea salt	Sigma-Aldrich
DPPH reagent (2,2-diphenyl-1-picrylhydrazyl)	————-
Ascorbic acid	————-
Streptomycin	————-
Terbinafine	————-
Ethanol	————-
Consumables	
Petri plates	
Test tubes	
Vials	
Micropipette	
Cotton plugs	
Cotton swabs	
Aluminum foil	
Falcon tubes 15 mL , 50 mL	
Eppendorf tubes	
Beakers 100 mL, 500 mL , 1000 mL	
Test tubes racks	
Discs	
Para film or masking tape	
Forceps	
Microorganism used	
Bacillus subtilis	*Aspergillus flavus*
Agrobacterium tumefaciens	*Aspergillus fumigatus*
Staphylococcus aureus	*Aspergillus niger*
Enterobacter aerogenes	*Mucor species*
Salmonella setubal	*Fusarium solani*
Micrococcus luteus	

3.2 Scheme of Methodology

FIGURE 3.1: Overview of Methodology

3.3 Synthesis of Silver Nanoparticles

3.3.1 Preparation of Silver Nitrate Solution

For silver nanoparticles synthesis, reported method was used with some modification [142]. of $AgNO_3$ solution was prepared for this purpose. Specific concentration of $AgNO_3$ salt was dissolved in 100 mL of de-ionized water along with different modifiers (Maleic acid and Citric acid) to attain different morphologies of silver nanoparticles (Table 3.2).

3.3.2 Preparation of Iron Sulfate Heptahydrate Solution

$FeSO_4.7H_2O$ solution was prepared by adding specific concentration of iron sulfate heptahydrate salt in 100 mL of distilled water. Both solution were cooled to 8-20 ^oC [142].

3.3.3 Synthesis of Silver Nanoparticles

Equal volume of $AgNO_3$ (silver nitrate) solution and $FeSO_4.H_2O$ (iron sulfate heptahydrate) solution were vigorously mixed according to the appropriate mass ratio (1:1) with high-speed stirring at room temperature.

This mixture was incubated at room temperature for 2 hours. Change in color appear and reduced aqueous components was subjected for the measuring of UV-Vis spectra of the solution.

The obtained AgNPs were separated from the supernatants through the centrifugation at 6000 rpm for 30 minutes. After that, nanoparticles was dried in hot air oven for 48 hours and later, synthesized silver nanoparticles was collected and stored in eppendorf tubes [142].

TABLE 3.2: Detailed Experimental Measurements for the Synthesis of Different Morphologies of Silver Nanoparticles

Morphology	Conc. of $AgNO_3$ (M)	Temperature ^oC	Conc. of $FeSO_4.7H_2O$ (M)	Modifiers (The type & conc. in $AgNO_3$) (M)
Spherical plates	0.1	17.5 ^oC	0.5	Maleic Acid 0.02
Flowerlike	0.1	12.3 ^oC	0.5	Citric Acid 0.02
Hexagonal (uncapped)	0.1	16.3 ^oC	0.05	None

3.4 Characterization of Silver Nanoparticles

3.4.1 UV-Vis Analysis

(a) Sample Preparation

Synthesized AgNPs were dissolved in distilled water and sample stock with final concentration of 25000 ppm was prepared. This stock was used for UV-Vis analysis.

Mixture was incubated at room temperature for 2 hours. Change in color appear and reduced aqueous components was subjected for the measuring of UV-Vis spectra of the solution.

(b) Procedure of UV-Vis Analysis

Silver nanoparticles optical properties was determined with the help of UV-Vis spectrophotometer (UV-1602). By setting distilled water as a blank reference, about 4 mL AgNPs solution were subjected to spectrophotometer to confirm the synthesis of silver nanoparticles [142]. Samples prepare for different morphologies were all scanned between the 300 - 700 nm for their optical properties.

3.4.2 Scanning Electron Microscope Analysis

Morphological features (size and shape) of AgNPs that were prepared from the chemical method were determined by scanning electron microscope (SEM) (JEOL-JSM-6490 LATM), effective at the voltage of 20 kV with the frequency of 2838 cps max. (Count per second). Magnified micrographs were taken up to the resolution of 5 μm in scale bar.

Scanning electron microscope slips were arranged by making a solution smear on to the slips. A skinny sheet of Pt (platinum) coat was made which helps the samples to become more conductive [123]. All samples of silver nanoparticles with different morphological features were analyzed at different resolutions for determining the size and shape.

3.4.3 X-ray Diffraction Spectroscopy Analysis

The phase identification and structural analysis of chemically produced silver nanoparticles was analyzed by X-ray diffraction spectroscopy (XRD) [124]. XRD is used to study the structure, alignments, composition and physical properties of nanoparticles.

3.4.4 Energy-dispersive X-ray Spectroscopy Analysis

Elemental arrangement and chemical characterization of synthesized AgNPs was performed by energy-dispersive x-ray spectroscopy (EDX). The signal for AgNPs was achieved at energy level of 3 keV which indicates the silver nanoparticles have been correctly recognized [125]. Semiconductor sensor measures energy of arriving photons in EDX. It should be chilled by Peltier cooling or with liquid nitrogen to maintain detector resolution and integrity. Energy dispersive x-ray spectroscopy is broadly engaged in electron microscopes where imaging is the main task and comparatively economical and convenient XRF units.

3.5 Biological Evaluation of Synthesized Nanoparticles

Antimicrobial Assays: Two kinds of antimicrobial assays were executed to evaluate the biological activity of synthesized silver nanoparticles.

- Antibacterial assay
- Antifungal assay

3.5.1 Antibacterial Assay

Six strains of bacteria were used for antibacterial assessment. Antibacterial properties of silver nanoparticles was analyzed by means of disc diffusion method as described by Ruparelia et al., [144].

3.5.1.1 Bacterial Strains Used

- *Bacillus subtilus*
- *Agrobacterium tumefaciens*
- *Staphylococcus aureus*
- *Enterobacter aerogenes*
- *Salmonella setubal*
- *Micrococcus luteus*

3.5.1.2 Preparation of Sample

Synthesized silver nanoparticles were dissolved in distilled water and final concentration of 25000 ppm was made. In this assay different dilutions of this stock were used (100 ppm, 50 ppm, 25 ppm, 5 ppm and 2.5 ppm).

