In Vitro Potential and Characterization of Bio Inspired *Viola odorata* Mediated Silver Nanoparticles

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Department of Biotechnology

Faculty of Biological Sciences

Quaid-i-Azam, University

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Supervisor

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A thesis submitted in the partial fulfilment of the requirements for the degree of

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DECLARATION

I, **Muhammad Waqas S/O Nasr Ud Din**, Registration no 02272011005, MPhil Biotechnology scholar, Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, hereby declare that the quoted data in the thesis entitled "**In Vitro Potential and Characterization of Bio Inspired *Viola odorata* Mediated Silver Nanoparticles**" is based on genuine work carried under the supervision of **Dr. Bilal Haider Abbasi** and has not been submitted or published somewhere else.

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DEDICATION

I dedicate my work to my parents who have always loved me unconditionally and whose good example has taught me to work hard for the things that I aspire to achieve. I also dedicate this thesis to my respectable and honorable teachers who have supported me in developing my personality as a competent professional.

Muhammad Waqas

Quaid-i-Azam University

Department of Biotechnology

CERTIFICATE

It is certified that the research work presented in this thesis titled "In Vitro Potential and Characterization of Bio Inspired *Viola odorata* Mediated Silver Nanoparticles" was conducted by **Mr. Muhammad Waqas** under the supervision of **Dr. Bilal Haider Abbasi**. This thesis is submitted to the Department of Biotechnology in partial fulfillment of the requirements for the degree of Master of Philosophy (MPhil) in **Biotechnology.**

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Dated: 30/03/2022

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Muhammad Waqas

List of Abbreviations

Abbreviation	Stands for
AgNP	Silver nanoparticles
CVD	Chemical vapor deposition
CNPs	Carbon-based nanoparticles
Cu NPs	Copper nanoparticles
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EDX	Energy dispersive X Rays
FTIR	Fourier-transform infrared radiation
eV	Electron volts
g	Gram
Mm	millimeter
mg/L	milligram per liter
NPs	Nanoparticles
NARC	National Agriculture research centre
PBS	Phosphate buffer saline
ROS	Reactive oxygen specie
SDR	Spinning disc reactor
TRP	Total reducing power
TEM	Transmission electron microscopy
TiO_2	Titanium dioxide
TAC	Total antioxidant capacity
UV-vis	UV-visible spectroscopy
µg/ml	Micro gram per milliliter
µl	microliter
µm	micrometer
XRD	X-ray diffraction

Abstract

Nanotechnology is one of the most promising technologies of the 21st century and has gained a dynamic interest in the field of life sciences. Although conventional chemical approaches have been employed to synthesize nanoparticles, however, they have some adverse effects. To minimize the shortcomings of chemical and physical synthesis of nanoparticles, we cynosure on green synthesis of silver nanoparticles (AgNPs). AgNPs were synthesized from aqueous leaf extract of *Viola odorata* commonly known as Gul-e-banafsha. Several techniques including Ultra Violet Visible Spectroscopy (UV-VIS), Fourier Transformed Infrared Spectroscopy (FTIR), X-Ray Crystallography (XRD), Dynamic Light Scattering (DLS) and Scanning Electron Microscopy (SEM) were used for the characterization of biosynthesized NPs. UV-VIS spectroscopy showed highest peak at 430nm, which confirmed formation of AgNPs. XRD pattern confirmed crystalline structure of NPs with average size of 11nm. FTIR showed capping of NPs with phytochemicals present in leaf extract of *Viola odorata*, which acts as reducing and stabilizing agent in NPs formation. Particle size distribution and zeta potential were analyzed by DLS. SEM revealed irregular shape in morphology. Furthermore, different biological activities have been done and the highest antioxidant activity recorded as 73.5%, 70.04%, and 78.3% at 200µg/ml for 2,2-diphenyl 1-picrylhydrazyl (DPPH), total reducing potential (TRP), and total antioxidant capacity (TAC), respectively**.** Highest hemolysis activity (6.9%) and brine shrimp cytotoxicity **(**60%) of AgNPs were observed at 200µg/ml. Cytotoxicity of AgNPs against liver cancer cell line (HepG2) was analyzed through Suforhodamine B assay and the results showed cell viability reduced to 35%, whereas for positive control (DOX), cell viability was recorded 40%. Green synthesized AgNPs also showed broad spectrum antibacterial and antifungal activity against various pathogenic strains. Zone of inhibition for gram negative strains *(Escherichia coli, Pseudomonas aeruginosa,* and *Klebseilla pneumonia*) was higher in comparison with gram positive strains (*Staphylococcus epidermidis* and *Bacillus subtilis*). This may be due to change in composition of cell wall. Our result suggested that AgNPs synthesized in this study are more compatible and have high anticancer activity and can be used for cancer therapy, drug delivery agent and other clinical products however further study is required to investigate and evaluate the effects of AgNPs in animal models.

Chapter 1

Introduction

Nanotechnology is one of the most promising technologies of the 21st century and has gained a dynamic interest in the field of life science. The word "Nano" in Greek means extremely small in size, ranging from 1 to 100nm, nanoparticles are also known as Nanocrystals. It is due to the tiny size and their greater surface to volume ratio that nanoscale materials have unique and superior physical and chemical properties compered to bulky structures (Aritonang *et al.,* 2019). The word nano was first introduced by Professor Norio Taniguchi in 1974 while the actual idea of nanotechnology was originated in the meeting of American Physical Society, December 1959 by Richard Feynman where he asked "What would happen if we could arrange the atom one by one the way we want them?" (Feynman, 1959). The word nano is the most popular terminology in modern science and has been introduced in the form of nanoscience, nanotechnology, nanometer, nanostructure, nanoscale, nanowire, nanotube and nanorobot. However, some other words have been published in Nature that still need worldwide recognition. These include, nanocrystals, nanoporous materials, nanomagnets, nanovalves, nanofibers, nanoelectronics, nanoantennae, nanocavity, nanoencapsulation, nanolithography, nanopatterning, nanoarraynae, nanoscaffolds etc. (Cristina *et al*., 2007).

Nanomaterials and nanoparticles are materials and structures with a diameter of less than one meter. Because of their discrete features i.e. large surface area and quantum size effects, nanoparticles can be regarded as a unique state of matter where solid, liquid, gaseous, and plasma states coexist. Traditional crystalline solids include graphite and diamond, while crystalline nanoparticles include carbon nanotubes. Some researchers limit the size of nanoparticles to 50 or 200 nanometers. The bulking behavior of some nanoparticles, when their size reaches these values, causes the specified limit of nanoparticle size (Borm *et al*., 2006). Discrete nanomaterials, nanoscale device materials, and bulk nanomaterials are the three categories of nanoparticles. Discrete nanomaterials (DN) materials are free-standing nanomaterials having a size between 1-10nm in at least one dimension, for example; carbon nanotubes and nanoparticles. Nanoscale devices (ND)

materials are made up of elements that are housed in a device and are nanoscale in size. These are commonly found as thin films, such as thin films on metal oxide. Bulk of nanomaterials are discrete nanomaterials or nanoscale materials that agglomerate and appear as a bulk form while maintaining their nanoscale dimension (K. T. Ramesh, 2009).

Chemical, physical, and biological methods are usually employed for nanoparticles synthesis. Chemical and physical methods might have shortcomings like contamination due to solvents, high energy consumption, low yield, time consuming, imbalance in particle size distribution, requirement of high pressure and temperature and cost associated with equipments (Elsupikhe *et al*., 2015; Siavash *et al*., 2013). Therefore efforts have been made to develop environmental friendly methods in which biological extract are mixed with salts solution followed by separation and purification of nanoparticles (Barabadi *et al*., 2019). The major advantage of biological methods is that reaction can occurred at room temperature without any harsh requirements and availability of secondary metabolites such as phenolic compounds, flavonoids, ascorbic acids, proteins and terpenoids in synthesis of nanoparticles (Gurunathan *et al*., 2009). Moreover, medicinal properties of plants (like anti-inflammatory and antioxidant properties) that are used in synthesis can be advantageous in the therapeutic applications of the resultant nanoparticulate biocomposites. The biological route in this perspective received much attention. Earlier microorganisms like bacteria (Lengke *et al*., 2007), fungi (Rautaray *et al*., 2003), algae (Govindaraju *et al*., 2008), and yeast (Kowshik *et al*., 2002) were extensively used for the synthesis of metal oxide nanoparticles but currently plant extract-based NPs synthesis has gained much importance. As plant extracts easily reduce the metal in a short time and give NPs a well-defined shape and size (Kumar *et al*., 2020)

Metallic nanoparticles have a wide range of applications like in cosmetics (Vinod & Jelinek, 2019), drug delivery (Busatto *et al*., 2019), gene delivery (Ansari *et al*., 2019), food and feed (Peters *et al*., 2016), biomedical research (Mondal & Jana, 2014) and environment (Mueller & Nowack, 2008). A large number of metallic nanoparticles, namely, silver, platinum, copper, cobalt, gold, cesium oxide, magnesium, and zinc oxide, occur naturally; however silver nanoparticle gained attention due to its unique physical, chemical and biological properties. Silver nanoparticles exhibit strong activity against

various drug resistance bacterial (Franci *et al*., 2015) and fungal strains (Medda *et al*., 2015). Due to unique antibacterial, optical and electronic properties AgNPs widely used in photonics (X. Hu & Chan, 2004), bio-sensing (Habouti *et al*., 2010) and electronics (Alshehri *et al*., 2012). The strong antimicrobial potential of AgNPs gives a major direction for the production of AgNPs products like in textile, antiseptic sprays, food storage containers and wound bandages.

Broad spectrum usage of antibiotics since 1945 has led to dramatic increase in multi-drug resistance (MDR) against microbial pathogens which is a serious threat to public health (Mortezaee *et al*., 2019). This has convinced researcher to find new antimicrobial agent to combat MDR. In order to tackle this problem nanomaterial base strategy is best approach because it cannot exert evolutionary pressure on bacteria (Qureshi *et al*., 2015). There is no genotypic resistance found in microbes for silver so AgNPs have a great potential to combat with MDR and therefore showed effective antimicrobial activity against various bacterial strains including *Pseudomonas aeruginosa, Acinetobacter baumannii*, *Staphylococcus* species and *Escherichia coli* also against fungal species (Medda *et al*., 2015; Panáček *et al*., 2018). Silver nanoparticles have good disinfectant and bacteriostatic properties and are used in water treatment to clean out microorganisms which cause water borne diseases (Clasen *et al*., 2004). Other application of AgNPs have been also reported such as thrombolytic effect (Mirmohammadi *et al*., 2015), anthelmintic (Sathiyaraj *et al*., 2020), anti-leishmania (Silva Viana *et al*., 2020), anticancer and antioxidant activity (Sathiyaraj *et al*., 2020) anticoagulant activity (Lateef *et al*., 2018) and tumor cell growth inhibition (Yanhua Li *et al*., 2020).

Different studies have reported the green synthesis of silver nanoparticles from the leaves, roots, flowers and seed extract of different plants such as *Azadirachta indica* (neem) (Verma & Mehata, 2016), marigold flower (Padalia *et al*., 2015), *Ficus benghalensis* (Banyan) (Saware & Venkataraman, 2014), *Acalypha indica* (Krishnaraj *et al*., 2010), *Sesuvium portulacastrum* (sea purselane) (Nabikhan *et al*., 2010), *Olea europaea* (olive) (M. M. Khalil *et al*., 2014), *Spirogyra varians* (Salari *et al*., 2016), *Ocimum tenuiflorum* (black Tulsi) (Banerjee *et al*., 2014), *Melia dubia* (Kathiravan *et al.,* 2014), *Erythrina indica* (Sre *et al*., 2015), *Solanum tricobatum* (Logeswari *et al*., 2013), *Ziziphora*

tenuior (Sadeghi & Gholamhoseinpoor, 2015), *Garcinia mangostana* (Mangosteen) (Veerasamy *et al*., 2011).

Viola odorata commonly identified as sweet violet in English and Gul-e-Banfasha in Indo-Pak belongs to family Violaceae (Siddiqi *et al*., 2012). It is mostly found in Europe and native America. In Pakistan, it is found in hilly areas of Kashmir, Kaghan, Chitral and Nathia Gali (Baquar, 1989). In Unani system of medicine *Viola odorata* is an important medicinal plant due to the presence of flavonoids, triterpines, tannins, anthocyanins, volatile oil, methyl salicylate, phenolic compounds, saponins, glycosides and sterols (Zaigham, 2019). This plant has been reported to have Antipyretic, Anti-inflammatory, Antioxidant, Anti-tubercular, Hepato-protective, Antimicrobial, Diuretic, Hypnotic, Anti-HIV, Anti-hypertensive and dyslipidemic activities (P. Mittal *et al*., 2015). The current study is focused on the biological synthesis of AgNPs from leaf extract of viola odorata. Characterization was done through UV-VIS, FTIR, XRD and SEM to confirm the nanoparticle size, morphology and functional group attached from the phytochemicals present in the extract of *Viola odorata*. Furthermore antioxidant assay like DPPH, TRP, TAC were done to check the potency of nanoparticles. Cell viability of synthesized NPs were also checked against cancer cell, brine shrimp and human RBCs.

1.1.Aims and Objectives

Main objectives of the current study include:

- To synthesized AgNPs from leaf extract of *Viola odorata*
- Characterization of green synthesized AgNPs through different techniques such as UV-VIS spectroscopy, FTIR, XRD, DLS and SEM.
- Evaluation the antioxidant potential of AgNPs (DPPH, TAC and TRP).
- Evaluation of AgNPs toxicity against brine shrimp and human RBCs.
- Evaluation of antibacterial and antifungal potential of bioinspired AgNPs.
- To study the potential of bio-inspired AgNPs against liver cancer cell lines (HepG2 cell lines).

Chapter 2

Literature review

2.1. Nanotechnology

The term nanotechnology was introduced by famous Nobel laureate Richard Feynman during his lecture in 1959 he said '*There's Plenty of Room at the Bottom'* (Feynman, 1992*)*. He demonstrated the idea of manipulating idea of matter at nanoscale at extremely small scale while definition of nanotechnology varies from field to field and as widely used for everything have very small size in range of nanometers (less than 100nm) to make products or material with elementary properties and new functions which can be used for various environmental and industrial applications (Roco, 2007).

2.2. Two main approaches

Two basic approaches are mainly used in nanotechnology:

i) Top down approach

ii) Bottom up approach.

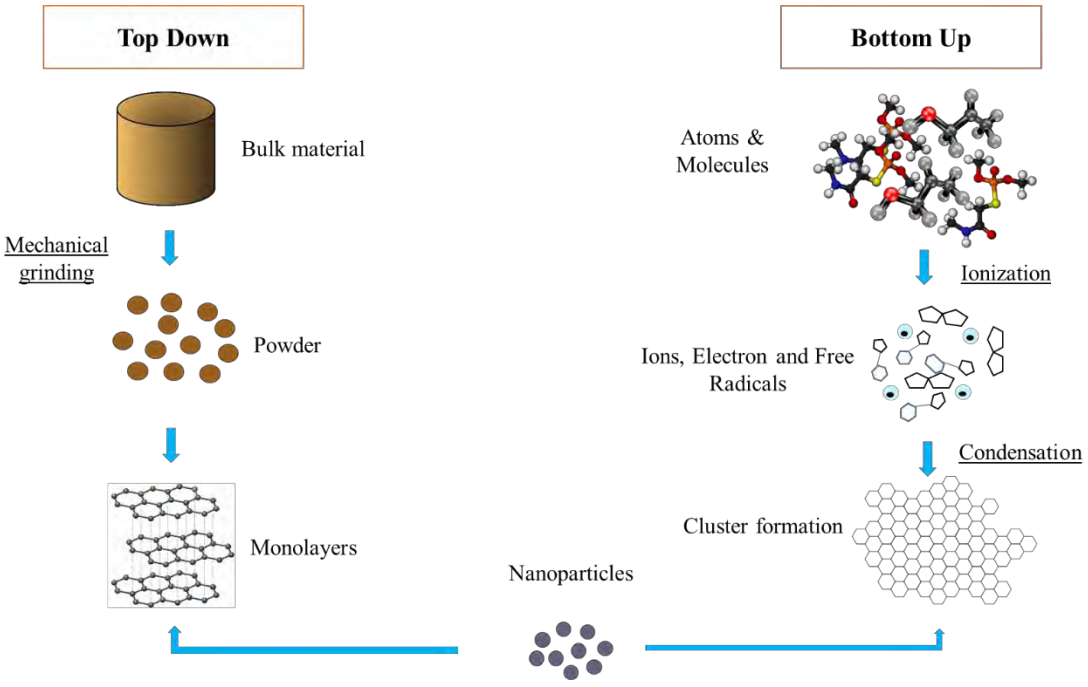


Figure 1: Mechanism of Top down approach and Bottom up approach

2.2.1. Top down approach

The approach in which bulk material is minimized to nanometer scale without disrupting their original properties. This approach consists of two steps; first is the conversion of bulk material into nanoscale and then conversion of nanoscale material into specific nanoparticles. Major advantage of top down approach would be its cost effectiveness and the major disadvantage is the imperfect structure of the final product. (Sanchez & Sobolev, 2010). Three methods are used in this approach namely Mechanical mining, Laser ablation and Nanolithography.