3.5.1.3 Media for Bacterial Growth

Luria broth agar was used for bacterial growth in petri plates. Its composition is as under:

a) NaCl	5g / 500 mL
b) Yeast	2.5g / 500 mL
c) Agar	7.5g / 500 mL
d) Bacto-tryptone	5g / 500 mL

3.5.1.4 Procedure

Petri dishes were prepared by pouring equal amount of Luria broth agar in all petri plates and allowed to solidify. After solidification, streaking of bacterial strains was done with cotton swabs and put the discs on it in a sequence. Each petri plate contains 6 discs, 4 discs of different samples of nanoparticles, 1 for positive control that was streptomycin 100 ppm and 1 disc of negative control that was distilled water. Finally, petri plates were sealed and incubated for 24 hours at 37 ^{o}C. After 24 hours, the clear inhibiting zones were identified around each disc and was measured with vernier calipers. Whole procedure was carried out in duplicate for each sample.

3.5.2 Antifungal Assay

For determining the antifungal activity of silver nanoparticles, tube dilution method was used [145].

3.5.2.1 Fungal Strains Used

Five strains of fungus were used for the antifungal assay.

- *Aspergillus flavus*

- *Aspergillus fumigatus*
- *Aspergillus niger*
- *Mucor species*
- *Fusarium solani*

3.5.2.2 Preparation of Sample

After centrifugation, pallet was dissolved in distilled water and final concentration of 25000 ppm was made.

3.5.2.3 Preparation of Media for Fungal Growth

For the fungal growth, Sabouraud dextrose agar was prepared. Its composition is given below

Sabouraud dextrose agar	26g / 400 mL of distilled water

3.5.2.4 Procedure

Firstly test tubes were striking to 10 cm. About 5 mL of sabouraud dextrose agar was added into the cotton plugged test tubes that was already autoclaved at 121 ^{o}C for 20 minutes. After that test tubes were loaded with 100 μL of different samples, distilled water as negative control, terbinafine as positive control and make a slant to the marked position at room temperature. As the media solidified, test tubes were inoculated with fungal strains and covered with the cotton plugs. The whole procedure were carried out in triplicate for each sample. Test tubes were incubated for 4 days at 37 ^{o}C. Readings was documented by measuring the fungal growth in slanting position. Growth inhibition was calculated with reference to the negative control linear growth. Following formula was used to calculate the percentage growth inhibition:

$$\text{Percentage inhibition} = \frac{\textit{Linear growth in negative control} - \textit{Linear growth in samples} \times 100}{\textit{Linear growth in negative control}}$$

3.5.3 Antioxidant Assay

Antioxidant capacity of synthesized silver nanoparticles was determined by using DPPH method (2, 2- diphenyl-1-picryl-hydrazyl-hydrate) that was described by Gyamfi et al. [141].

3.5.3.1 Sample Preparation

Stock was prepared by adding distilled water in to synthesized nanoparticles. Different dilutions were used (20, 40 and 80 μM).

3.5.3.2 Preparation of DPPH Solution

By adding about 12 mg of DPPH in 100 mL of ethanol, the reagent solution was prepared

3.5.3.3 Procedure

200 μL of the serial solution of nanoparticles along with the 2.8 mL of reagent (DPPH) was added in a vial. Whole procedure was carried out in triplicate. Ascorbic acid along with the reagent was used as positive control and ethanol as negative control. Vials were placed at dark place for 45 minutes. After that absorbance of samples was measured at 517 nm by using distilled water as a blank reference. To calculate the free radical percentage scavenging, following formula was used:

$$\% \text{ scavenging} = \frac{\textit{Control absorbance} - \textit{Nanoparticle sample absorbance} \times 100}{\textit{Control absorbance}}$$

3.5.4 Cytotoxic Assay

Brine shrimps cytotoxic assay was performed to determine the level of toxicity of synthesized nanoparticles as reported earlier. [146].

3.5.4.1 Preparation of Sample

Stock was prepared by adding distilled water in the synthesized nanoparticles and final concentration of 25000 ppm was made. Different dilutions were used for cytotoxic assay (5, 25, 50, 100 μM).

3.5.4.2 Sea Salt Preparation

Sea salt water was prepared according to the given concentration:

Sea salt	34g / L

3.5.4.3 Hatching of Eggs

Brine shrimps eggs were hatched in sea salt water (34 gL^{-1}).

3.5.4.4 Procedure

Vials were prepared with the nanoparticle samples 5, 25, 50, 100 μM were added in each vial and final volume of 5 mL was made by adding seawater. Distilled water was used as negative control. After 1 day, shrimps eggs were hatched and shifted to the vials (about 15 shrimps in each vial).

Whole process was carried out in triplicate. Vials were kept under light and room temperature at 25 ^{o}C. After 24 hours, alive or surviving shrimps were counted by pasture pipette (3x magnifying glass). By using this formula, percentage viability was calculated:

$$\text{Percentage Viability} = \frac{(Negative\ control - test)}{Negative\ control} \text{ x } 100$$

Chapter 4

Results and Discussions

This chapter includes all the findings achieved in the synthesis of silver nanoparticles through chemical reduction method and their characterization by using UV-Vis spectrophotometer analysis, SEM, XRD and EDX. These synthesized silver nanoparticles were also biologically evaluated through different assays; antibacterial, antifungal, cytotoxic and antioxidant. Results are given below.

4.1 Synthesis of Silver Nanoparticles

Change of color was the first visual confirmation of synthesis of AgNPs. Both solutions of silver nitrate *($AgNO_3$)* and iron sulfate heptahydrate *($FeSO_4.7H_2O$)* were mixed in equal ratio (1:1). 100 mL of 0.1 M $AgNO_3$ and 100 mL of 0.5 M $FeSO_4.7H_2O$ along with the 0.02 M of maleic acid were mixed and wait until the color of solution was changed from white to blackish brown. Similarly, 100 mL of 0.1 M $AgNO_3$ and 100 mL of 0.5 M $FeSO_4.7H_2O$ along with the 0.02 M of citric acid were vigorously mixed until the color of solution was changed from white to blackish grey. For uncapped silver nanoparticles, 100 mL of 0.1 M of $AgNO_3$ and 100 mL of 0.5 M $FeSO_4.7H_2O$ were mixed without any modifier. Color changes steadily from white to yellowish orange and then blackish grey and

blackish brown were witnessed which confirms the formation of silver nanoparticles (AgNPs). Change of color indicates the metallic nanoparticle synthesis [147].