2.2.1.1 Mechanical mining

Mechanical milling is the most used process in top down approach. It uses high energy to reduce particle size. During this process a high energy ball rolling on the surface of nanomaterial in parallel wise pattern and smashed them into small size. For large scale production of nanoparticles mechanical milling is preferred as it is cost effective (Yadav *et al*., 2012).

2.2.1.2. Laser ablation

Laser ablation is used for fabrication of nanoparticles by dipping metals in a solution and then irradiating them through laser causing them to fabricate. This is the most used method due to its cost effectiveness as it doesn't require any costly chamber and high pressure vacuum pump. The size of the synthesized material can be controlled by different parameters like change duration of laser irradiation, pulse of laser, pH of solution and addition of surfactant. It is also contamination free protocol. (Poondi *et al*., 2000).

2.2.1.3. Nanolithography

Nanolithography is used for fabrication of nanomaterial in three dimensions using properties of light or electron to draw patterns in the substrate. Two basic techniques that are used for fabrication of surface of nanomaterials are Soft lithography and Dip pin lithography (Arango-Santander *et al*., 2018). Various lithographic techniques include electrostatic atomic force microscope nanolithography (Lyuksyutov *et al*., 2003), optical and scanning lithography and nanoimprinting (Yadav *et al*., 2012). Major advantages of lithography include direct writing, high resolution and desired shape and size of the end

product. White the drawbacks include inapplicability on nonpolar surfaces, high cost of labor and lack of chemical control (Tran & Nguyen, 2017).

2.2.2. Bottom up Approach

In this approach, small molecules (atoms) are clustered into large molecule in the macro-scale range (E. L. Hu & Shaw, 1999). The geometry of molecule is restrained by varying conditions of pH, temperature, concentration of solute and time of incubation. The molecular interactions like Van der Waals forces and relatively stronger Hydrogen bonding and Hydrophobic-Hydrophilic interactions are responsible for assembling small molecule into well-organized structures. Methods used in these techniques are further discussed below.

2.2.2.1. Sol gel technique

A very unique chemical based technique through advanced nanomaterial can be produces for various purposes. Sol gel process involves hydrolysis of a precursor into colloidal suspension that forms a freeze gel network and on heating convert into crystalline phase (Rajput, 2015). Commonly used precursors are tetraethoxysilanes (TEOS) and tetramethoxysilane (TMOS) which form silica gels. This process has some benefits including easy to follow protocol, low cost and high purity of end product. (X. Guo *et al*., 2016). This process attracts more attention because of its wide range of applications and advantages and will soon become a widely adopted technique for the production of most advanced nanomaterials.

2.2.2.2. Spinning

A spinning disc reactor SDR is used for synthesizing nanoparticles. It consists of a rotating disc inside a chamber/reactor. Physical factors such as temperature can be adjusted within the chamber. For avoiding the chemical reactions, oxygen is removed by filling the reactor with inert gases or nitrogen (Tai *et al*., 2007). The liquid (precursor and water) is pumped into the chamber at different speed levels. The atoms or molecules fuse together as a result of the spinning followed by their precipitation, collection, and drying (Mohammadi *et al*., 2014). The properties of nanoparticles synthesized via SDR are determined through many operating factors such as liquid flow rate, disc rotation speed, liquid/precursor ratio, feed location, disc surface etc.

2.2.2.3. Chemical vapors deposition (CVD)

CVD is a process in which a chemical reaction is carried out for depositing a solid onto a heated surface from a pre-existing gaseous phase. The activation energy required for CVD is provided by different methods. Major advantage of CVD is that we can obtain nanoparticles with uniform size, shape, strength and purity while one of the major disadvantage is the production of by products from toxic gases (Adachi *et al*., 2003).

2.3. Nanoparticles

Man has created and developed the material world and its components in larger than the largest and smaller than the smallest dimensions of mass, length, and time in his search for knowledge. Though an atom of an element was discovered to be the smallest thing with unique characteristics, realizing the single atom in physical form and helping humanity has only been a dream (Rajput, 2015). For decades, nanoparticles have been the limelight of research in various fields because of its small size, unique physical and biological properties. The particles that vary in size from 1-100nm are considered nanoparticles the bulk of which have been reported to have uses in food industry, medicine and cosmetics for different purposes (Semenzin *et al*., 2015).

2.4. Classification of nanoparticles

Nanoparticles vary in shapes, sizes, and structures. They may be spherical, hollow core, cylindrical, tubular, flat, spiral, conical or irregular in shape having a size range of 1-100nm. Some nanoparticles are crystalline or amorphous, with single or multi crystal solids that might be loose or clumped together (Machado *et al*., 2015). The nanoparticles being fixed at a single point and having no dimensions with respect to length, breadth and height are referred to as **zero dimensional,** for example, nano dots. **One dimensional** nanoparticles comprise only one dimension (one parameter only), for example, graphene. The nanoparticles having parameters of length and breadth are **two dimensional nanoparticles** like carbon nanotubes. The nanoparticles with the parameters of length, breadth and height are **three dimensional nanoparticles,** for example, gold nanoparticles (Cho *et al*., 2013). Further four main categories of nanoparticles are organic, inorganic,

semiconductor and metallic nanoparticles the most broadly used nanoparticles are metallic nanoparticles.

2.4.1. Organic nanoparticles

These are biodegradable nanoparticles which are also known as nanocapsule. Apart from their size, shape and composition these nanoparticles are ideal for drug delivery because of its drug carrying capacity and stability (Tiwari *et al*., 2008). Organic nanoparticles are mostly utilized in the biomedical field due to its nontoxic nature and high sensitivity to heat and electromagnetic radiations. Dendrimers, Liposomes, Ferritin and Micelles are few examples of organic nanoparticles.

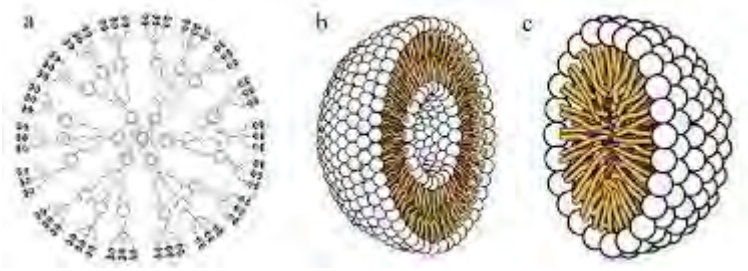


Figure 2: Organic Nanoparticles (a. Dendrimers, b. Liposomes, c. Micelles) (Ealia & Saravanakumar, 2017)

2.4.2. Inorganic nanoparticles

Inorganic nanoparticles have a variety of qualities that make them ideal for cellular delivery, including vast availability, extensive functionality, strong biocompatibility, the potential for targeted distribution (e.g., selectively eliminating cancer cells while sparing normal tissues) and controlled drug release (Xu *et al*., 2006). These nanoparticles are made of carbon and categorized into metal and metal oxide base nanoparticles.

2.4.2.1. Metal based nanoparticles

Nanoparticles synthesized from metal either through bottom up or top down approach are metal based usually all metals can be synthesized into their nanoparticles. Common metal nanoparticles are Silicon (Si), Aluminium (Al), Cadmium (Cd), Cobalt (Co), Copper (Cu), Iron (Fe), Gold (Au), Zinc (Zn), Lead (Pb) and Silver (Ag). Metal based nanoparticles have been widely used for their novel antiviral, antibacterial and antifungal properties as an alternative treatment for biocides and antibiotics. (Morones *et al*., 2005).

To impart antimicrobial qualities, (Ag NPs) are currently being added to many common household products, including humidifiers, bedding, water purification systems, washers, tooth paste, textiles, shampoo, filters, deodorants, paints, toys and culinary utensils (Samuel & Guggenbichler, 2004). Copper nanoparticles (Cu NPs) are being used as fillers in a number of industrial applications to improve conductivity, wear resistance and ductility, reduce friction, and function as catalysts on activated carbons to lower nitrate levels in water (G. Liu *et al*., 2004).

2.4.2.2. Metal oxide based nanoparticles

The synthesis of metal oxide based nanoparticles is carried out by altering the properties of their respective metal based nanoparticles. For instance, oxygen at room temperature causes the rapid oxidization of iron nanoparticles to iron oxide (Fe_2O_3) nanoparticles, which possesses high reactivity than iron (Fe) nanoparticles. Nanoparticles of metal oxides like that of Silicon dioxide (SiO_2), Aluminum oxide (Al_2O_3), Cerium oxide (CeO_2), Magnetite (Fe_3O_4), Iron oxide (Fe_2O_3), Zinc oxide (ZnO) and Titanium oxide (TiO_2) are synthesized commonly. Such nanoparticles possess unique properties in contrast to their metal counterparts. Silicon dioxide is used in molecular sieves, gene therapy and drug delivery (Roy *et al*., 2005). TiO_2 is utilized in cosmetics, filters with strong germicidal characteristics and odour removal, and as an antibacterial agent in combination with Ag (Ellsworth *et al*., 2000). TiO_2 also used for skin protection in combination with ZnO.

2.4.3. Carbon Based nanoparticles

The nanoparticles entirely made up of carbon and have different geometrical shape and size in range of nanoscale. Classified into fullerenes, spherical in shape, graphene being hexagonal in shape, Carbon Nano Tubes (CNT) and nanofoil fold into hollow cylindrical shape, carbon nanofibers configure into cup or cone shape and carbon black takes the configuration of a sphere but due to higher interaction between the particles they are mostly found clustered together in size range of >500nm (Bhaviripudi *et al*., 2007). CNPs are extensively applied in a variety of fields. It is most adaptive in numerous technical and scientific applications such as photo-catalysis, biological imaging, biomedical, chemical, and optical sensing because of its qualities such as strong biocompatibility, low cost, low toxicity, and larger surface area (Asadian *et al*., 2019).

Table 1: Properties of different nanoparticles

Nanoparticles	Properties	References
Metallic Nanoparticles		
Silver	Acts as disinfectant, scatters light, antibacterial, wound healing,	(Hulteen *et al*., 1999)
Cadmium	Insoluble and semiconductor of electricity	(Osuntokun & Ajibade, 2016)
Zinc	Anticorrosive , antibacterial, UV filtering, antifungal	(Bogutska *et al*., 2013)
Aluminum	Sensitive to heat, light and moisture, highly reactive	(Geetha *et al*., 2016)
Lead	Stable, highly reactive and toxic	(Tyszczuk-Rotko *et al*., 2016)
Copper	Flammable, good conductor of electricity, soft	(Ryu, Joo, & Kim, 2016)
Metallic oxide Nanoparticles		
Silicon dioxide	Nontoxic, less stable, large surface area	(Kaynar *et al*., 2016)
Aluminum oxide	Highly reactive, sensitive to heat and sunlight	(Z. Guo *et al*., 2006)
Titanium oxide	Antibacterial, magnetic, biocompatible	(Laad & Jatti, 2018)
Iron Oxide	Reactive and less stable	(Ruales-Lonfat *et al*., 2015)
Zinc oxide	Antifungal, antibacterial and UV filtering	(Bajpai *et al*., 2016)

Cerium oxide	Low reduction potential and antioxidant	(Kim & Chung, 2016)

Carbon base Nanoparticles

Carbon nanotube	Good conductor of electricity, good conductor of heat, flexible, high tensile strength	(De Volder *et al*., 2013)
Fullerenes	Inert and safe, superconductor of heat, transmit light	(Tenne, 2002)
Carbon nanofiber	Stable, good conductor of electricity	(Qian *et al*., 2007)
Graphene	Absorb light, good conductor of heat and electricity, high stable	(HUANG *et al*., 2010)
Carbon black	UV resistance, good conductor, large surface area	(Fawole *et al*., 2016)

2.5. Methods for Synthesizing Nanoparticles

Nanoparticles synthesis can be categorized into two main approaches, i.e., top down approach and bottom up approach. Top down approach is also known as destructive approach in which bulk material are used to produce nano scale particles. Mechanical milling, thermal decomposition, laser ablation and nanolithography are common methods in top down approach. Bottom up approach, also termed as constructive method in which small atom are clustered to form nanoparticles. For example: Chemical Vapour Deposition (CVD), spinning, pyrolysis, sol gel and biosynthesis are common methods of bottom-up approach (Daraio & Jin, 2012). Physical, chemical and green synthesis or bio-assisted method are general protocols for synthesis of nanoparticles.

2.5.1. Physical methods

Physical methods involved the use of mechanical pressure like high energy ball milling, high thermal and electric energy to generate nanoparticles. Laser ablation, condensation and evaporation are physical methods and fall under top down approach.

Because of absent of solvent the nanoparticles produce is uniform in size and contamination free (Tsuji *et al*., 2002). One of the major disadvantage of physical method is the used of high energy that increase the temperature of source material and take more time to achieve thermal stability. Through physical methods we can produced a bulk amount of nanoparticles. Physical methods are mostly suitable for production of nanoparticle used for long term experiment especially for toxicity studies because of its stability (Jung *et al*., 2006).

2.5.2. Chemical methods

In chemical method, chemicals are employed as reducing agent for synthesizing nanoparticles. Both organic and inorganic reducing agent are used for reduction for example: sodium borohydride ($NaBH_4$), elemental hydrogen, Sodium citrate, ascorbate, N, N-dimethylformamide (DMF), Tollens reagent and poly (ethylene glycol). Reducing agent reduces ions of particle salts and agglomerates them into culture leads for the formation of nanoparticles (Siavach Iravani *et al*., 2014). In order to stabilize nanoparticles some stabilizing agent are use they can protect nanoparticles from agglomeration, sedimentation and surface properties of particles (Oliveira *et al*., 2005). For example: poly (vinylpyrrolidone), poly (ethylene glycol), polymethylmethacrylate, poly (methacrylic acid), and poly (vinyl alcohol) are the effective stabilizing agents. Sol gel, micro emulsion, hydrothermal, polyol and chemical vapor deposition are the common techniques in chemical synthesis of nanoparticles.

2.5.3. Biological methods

A number of studies have reported that the chemical methods of synthesis are toxic, not environmental friendly and expensive. So there is the need of a method which is easy, cost effective and environmental friendly. Bio-based methods are cheap, environmental friendly, less toxic, more efficient and do not require the use of hazardous chemicals. Organism have the potential for the synthesis of nanoparticles ranging from a simple prokaryotic cell to a complex eukaryotic cell for example nanoparticle can be synthesized from plant, bacteria, fungi, yeast, viruses, actinomycetes and algae etc. (Mohanpuria *et al*., 2008). Bio-assisted nanoparticles are well characterized and highly stable. Their size and morphology can be controlled by changing various conditions, including temperature, pH,

light, exposure time, incubation period, buffer concentration, mixing speed, reducing agents and substrate etc. (Siavach Iravani *et al*., 2014).

2.6. Characterization of Nanoparticles

Various methods has been used for characterization of nanoparticle that determine application and potential of nanoparticles. It is challenging for every research group to get access to a large range of characterization tools because of multidisciplinary nature of nanotechnology. In fact, a broader characterization of NPs is frequently required, necessitating a complete strategy involving the use of complementary approaches. Following techniques are used for Characterization of nanoparticles: X-ray diffraction (XRD), UV-visible spectroscopy (UV-VIS), Fourier transform infrared spectroscopy (FTIR), Dynamic light scattering (DLS), Scanning electron microscopy (SEM), Zeta potential, Transmission electron microscopy (TEM), Thermogravimetric analysis (TGA), Atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and Nuclear magnetic resonance (NMR) (Mourdikoudis *et al*., 2018).