After centrifugation and washing, pellet of nanoparticles was placed in hot air oven for drying at 60 ^{o}C up to 48 hours. Finally, silver nanoparticles was collected and saved for further analysis.

4.2 Characterization and Biological Evaluation of Silver Nanoparticles

4.2.1 Silver Nanoparticles Analysis Through UV-Vis Spectrophotometer

UV-Vis spectrophotometer was used to confirm the silver nanoparticles synthesis and to analyze the optical properties of synthesized silver nanoparticles. Silver showed a solid wide-ranging peak at 400 nm [148], and widening of the peak indicates the formation of poly dispersed silver nanoparticles (Fig 4.1). The peak of the citric acid capped and uncapped AgNPs was broader than that of the maleic acid capped AgNPs that may be because of their larger size range [149]. The spectrum did not contain any additional peak indicating that the sample did not contain any contaminants. UV-Vis spectroscopy is extensively used for silver nanoparticles characterization which are known to exhibit a UV-visible absorption maximum in the range of 400-500 nm because of surface plasmon resonance excitation [148].

Nanoparticles contain optical properties which are sensitive to shape size accumulation state, concentration and surface chemistry of nanoparticles which makes UV-Vis spectroscopy an important device for the identification and characterization of nanoparticles [150]. Silver nanoparticles have free electrons, which offer rising to absorption band of SPR (surface plasmon resonance) [151], because of the joined vibration of electrons of silver nanoparticles in vibration with the light wave [152].

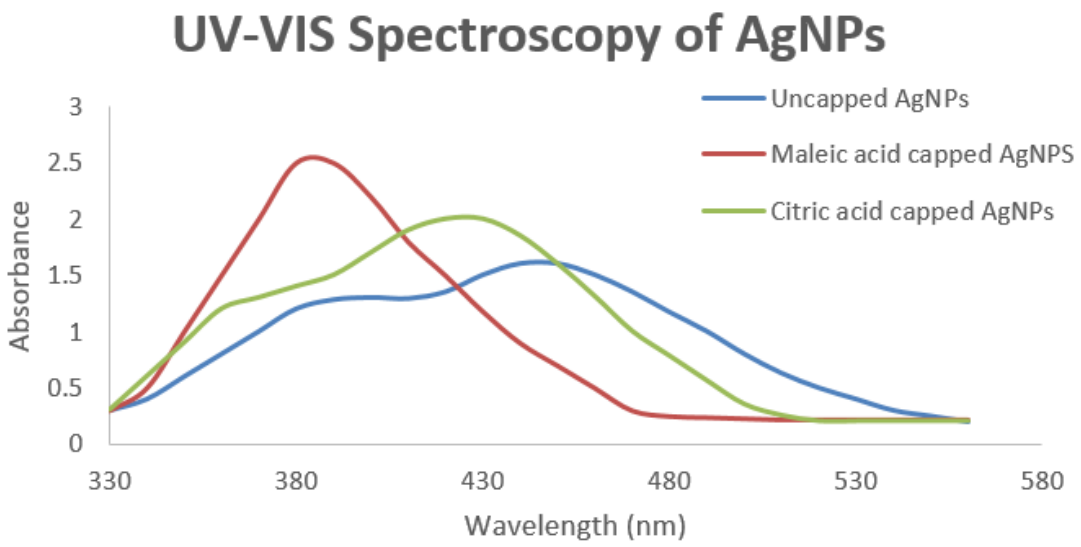


FIGURE 4.1: Spectra of Maleic Acid Capped, Citric Acid Capped and Uncapped Silver Nanoparticles

4.2.2 Silver Nanoparticles Analysis Through SEM

For further size and morphological analysis of synthesized silver nanoparticles, SEM was used. Magnified micrographs were taken up to the resolution of 5 μm in scale bar.

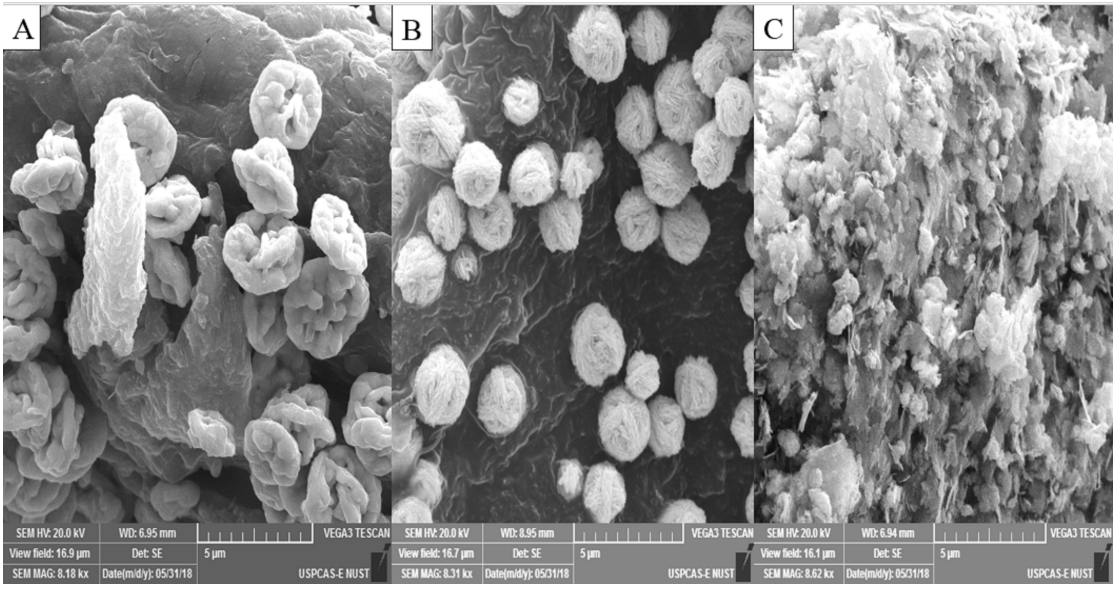


FIGURE 4.2: SEM Analysis; (A) Uncapped Silver Nanoparticles, (B) Maleic Acid Capped Nanoparticles (C) Citric Acid Capped Nanoparticles