X-ray diffraction (XRD) is among the commonly used techniques for the characterization of nanoparticles. The crystalline structure, crystalline particle size, phase nature and phase parameters are determined by XRD. The Scherrer equation is used to calculate the size from the most intense peak of an XRD measurement for a specific sample (Véron *et al*., 2013). FTIR is used to confirm functional group attached on the surface of nanoparticles either through some stabilizing agents or through absorption. UV-VIS spectroscopy is widely used for confirmation of nanoparticle synthesis, average particle size and agglomeration in particles and most economical method (Rajasekharreddy *et al*., 2010). SEM is used for morphology and size using high energy photon that transmit through the surface of nanoparticles. AFM is high resolution method used for elemental composition, dispersion of nanoparticle in cell and morphology (Hassellöv *et al*., 2008). DLS is used for size, charge and potential distribution of nanoparticles. NMR used for size, shape, detection of ligand attached on the surface of NPs, growth kinetics, surface area and atomic composition. NMR is not preferable for ferromagnetic particle because it involves the use of electromagnetic field (Lu, 2011). TGA is used to calculate mass and composition of nanoparticles by heating and different component are produced as vapours and detected

by TGA device. Pros of TGA include no need of sample preparation and sample used in dry powder while cons includes large amount of nanoparticle are required up to few milligram which rise the cost and production feasibility issues (Mourdikoudis *et al*., 2018).

2.7. Silver nanoparticles

Silver nanoparticles have high antibacterial potential and unique physical and chemical properties and widely used in number of applications. Nanotechnology has opened up new possibilities for AgNPs throughout the last two decades. AgNPs, because of their nanoscale dimension, have several unique characteristics compared to bulk metal, which has sparked great attention in the construction of novel applications. Nanosilver is not a new concept, Lea published the first synthesis of a silver colloid stabilized by citrate in 1889 (J. Liu & Jiang, 2015). Silver colloids have been utilized in the medical field for more than 100 years under the name "Collargol" despite not being properly recognized or under the term "nano." In 1954, the United States registered the first biocidal silver product, "Algaedyn," which is still used in disinfectants today (J. Liu & Jiang, 2015).

Nanosilver's potential to kill pathogenic bacteria has drawn attention in recent years, making it popular for use in a variety of products. Nanosilver has a wide range of antibacterial action and can stop growth of both Gram-positive and Gram-negative bacteria (including *Pseudomonas aeruginosa, Escherichia coli*, and *Staphylococcus aureu*s) (W.-R. Li *et al*., 2011). AgNPs have strong antifungal activity against various strains such as *Saccharomyces cerevisiae*, *Spergillus fumigatus, Aspergillus fumigatus*, and *Candida tropicalisand* (Wright *et al*., 1999). Antiviral activity has also been reported against HIV, HBV and herpes simplex virus (Wright *et al*., 1999; Du *et al*., 2018). Nanosilver is the emerging field and hot topic of research, according to a report 313 out of 1317 nanomaterials in market are claimed to include AgNPs. Silver nanoparticles has been widely used in medical applications such as wound healings, bone cements, female hygiene products and surgical instruments (Beamer, 2020). AgNPs are also used in electronic devices, disinfectant, and textile industries to increase the durability and wrack resistance clothes. During synthesis of nanoparticles some may be released to environment that raise some public concerns.

2.8. Techniques for silver nanoparticles synthesis

Various methods have been brought under consideration for synthesizing silver nanoparticles. The size and shape of nanoparticles varies by altering conditions like temperature, pH, salt concentration, and capping agents. Properties are associated with size, geometrical shapes, surface chemistry, reactivity of nanoparticles and stability. Methods commonly used for AgNPs are sol gel, chemical precipitation, hydrothermal method and biological method.

2.8.1. Sol gel method

Sol gel method is mostly used for AgNPs because it produce pure, ultrafine powder of nanoparticles, rapid productivity and high production rate and is cost efficient. In sol gel method wet-chemical process consisting of a chemical solution serves as a precursor for a discrete particle system. In the sol-gel process, chlorides and metal oxides are often used as precursors (S. Ramesh, 2013). The precursor were dipped in liquid solution and mixed by stirring or sonicating them to separate phases. The nanoparticles in solid phase were than separated through filtration or centrifugation. Ions of aloxysilanes and metal alkoxides are used as precursor for synthesizing of these collides. For example: Tetraethoxysilanes (TEOS) and tetramethoxysilane (TMOS) are most commonly used.

2.8.2. Chemical precipitation method

Chemical method of silver nanoparticle is well established method. Three components are involved in synthesis of silver nanoparticles a) reducing agent; b) metal precursor; c) stabilizing agent. The formation, size and morphology depends on two processes during reaction and can be controlled by changing parameters like pH, temperature and concentrations etc. The two mechanisms are i) nucleation and ii) growth, the former depends on high activation energy while the later requires low activation energy (Evanoff Jr & Chumanov, 2005). Turkevich method and Creighton method are commonly used for synthesis of silver nanoparticles, in turkevich method $AgNO_3$ is used as a metal precursor and sodium citrate as a reducing agent and nanoparticles thus obtained are larger in size. In Creighton methods, $NaBH_4$ is used as reducing agent in place of sodium citrate and nanoparticles thus obtained are smaller in size in range of 10nm (García-Barrasa, López-de-Luzuriaga, & Monge, 2011). For synthesis of silver nanoparticles ascorbic acid

(Velikov *et al*., 2003), polyol (Jacob *et al*., 2007) and monosaccharide (Raveendran *et al*., 2003) have also been reported to be used as reducing agents. Dimethylformamide (DFM) has also been used in combination with silver salt as a reducing agent at different reaction conditions and nanoparticles of different sizes and shapes can be obtained (Pastoriza-Santos & Liz-Marzán, 2008).

2.8.3. Bio-Assisted methods

Biosynthesis of nanoparticle is a green method and is ecofriendly. Every organism has the potential to produce various kinds of nanoparticles such as cadmium, platinum, palladium, gold, silver, zirconium, titanium oxide, copper oxide and zinc oxide etc. These organisms include plants, bacteria, fungi, viruses, algae and yeast. These organisms produce nanoparticles that may be extracellular or intracellular (Hasan, 2015).

Table 2: Sources from which silver nanoparticles synthesized (Siavach Iravani *et al*., 2014)

Plants	Bacteria	Algae	Fungi
Medicago sativa	*Bacillus licheniformis*	*Lyngbya majuscule*	*Fusarium oxysporum*
Camellia sinensis	*B. subtilis*	*Tetraselmis gracilis*	*Fusarium acuminatum*
Platanus orientalis	*Pseudomonas stutzeri*	*Oscillatoria willei*	*Phanerochaete chrysosporium*
Capsicum annuum	*Escherichia coli*	*Padina pavonica*	*Aspergillus flavus*
Acalypha indica	*Klebsiella pneumonia*	*Chlorella vulgaris*	*Aspergillus fumigatus*
Euphorbia hirta	*Enterobacter cloacae*	*Rhizoclonium heiroglyphicum*	*Cladosporium cladosporioides*
Ficus bengalensis	*Lactobacillus sp*	*Cladophora prolifera*	*Penicillium fellutanum*
Nelumbo nucifera	*Aeromonas sp*	*Spirulina subsalsa*	*Coriolus versicolor*
Ginko biloba	*corynebacterium*	*Sargassum fluitans*	*Verticillium sp*
Aloe vera		*Chlorella salina*	

2.9. Applications of AgNPs

Due to the unique properties such as magnetic, electrical, optical, size and shape silver nanoparticles have wide range of applications and can be incorporated into biosensor materials, antimicrobial, cosmetic products, biosensor materials, textile and electronic components (Albrecht *et al*., 2006). AgNPs also have applications in medical field for targeted drug delivery, medical imaging and nano composites (Tan *et al*., 2006). AgNPs have also been used widely in integrated circuits (Kotthaus *et al*., 1997), bio-labelling filters (Cao, 2004), paper batteries (L. Hu *et al*., 2009) and cell electrodes (Klaus-Joerger *et al.,* 2001).

AgNPs are known to have strong toxicity against wide range of bacteria and are used as an antibacterial agent in different products such as socks, detergents, sprays, food containers and cosmetics to stop or kill germs. AgNPs promote cell lysis to break cell wall of bacteria and become integrated to stop the growth of bacteria or to kill bacteria that is why AgNPs is considered as strong antibacterial agent (Gupta & Silver, 1998). AgNPs are also used in medicines for sunblocks, burn treatments, dental items, wastewater treatments etc.

Electrochemical property of AgNPs integrate them in sensor design that increase its durability and high sensitivity to offer faster response. Moreover AgNPs also have catalytic properties that can be used to degrade organic dye, (Köhler *et al*., 2008) reported that the degradation of organic dye can be increased by silver nanoparticles in the presence of potassium peroxodisulphate. The optical property of AgNPs purely depends on plasmon resonance where plasmon are free electron in nanoparticles. Peaks obtained from plasmon resonance are highly sensitive to shape and size of nanoparticles and could be potentially used for biological labelling (Kelly *et al*., 2003). Affinity of AgNPs to react with biomolecule containing sulfur or phosphorus is high and therefore protein in cells or phosphorus elements like DNA are the favorable site for binding of silver (Chung *et al.,* 2016).

2.10. Nanotoxicology

The branch of bioscience that deals with toxicity of nanoparticles and determines how much of a threat these nanoparticles are to humans and environment. In order to regulate the toxic effect of nanoparticle, nanotoxicology is concerned to eliminate adverse threats to humans, plants and animals (Rana & Kalaichelvan, 2013). Since 2000s, potential health and environmental effects of nanoparticle have been reported by many scientists, NGOs and regulatory authorities because of their toxic behaviors, and hazardous chemical and magnetic properties (Santamaria, 2012).

In 1998, a study has shown the toxic effects of nanoparticles on respiratory and inflammatory system (Sato & Donaldson, 1998). Toxicity of nanoparticles depends upon their shape, size, chemistry, crystalline structure, durability and solubility (Schlesinger, 1995). Size of NPs are directly proportion to toxicological effects. As the size increases, the toxicity of NPs also increases because of the possibility of attaching more molecules on the surface. Due to small size they can easily penetrate into cell membrane and because of large surface to volume ratio they carry more molecules which may increase the toxicological effects of nanoparticles (Linkov *et al*., 2008). As well as microorganism. This give us the motivation to use nanoparticles in industries and medicine for example the antibacterial properties of various nanoparticles has been used in textile industry and in medical instruments. Antibacterial properties of TiO_2 has been used in coating of medical instrument to protect them from any kind of microbial contamination (Okuda-Shimazaki *et al*., 2010). With the increase of research interest in engineered nanoparticles the safety measurement of nanoparticles is a concern, such as proper handling and disposal of metal nanoparticles to reduced hazard to environment and human health.

2.11 *Viola odorata*

Viola odorata commonly known as Gul-e-Banafsha and belongs to family Violaceae native to Europe, North America and Asia in Pakistan it is mostly found in Nathia Gali, Kashmir, Swat, Kaghan and Hazara. Around 500BC, this plant was used to reduce pain, antidepression, and for normalizing of blood pressure (Siddiqi *et al*., 2012). It has heart shaped leaves with toothed margins, thick and underground stem, height is about 6 inches, purple or blue coloured flowers with green sepals and some leaves grow on the

base of the plant and dark green in colour. It is an ever green plant and flowering season is winter and mostly grows on river banks and places which are exposed to sunlight (Erhatic *et al*., 2010). In Unani medicine system, it is used for migraine, headaches, laxative and antipyretic, anti-inflammatory, diuretic, visominia, and expectorant. It is also used for the treatment of diabetes, digestive disorders, bronchitis, tumor metastasis, antioxidant, anti-inflammatory, antifungal, antipyretic agents, antiasthmatic and anti HIV (P. Mittal *et al*., 2015). The phytomedicine of *Viola odorata* is due to the presence of essential phytochemicals such as flavonoids, glycoside, vitamin C, methyl silicate and saponins.

Essential oil has also been extracted from *Viola odorata* oil from leaves are green in colour and yellow from flower which are used as aroma in perfumes. The common extracted from *Viola odorata* are geraniol, linalool, citronella, and salicylaldehyde that are used for wound healing and anti-inflammatory. Other compound that are found in *Viola odorata* are pinene, 2-hexenal, 3-hexenol, hexadecane, linalool, pinene, citronellal, dodecanol, 1,8-ocimene, tridecane, methyl salisylate, spathulenol, salicylaldehyde and geraniol (Cu, Perineau, & Gaset, 1992).

Table 3: Taxonomy of *Viola odorata*

Kingdom:	Plantae
Order:	Malpighiales
Family:	Violaceae
Genus:	*Viola*
Species:	*V. odorata*
Botanical Name	*Viola odorata*
Common Name	Gul-e-Banfsha

Chapter 3

Materials and Methods

3.1. Preparation of plant extract

Viola odorata was harvested from National Agriculture Research Centre (NARC), Islamabad. Distilled water was used for washing the leaves various times for the removal of dust and were stored at room temperature for the removal of water. 20g of fresh leaves were chopped in pestle and mortar to make a paste. 200ml of dH_2O was added and boiled for 20 minutes at 100°C. After cooling at room temperature filter extract through whatman filter paper and then centrifuged at 5000 rpm for 10 minutes and stored supernatant at 4°C for further usage.

3.2. Synthesis of AgNPs

For silver nanoparticles, 1mM of $AgNO_3$ were prepared and gently added to plant extract drop by drop in the ratio of 1:5. The mixture was then exposed to sunlight until the color changed to dark brown, followed by incubation at room temperature for 24 hours for reducing all Ag to AgNPs. AgNPs were confirmed by recording absorbance at 430nm through UV-visible spectroscopy. The mixture was centrifuged at 20000rpm for 15 minutes after the completion of AgNPs synthesis. After centrifugation, the supernatant was discarded and pellet containing AgNPs were washed thrice with distilled water. Thick suspension obtained was poured into petri plate and dried overnight in hot oven at 40°C. The dried particles were then grinded in pestle mortar into fine powder. Prepared AgNPs was used for further physical characterization and biological application.

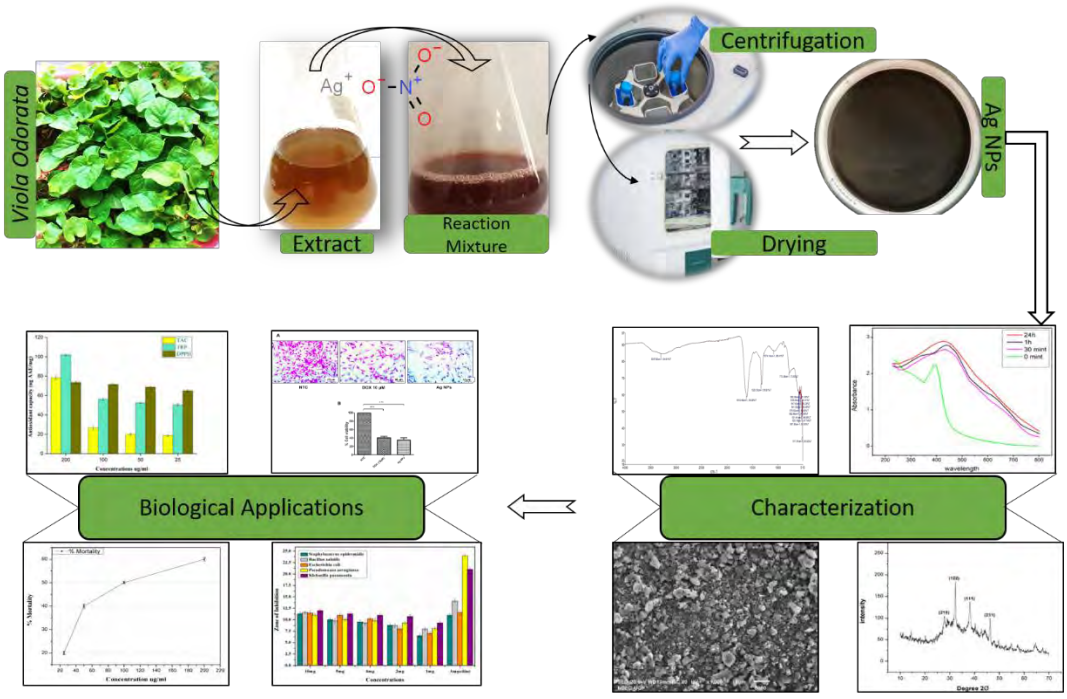


Figure 3: Graphical representation for synthesis, characterization and biological application of AgNPs from *Viola odorata* leaf extract

3.3. Characterization

Synthesized AgNPs were characterized through various techniques includes UV Vis spectroscopy, XRD, FTIR, DLS, EDX and SEM.

3.3.1. UV Visible Spectroscopy

The analysis of the optical property of biosynthesized silver nanoparticles was carried out by UV Visible spectroscopy (HALO DB-20S UV-VIS Double Beam, Australia) at the absorbance range of 200nm to 800nm at zero, 30mintues, 60 minutes and 24h.