Experimental outcomes exposed that the diameters of synthesized silver nanoparticles have adjusted sizes between 39 ± 4 nm for maleic acid capped nanoparticles (Fig 4.2 B), 250 nm for citric acid capped nanoparticles (Fig 4.2 C) and 500± 4 nm for hexagonal uncapped silver nanoparticles (Figure 4.2 A). Size of nanoparticles is controlled by the ratio of the silver ions to stabilizing agents (in current studies, maleic acid and citric acid). Apart from this, reducing agent concentration, time and metal salts played vital role in the silver nanoparticle formation [153], however, in controlling the morphological features of synthesized nanoparticles, modifiers played an important role by avoiding their accumulation [154].

Maleic acid showed a distinctive reaction towards both carboxylic acids and olefins because it is an unsaturated organic dibasic acid. Citric acid also showed a typical reactions of carboxylic acids because it has one hydroxyl and three carboxyl groups. Citric acid and maleic acid can be absorbed on silver colloid surfaces through double bonds and carboxylic acid groups. Tamasa Panigarhi also reported similar results in which the silver nanoparticles are greater than the usual size [155]. The shape of synthesized silver nanoparticles was round plates with multiple facets in case of maleic acid capped nanoparticles, rough flowerlike shape in case of citric acid capped nanoparticles and hexagonal in case of uncapped silver nanoparticles. Images also showed that synthesized silver nanoparticles are considerably accumulated in case of citric acid capped nanoparticles. The reason behind this was may be due to magnetic behavior of silver nanoparticles and their larger surface to volume ratio which incline them to cumulative with the purpose to reduce energy [156]. To remove accumulation, the chemically synthesized silver nanoparticles are be coated with the biocompatible polymer [156].

4.2.3 Silver Nanoparticles Analysis Through EDX

EDX equipped with SEM was used to determine the chemical characteristics and elemental composition and arrangements of synthesized AgNPs. The EDX spectrum indicated the silver as a major constituent of synthesized silver nanoparticles (Figure 4.3) by producing a strong silver signal. It was confirmed from the

EDX profile that the synthesized samples are purely silver without any contaminants (with sharp silver peak). Corresponding peaks except oxygen and silver are might indicate that iron sulfate heptahydrate remains in the nanoparticles and carbon-coated copper grid elements utilized in the scanning electron microscope measurement [157].