3.3.2. X rays Diffraction (XRD)

X rays diffraction was performed for the confirmation of the crystalline nature and size of biosynthesized AgNPs in the range of 2 θ (10°-70°). Diffraction data were obtained through Cu_{Ka} radiation (wavelength, 1.5406Å; generator voltage, 40kV and tube current, 30mA). Then the calculation of crystallite silver nanoparticles (biosynthesized) was done using Scherer's equation (Holzwarth & Gibson, 2011).

$$D = K \lambda / \beta \cos \theta$$

Where D represents crystallite size, k is the shape factor (0.94), λ illustrates the X-ray wavelength, which was 1.5421Å, and β and θ refer to the full width at half maximum in radians and Bragg's angle, respectively.

3.3.3. Fourier transform infrared (FTIR) spectroscopy

The determination and evaluation of bio-molecules and functional groups involved in the synthesis of silver nanoparticles was executed by Fourier transform infrared (FTIR) spectroscopy (SHIMADZU 8100 M FTIR, Shimadzu, Kyoto, Japan) in the spectral span of 500–4000cm^{-1})

3.3.4. Dynamic light scattering

The calculation of Zeta potential and particle size distribution was done by using Zeta sizer Nano-ZS (Malvern Instruments UK). 1mg of silver nanoparticles was dissolved in 1ml of deionized water followed by room temperature analysis.

3.3.5. Scanning electron microscope (SEM)

Morphological and particle size were determined by using scanning electron microscope. Samples were mixed in isopropanol for uniform distribution of nanoparticles on stubs and to prevent powder from flying off because of vacuum and beam.

3.3.6. Energy Dispersive X-rays (EDX)

Energy dispersive X rays is an instrument generally attached with SEM and used to confirm the elemental composition of the particles. Same procedure was followed as SEM.

3.4. Biological Assays

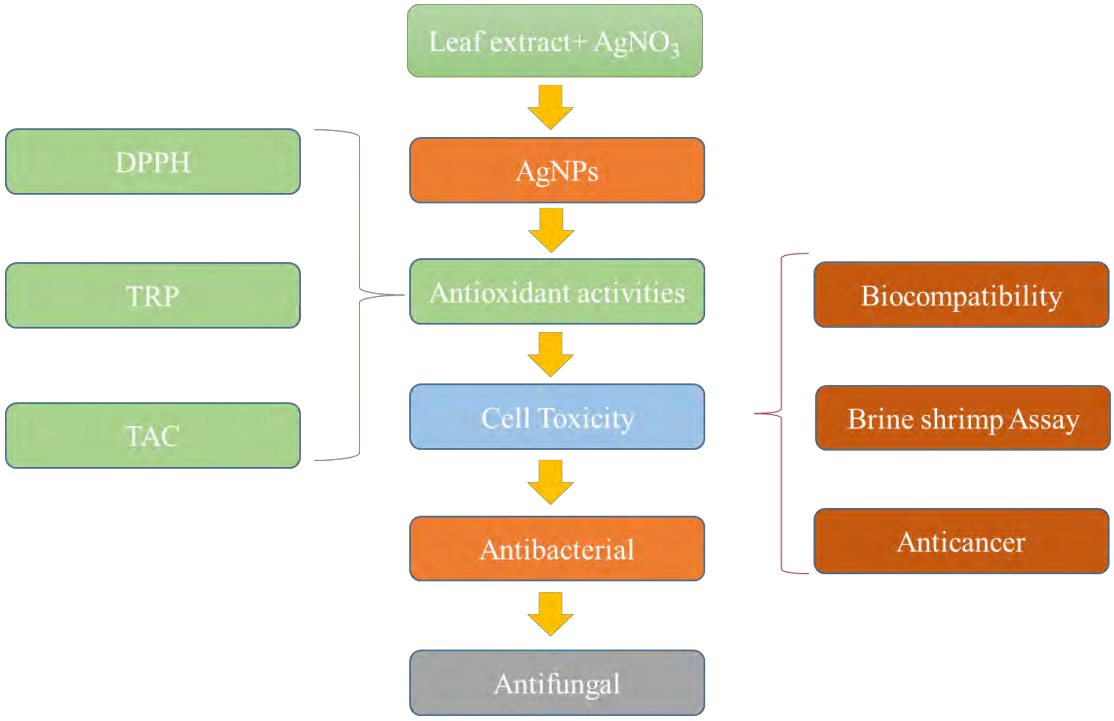


Figure 4: Schematic diagram of Biological activities for AgNPs synthesized from leaf extract of *Viola odorata*

3.4.1. Free radical scavenging assay:

To determine the antioxidant activity of green synthesized silver nanoparticle 2,2-diphenyl 1-picrylhydrazyl (DPPH) free radical were used (Shah *et al*., 2020). Different concentrations of silver nanoparticle 200, 100, 50, 25µg/ml and DPPH solution (4.80mg/50ml methanol) were made and used in the reaction mixture. Furthermore 20µl of sample and 180µl of DPPH solution were added into each well of microplate. Incubate the plate for 90 minutes at room temperature in dark. DMSO and ascorbic acid were used as negative and positive controls respectively. The reaction was performed three times to reduce the uncertainty. Absorbance were measured at 515nm using microplate reader and scavenging potential of silver nanoparticles was calculated using following formula.

$$\%FRSA = \frac{1 - Abs}{AbN} \text{ X100}$$

Here,

Abs = Absorbance of DPPH with addition of sample

AbN = Absorbance of negative control.

3.4.2. Total Reducing Power (TRP)

Potassium ferricyanide based assay was utilized to determine the total reducing power of silver nanoparticles (Nadeem *et al*., 2019). Different concentrations of test sample (200, 100, 50 and 25µg/ml) were prepared and used in the assay. Potassium Ferricyanide (1%) [$K_3Fe(CN)_6$] 400µl was mixed with 400µl of 0.2M phosphate buffer (pH 6.6) and 100µl of test sample from each concentration were added to eppendorf tube followed by Incubation for 20 minutes at 50°C. After incubation, 100µl of Trichloroacetic acid (10%) were added into each tube. Centrifuge tubes at 3000rpm for 10 minutes at 25°C. 150µl supernatant were collected from each tube and poured into each well of 96 wells plate with addition of 50µl ferric chloride (0.1%). Microplate reader was utilized to determine the optical density at 630nm. Experiment was performed three times to reduce uncertainty. Ascorbic acid were used as positive control and DMSO were used as negative control. The total reducing power of green synthesized silver nanoparticles were expressed as (AAE µg/mg).

3.4.3. Total antioxidant capacity (TAC)

Phosphomolybdenum based assay was used to estimate the total antioxidant potential of green synthesized silver nanoparticles (Nadeem *et al*., 2019) TAC reagent were prepared by mixing of 1.63ml (0.6M) H_2SO_4, 1.6795g (28mM) NaH_2PO_4 and 0.0247g (4mM) Ammonium molybdate. A 100µl of test sample with different concentration were added to 900µl of TAC reagent and incubated tubes at 95°C for 90 minutes in water bath. After incubation the reaction mixture was cooled down to room temperature and 200µl sample were taken from each tube and transfer into each well of 96 well plate. Absorbance were measured at 630nm using microplate reader. Ascorbic acid and DMSO served as positive and negative control respectively. The experiment was performed three times to reduce uncertainty. The antioxidant capacity were expressed in AAE µg/mg.

3.5. Antibacterial Assay

Antibacterial potential of the green synthesized silver nanoparticles was performed using disc diffusion method with slight modification (Ali *et al*., 2020). The activity was assessed against two gram positive (*Staphylococcus epidermidis* and *Bacillus subtilis*) and three gram negative bacteria *(Escherichia coli, Pseudomonas aruegonosa, and Klebseilla pneumonia).* Stock solution of AgNp were prepared by dissolving 20mg of AgNPs in 1ml water. Further, 5 dilutions were prepared from stock solution i.e. 10,5,4,2, and 1mg/ml respectively. Tryptone Soy Agar (TSA) media was prepared and significant growth was achieved by swabbing overnight culture already prepared in broth. 6µl of prepared dilutions were loaded in each disc and plates were labeled accordingly. The plates were incubated for 24 hours at 37°C. Ampicillin was utilized as positive control whereas DMSO was utilized as negative control. After 24 hours of incubation, zone of inhibitions were measured using Vernier caliper. Experiment was executed in triplicates.