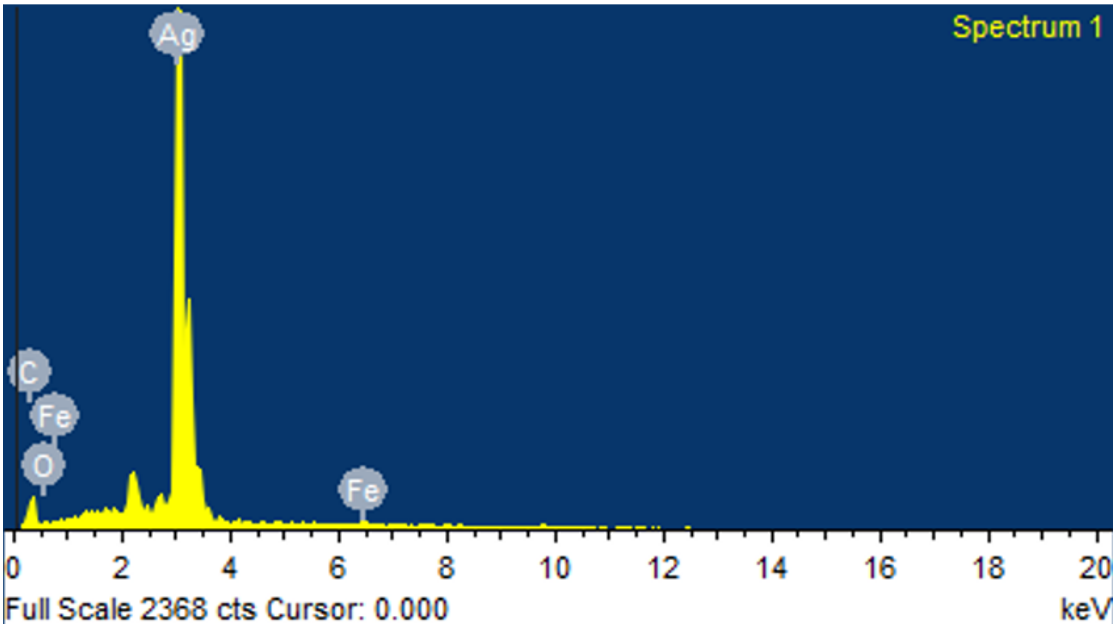


FIGURE 4.3: EDX Spectrum of Synthesized Samples of Silver Nanoparticles

4.2.4 Silver Nanoparticles Analysis Through XRD

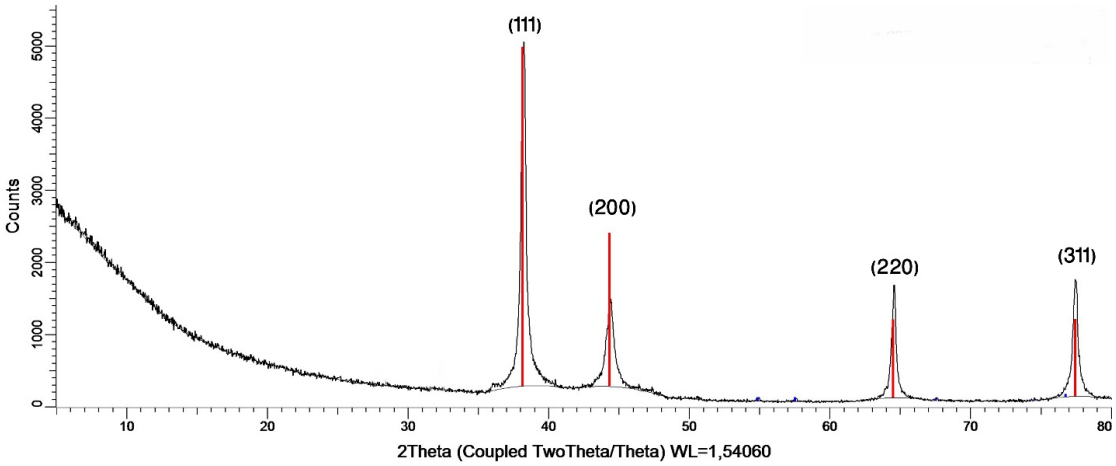


FIGURE 4.4: XRD Peak Diffractogram

X-ray crystallography technique was used to determine the phase identification, phase arrangements and crystalline alignment of synthesized AgNPs. XRD has used to identify and find out the unpacked materials and nanoparticles [150]. The results exhibited that the synthesized AgNPs are of crystal-like nature with identified peaks [38.23 (1 1 1), 44.41 (2 0 0), 64.38 (2 2 0), 77.5 (3 1 1)] (Fig 4.4). The XRD outcome was found in according with the standard ICSD (inorganic crystal structure database) No. 98-018-0878 [158, 159].

4.2.5 Antibacterial Assay

To assess the antibacterial activity of chemically synthesized silver nanoparticles, disc diffusion method was used. Six different strains of bacteria were used for this activity. Results are shown in Table 4.1 and Figure 4.5. It was clearly perceived that by increasing the concentration of silver nanoparticles, growth inhibition was also progressively increased. Vernier caliper was used to measure the inhibition zone.

To calculate the minimum inhibitory concentration (MIC) 100 ppm, 50 ppm, 10 ppm, 5 ppm and 2.5 ppm concentrations of silver nanoparticles were used. MIC for each strain is the concentration at which nanoparticles showed the least activity. For each bacterial strain (counting both the gram positive and gram negative), AgNPs at 100 ppm concentration showed highest zone of inhibition, whereas at the concentration of 2.5 ppm no zone of inhibition was observed.

This indicates that with the decreasing concentrations of AgNPs, the activity was also decreased and ultimately at lowest concentration (2.5 ppm), there was no activity. Findings revealed that silver nanoparticles appeared more effective against gram-negative bacteria (MIC as low as 5 ppm) as compared to gram-positive (MIC 25 ppm), this can be attributed to the less peptidoglycan in case of the gram-negative bacterial cell wall [160].

Moreover maleic acid capped nanoparticles showed better activity than the citric acid capped nanoparticles even at low concentration. The reason is that maleic

acid capped nanoparticles have smaller size, smooth surface and spherical shape than the citric acid capped and uncapped silver nanoparticles that had large sizes and rough surfaces.

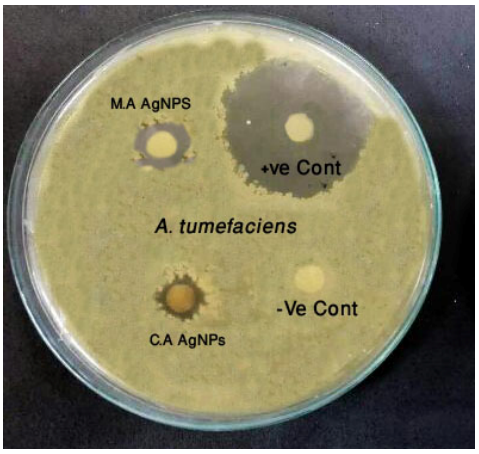
Silver nanoparticles have recently acknowledged a great deal of interest and concerns because of their antimicrobial activity [161]. The antibacterial activity of silver nanoparticles results from their penetration into a bacterial cell, and then their attachment to their cell membrane surface, and producing disruption of energy production by producing cell membrane damage, that is followed by the release of cell substances [162].

Silver nanoparticles attack the gram-negative bacteria by attaching on the surface and penetrating into their cell wall, leading to a cell wall structural modifications that increase the permeability [163]. This antibacterial mechanism is strongly associated with the free radicals formation of AgNPs and with the main cause of cell membrane damage. Ag ions strongly interact with the thiol groups that are present in the phosphorus-containing bases and enzymes, however the silver nanoparticle interaction with the DNA may inhibit the cell division and DNA replication of bacteria that eventually leads to the cell death [164].

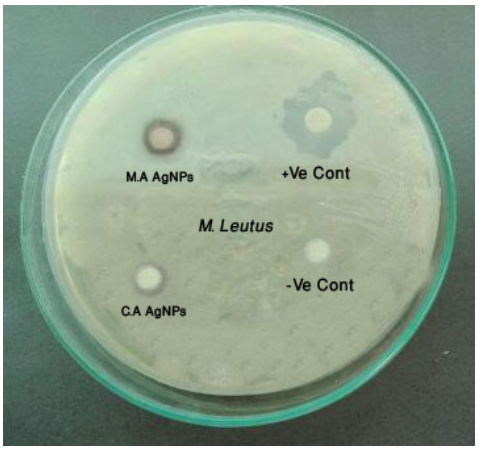
Our results showed that the both types of silver nanoparticles have capacity of antibacterial activity so they are considered for antibacterial drugs after capped with a biocompatible material because they are effective for both gram-negative and gram-positive bacteria.

It is previously reported that this bactericidal activity is because of the physical interaction of AgNPs with the bacterial cell surfaces, particularly in case of gram-negative bacteria, where accumulated nanoparticles were reported there on bacterial surfaces [165].

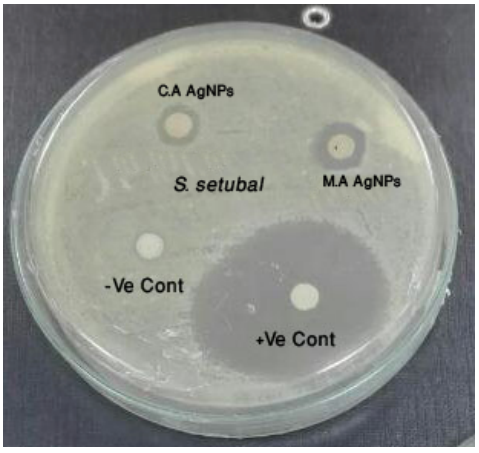
Moreover, some studies also report that AgNPs cause damage to the cellular membrane resulting modified structure, making bacteria more penetrable [166, 167]. This influence is highly predisposed by the nanoparticles morphology and concentration [166, 168].



(A)



(B)



(C)

FIGURE 4.5: Antibacterial Activity of Synthesized AgNPs Performed Using Disc Diffusion Method. (A)Antibacterial Activity Against Gram-negative Strain A. tumefaciens (B) Antibacterial Activity Against Gram-positive Strain M. Leutus and (C) Antibacterial Activity Against Gram-negative Strain S. setubal

TABLE 4.1: Results of Antibacterial Assay of Maleic Acid Capped, Citric Acid Capped and Uncapped AgNPs Against Different Bacterial Strains

AgNPs Conc (ppm)	**Zone of inhibition (cm)±S.E**																	
	Gram Positive Strains									**Gram Negative Strains**								
	M. luteus			***S. aureus***			***B. subtiles***			***A. tumefaciens***			***S. setubal***			***E. aerogenes***		
	M.A.	***C.A.***	***C***	***M.A***	***C.A.***	***C***	***M.A***	***C.A.***	***C***	***M.A***	***C.A.***	***C***	***M.A***	***C.A***	***C***	***M.A***	***C.A***	***C***
100	1.25± 0.9	0.88±0 .5	0.4± 1.2	1.21 ±0.7 5	0.63 ±0.8 9	-	1.31 ±0.2 3	0.74 ±0. 60	-	3.1± 1.33	2.01 ±1. 3	0. 7± 1	1.66 ±0.5	1.2 ±1. 3	0. 54 ±1 .3	3.23± 0.34	2.4±1. 3	0.5±0. 9
50	0.98± 1.1	0.56±1 .21	-	0.68 ±0.6 6	-	-	0.64 ±1.1 5	0.52 ±0. 98	-	2.5± 1.55	0.85 ±0. 89	-	1.16 ±1.1	0.66 ±0. 56	-	2.24± 1.3	1.4±1. 12	-
25	0.74± 1.7	0.42±0 .82	-	0.46 ±1.3	-	-	0.4± 01.2	-	-	0.87 ±0.7 8	0.54 ±0. 44	-	0.76 ±0.9 8	0.45 ±1. 2	-	1.76± 0.78	0.72± 0.98	-
5	-	-	-	-	-	-	-	-	-	0.51 ±1.2	-	-	0.51 ±0.2	-	-	0.79± 1.2	0.54± 0.23	-
2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$AgNO_3$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Distilled H_2O (Negative Control)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptomycin (Positive Control)	2.1			2.42			2.4			3.4			2.72			4.2		

Abbreviations: AgNPs; silver nanoparticles, conc; concentration, SE; standard error, M.A; maleic acid, C.A; citric acid, C: uncapped control, M. luteus; Micrococcus luteus, S. aureus; Staphylococcus aureus, B. subtilis; Bacillus subtiles, A. tumefaciens; Agrobacterium tumefaciens, S. Setubal; Salmonella Setubal, E. aerogenes; Enterobacter aerogenes.

4.2.6 Antifungal Assay

Synthesized particles were also found effective against fungal strains except uncapped silver nanoparticles which did not show inhibition against any fungal strain. Results are showed in Table 4.2 and Figure 4.6. The highest activity was observed against *Aspergillus flavus* 50.56% (growth inhibition) in the case of maleic acid capped silver nanoparticles and 40.11% for those of citric acid capped silver nanoparticles at 500 ppm. Whereas, least activity was observed against *Aspergillus niger* i.e. maleic acid capped AgNPs showing 25.72% while citric acid capped AgNPs showing 18.2% inhibition at similar concentration. Fungal species showed less growth inhibition as compared to bacterial species because of the rigidity of their cell wall which is made up of chitin than peptidoglycan containing cell wall of bacteria [160]. Similar to antibacterial activity, maleic acid capped silver nanoparticles were found more fungicidal mainly because of their smaller size range and round shape with multiple sharp facets providing more surface area to interact with pathogens to act more effectively as compared to citric acid capped nanoparticles which were comparatively larger in size and rough flower shaped [169]. There are several reports which proved the findings that small spherical nanoparticles have better antimicrobial potential than other morphologies as these are provided with a greater surface area to volume ratio thus proven more effective [160, 170]. Large surface to volume ratio and high-atomic-density aspects possibly boosted the pathogen killing proficiency [170]. It is also understood that more silver ions are released by smaller sized particles than larger ones to kill more pathogens [171]. Other reports also support these findings confirming that antimicrobial activity is reliant on size and shape of silver nanoparticles, mainly because, diverse morphologies offer different areas to intermingle with microbes and as a consequence end up with different competence [171, 172].

Actually, interaction of AgNPs with microorganisms (bacteria, fungi, and viruses) releases silver ions (Ag^+) that may damage the microorganism by different means; for example, they target the microbial cell wall (negatively charged) to disable

cellular enzymes and interrupt membrane penetrability; subsequently, cell disintegrates and cell death takes place [173]. Another justification being that, silver ions also interact with enzymes and protein's thiol groups and playing a vital part in its antimicrobial act [174]. As nanoparticles resistance against pathogens has not been reported yet, thus they may have significant benefit over orthodox antimicrobial agents [175].

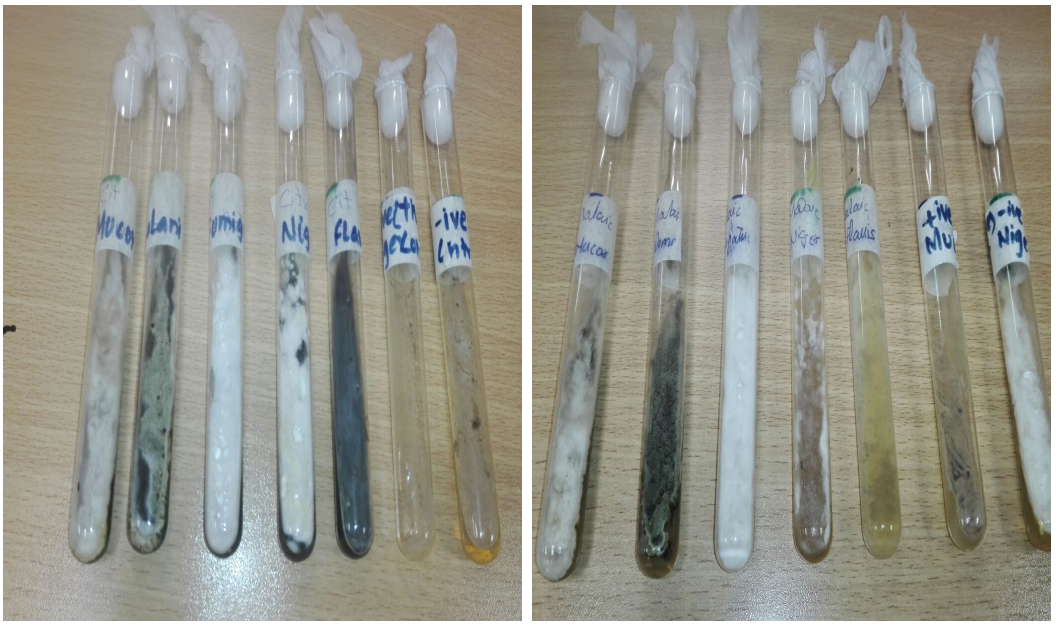


FIGURE 4.6: Antifungal Activity of Maleic Acid Capped and Citric Acid Capped Silver Nanoparticles Against Different Fungal Strains

TABLE 4.2: Percentage Inhibition of Maleic Acid and Citric Acid Capped Silver Nanoparticles Against Fungal Species

S.No	**Samples**	***Percentage Inhibition Against Fungal Species (%)***				
		Mucor.sp.	***F. solani***	***A.fumigatus***	***A. flavus***	***A. niger***
1	Malic Acid Capped AgNPs	33.76	38.44	43.67	50.56	25.72
2	Citric Acid Capped AgNPs	26.23	25.56	35.41	40.11	18.2
3	Uncapped AgNPs	-	-	-	-	-
4	Distilled H_2O (-ve Cont)	-	-	-	-	-
5	Terbinafine (+ve Cont)	100	100	100	100	100

4.2.7 Antioxidant Assay (DPPH)

To examine the antioxidant capacity of synthesized silver nanoparticles, DPPH (2, 2 diphenyl-1-picrylhydrazyl) assay was used. The results showed the moderate free radical scavenging activity exhibited by both types of capped silver nanoparticles (Figure 4.7). DPPH is a stable free radical and become a stable diamagnetic molecule by accepting a hydrogen radical or an electron. The antioxidant impact is showed by the synthesized nanoparticles having hydrogen donating property [176]. In this study, the silver nanoparticles showed different inhibition rate at 80 μM (74% in case of maleic acid capped silver nanoparticles and 56% in case of citric acid capped silver nanoparticles) (Fig 4.7). Whereas, no scavenging activity was observed in case of uncapped silver nanoparticles. The results were significant with p value < 0.05 (Table 4.3) which is concentration dependent. In case of maleic acid capped nanoparticles, the inhibitory concentration (IC50) value 32 μM was observed which is low as compared to the citric acid capped nanoparticles with IC50 value 50 μM. It was suggested that the modifiers or capping agents played vital role in exhibiting antioxidant activity. They might be absorbed at the silver nanoparticle surface and hold the antioxidant property because of their capability to chelate metals, therefore categorized as synergistic [177].

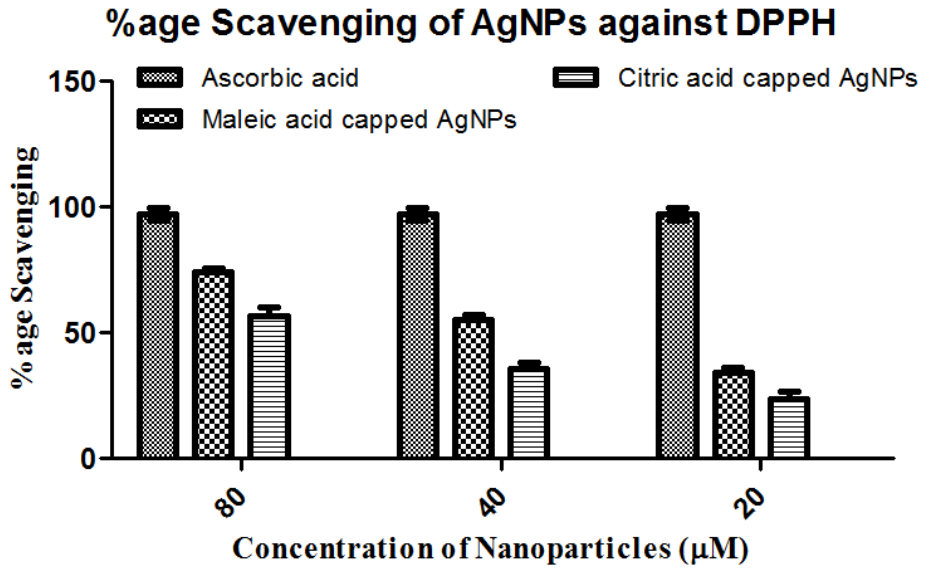


FIGURE 4.7: %age Scavenging of Nanoparticles Against DPPH

Previously, a study in which Kumar et al. described the great antioxidant activity of green synthesized silver nanoparticles (at 0.1 μM concentration> 78% activity was observed) [158]. One more study reported the 74% inhibition rate of silver nanoparticles against the DPPH antioxidant assay [178].

TABLE 4.3: Analysis of Variance for Factors Effecting the Free Radical Scavenging Activity of Nanoparticles

Source of Variation	**Df**	**Sum-of-Squares**	**Mean Square**	**F-Value**	**P Value**	**Significant**
Interaction	6	1339	223.2	21.02	<0.0001	Yes
Types of Nanoparticles	3	27620	9206	866.8	<0.0001	Yes
Concentration	2	3197	1599	150.5	<0.0001	yes
Residual	24	254.9	10.62			

4.2.8 Cytotoxic Assay

To evaluate the toxic behavior of silver nanoparticles, brine shrimp cytotoxic assay was used. The results showed the AgNPs toxic effect with significant mortality rate. In this research, four different concentration 5, 25, 50 and 100 μM of synthesized silver nanoparticles were used to test their toxic effect by using brine shrimp cytotoxic assay. Results are shown in Figure 4.8.

It was observed that the viability of shrimps was considerably decreased (75.3% in the case of uncapped silver nanoparticles, 44.3% in the case of citric acid capped silver nanoparticles and 22% in the case of maleic acid capped nanoparticles at the 100 μM concentration).

Higher concentration had more mortality rate than lower concentrations of silver nanoparticles (Figure 4.8). Moreover, nanoparticles that were synthesized by using maleic acid as modifier had more mortality rate than the nanoparticles that were synthesized by using citric acid as modifier. These results were found quite significant statistically with ED50 30.41 μM and 19.14 μM for citric acid capped silver

nanoparticles and maleic acid capped silver nanoparticles respectively. However, uncapped silver nanoparticles had greater ED50 45.1 μM that proved the uncapped nanoparticles less effective.

Earlier, a study in which silver oxide nanoparticles synthesized from Callistemon lanceolatus showed the concentration and time-dependent cytotoxicity with LC90 value of 221.8 ppm and IC50 value of 85.32 ppm against brine shrimp eggs [179]. The results were found statistically significant with p value <0.05 (Table 4.4).

Silver ions majorly subsidize to stress associated effects and toxicity. Also nano scale size has distinctive lethal effect on the cells, which put forward that both the dissolved ions and nano scale particles can synergically effect the cellular response. [180]. Anticancer activity of nanoparticles can be assessed by the toxicity effects towards brine shrimp's larvae [181].

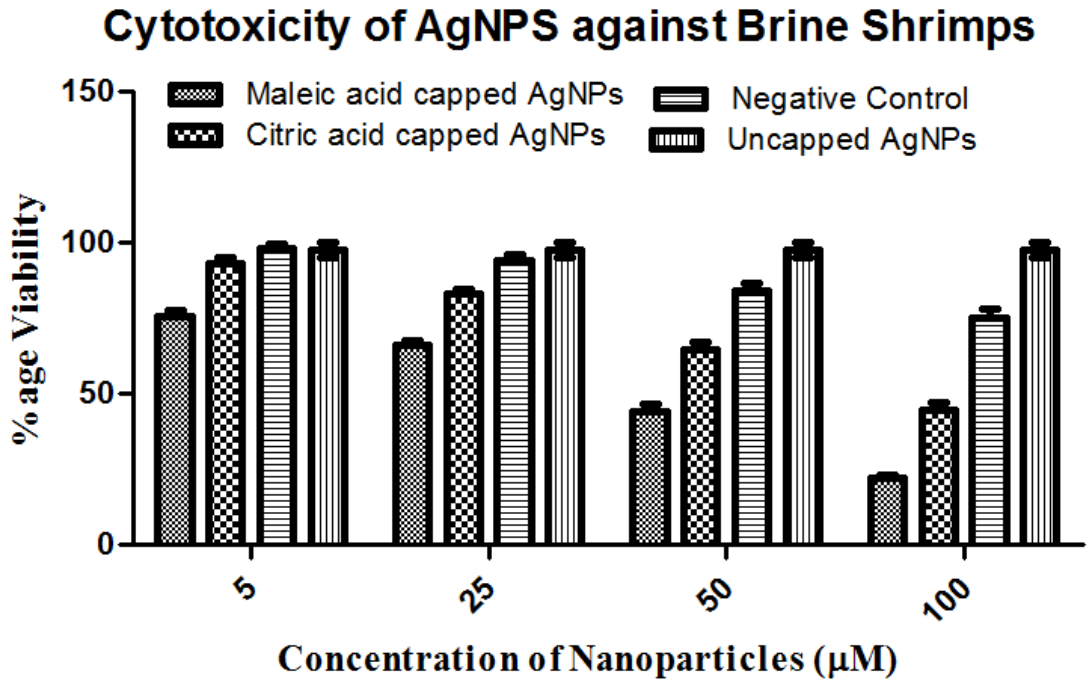


FIGURE 4.8: Total Cytotoxicity of AgNPs Against Brine Shrimps, Maleic Acid Capped, Citric Acid Capped and Uncapped Silver Nanoparticles

Table 4.4: Analysis of Variance for Factors Effecting the Viability of Brine Shrimps

Source of Variation	**Df**	**Sum-of-Squares**	**Mean Square**	**F**	**P Value**	**Significant**
Interaction	9	3091	343.5	117.4	<0.0001	Yes
Types of Nanoparticles	3	14280	4761	1628	<0.0001	Yes
Concentration	3	6649	2216	757.7	<0.0001	yes
Residual	32	93.6	2.925			

Chapter 5

Conclusion and Future Work

The synthesized AgNPs by means of chemical reduction method have shown that the modifiers plays significant role in regulating the morphological features (size and shape) of nanoparticles. Moreover, it was perceived that maleic acid capped silver nanoparticles are more effective than the citric acid capped and uncapped silver nanoparticles because they have smooth spherical surface and smaller size (39±4) that makes them more effective as antimicrobial and cytotoxic agent.

Additionally, synthesized silver particles were found very significant as antimicrobial, cytotoxic and antioxidant agent giving us an opportunity to further discover them in the field of nano medicine as potential antibiotics and anticancer drugs. Moreover, silver nanoparticles can also be used as carriers for different drug molecules in controlled and targeted drug delivery systems.

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