3.6. Antifungal Assay

Antifungal potential of green synthesized silver nanoparticles were evaluated by agar well diffusion method with slight modification (Ahmad *et al*., 2016). Two pathogenic strain of fungi *Candida albicans* and *Aspergillus niger* were used for assay. Sabouraud Dextrose Agar (SDA) media was prepared autoclaved and 100µl suspension of fungal spore were swabbed on petri plate. Make a well of 5mm using sterile borer and 20µl of test sample was poured into wells and labelled accordingly. Nilstat and DMSO served as positive and negative control respectively. The plates were then incubated at 28°C for 24 hour. The experiment was performed three times to reduce error. After 24h zone of inhibition were measured using Vernier caliper in millimeter.

3.7. Biocompatibility of AgNPs Nanoparticles with RBCs

The biocompatibility and bio safe nature of AgNPs was assessed by utilizing AgNPs against human RBCs (Jan *et al*., 2020). About 2ml of fresh blood was collected from healthy individual in an EDTA tube to prevent blood coagulation followed by centrifugation at 13000rpm for 10 minutes to separate RBCs from plasma. Supernatant was discarded and phosphate-buffered saline (PBS) was used to for triple washing of the pallet.

9.8ml of PBS (pH 7.2) were mixed with 200µl of isolated RBCs and gently mixed it to make a suspension for further used. 200µl of RBCs suspension were taken in eppendorf tube and add AgNPs of different concentration (200, 100, 50 and 25µg/ml) at 1:1 followed by incubation at 35°C for 1 hour. After incubation the reaction mixture were centrifuge at 10000rpm for 10 minutes. Take 200µl supernatant and transfer into each well of 96 well microplate. Absorbance was measured at 540nm to detect hemoglobin released. Triton X 100 (1%) served as positive control and negative control was induced by using DMSO. The experiment was repeated thrice for minimizing any error and % hemolysis was calculated using formula.

$$\%Hemolysis = \frac{Sample - Negative\ control}{Positive\ control - negative\ control} \text{ X } 100$$

3.8. Brine Shrimp Assay

This activity was carried out to check the cytotoxicity effect of AgNPs. Purchased *Artemia salina* (brine shrimp) eggs from ocean star international and stored at 28°C. The eggs were placed in tray having artificial sea water (34g/L) and were allowed to hatch near a light source at 37°C. This experiment was performed by using 96 well plate as previously described (A. T. Khalil *et al*., 2017). 10 newly hatched shrimps were taken and transfer to each well of microplate with addition of different concentrations (200, 100, 50 and 25 µg/ml) of green synthesized AgNps. Adjust the volume to 300µl at this stage and leave it for 24 hrs. After 24 hrs of exposure shrimp were observed under light microscope and count the dead and live shrimps. Percent mortality was determined by following formula:

$$\%\ Mortailty = \frac{Number\ of\ Dead\ Artemia\ salina}{Number\ of\ Initial\ Artemia\ salina} \text{ X } 100$$

3.9. Cytotoxicity against Liver Cancer cells (HepG2)

3.9.1. Cell culture

Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Calf Serum (FCS), supplemented with 2mM L-glutamine, 1mM Na-pyruvate, 100U/mL penicillin, 100µg/mL streptomycin at 37˚C in a humidified 5% CO_2 atmosphere was used to culture human hepatocellular carcinoma cells (ATTC HB-8065). Cell harvesting was done with 0.5 mM trypsin/EDTA at room temperature for 1 min.

3.9.2. Cell viability assay

Cell toxicity of green synthesized silver nanoparticles against HepG2 cell lines was assessed through Sulforhodamine B (SRB assay) (Siddiquah *et al*., 2018). Stock solution (1mg/ml) in deionized water and were dissolved through Sonication. HepG2 cells (> 90% confluency) were planted in a 96-well plate at a density of 12000 cells/well and allowed to adhere for 24 hrs at 37˚C. Cells were then treated with 50µg/ml of AgNPs for 24 hrs. In order to fixed the cell 50% pre-chilled Trichloroacetic acid (TCA) and incubate at for 4 hrs at 4˚C followed by washing the plate with deionized water. Cells in plate were then stained with 0.05% SBR dye and incubated for 30 minutes at room temperature. After incubation the remaining unbounded dye was removed by washing with 1% acetic acid. Doxorubicin drug used as positive control and Non-treated Cells (NTC) used as negative control in experiment and sample only and media only representing background optical density. Experiment were performed three times to reduce error. Olympus CK2 light microscope equipped with digital camera were used to take photographs. A 100µl of 10mM Tris (pH 8) were added into each well at room temperature for 5 min for Solubilization of SBR dye. Absorbance was evaluated at 565nm utilizing microplate reader. Percent (%) viability was determined relative to the NTC sample using the following formula:

$$Cell\ viability\ \% = \frac{Absorbance\ of\ sample - Absrobance\ of\ sample\ control}{Absorbance\ of\ NTC - Absorbance\ of\ media\ only}\ \text{X}\ 100$$

Chapter 4

Results and Discussion

For the first time *Viola odorata* was successfully used for synthesizing silver nanoparticles (AgNPs). Following various steps of sterilization, drying, grinding, and addition of salts, a greenish gray colour AgNPs were obtained that was stored at room temperature for further analysis. The vial was covered with aluminum foil because of light and sensitive nature of AgNPs.

4.1. Characterizations

Synthesized AgNPs were characterized through various techniques includes UV Vis spectroscopy, XRD, FTIR, DLS, EDX and SEM.

4.1.1. Uv-Vis Spectroscopy

Aqueous leaf extract of *Viola odorata* was added into silver nitrate solution, results changed in color from yellow to dark brown indicating formation of AgNPs shown in Figure 5 (a). The color was changed because of the reduction of Ag^{+} due to excitation of Surface Plasmon Vibration (SPV) (Veerasamy *et al*., 2011). The solution was kept for 24 hrs and no further color change and absorption peaks were observed. Synthesized AgNPs were further confirmed through UV-Vis spectroscopy by setting range between 200-800nm and the highest peaks were observed at 430 nm. Readings were taken at 30 min, 1hr, and 24 hrs and no further change in absorbance intensity occurred after 24 hrs as shown in Figure 5. Our results were corresponding with previous reports (Fayaz *et al*., 2010).

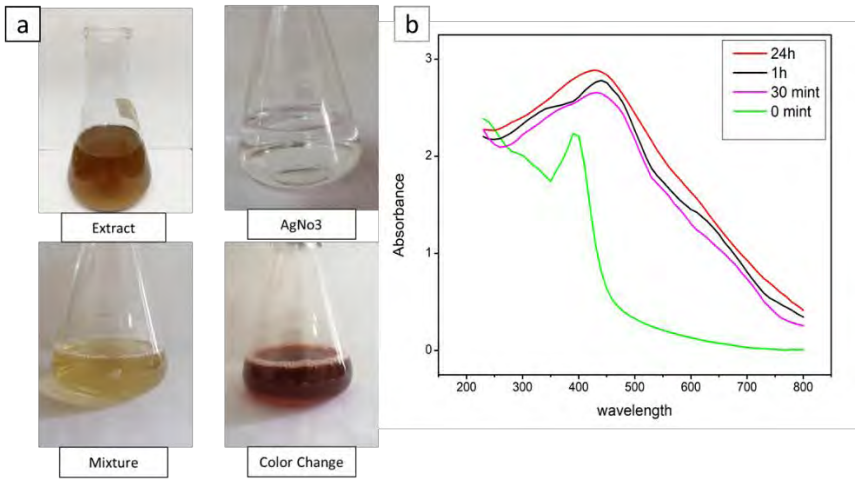


Figure 5: (a) Showed color changed (b) showed UV-Vis spectroscopy of Ag nanoparticles

4.1.2. X-rays Diffraction (XRD)

Crystalline nature of biosynthesized AgNPs is confirmed by XRD analysis in the range between 10 to 70˚ at 2θ angles. The XRD pattern (Figure 6) shows four main peaks at 27.9˚, 32.23˚, 38.1˚ and 46.3˚ corresponding to lattice planes (210), (122), (111) and (231), respectively. Same pattern also reported in previous literature (Arshad, Sami, Sadaf, & Hassan, 2021; Tahir *et al*., 2015). Debye-Scherrer equation was used for the determination of average grain size of biosynthesized AgNPs.

$$D = k \lambda / \beta \cos \theta$$

D = The average crystalline size perpendicular to the reflecting planes

K = Scherrer coefficient (value=0.85)

λ = X-ray wavelength (i.e.1.5406 A°)

β = Angular full width at half maximum (FWHM) in radians

θ = Bragg's angle/Diffraction angle.

The average particle size calculated was 11nm. Our results are in correspondence with previous reports (Ukkund *et al*., 2019).

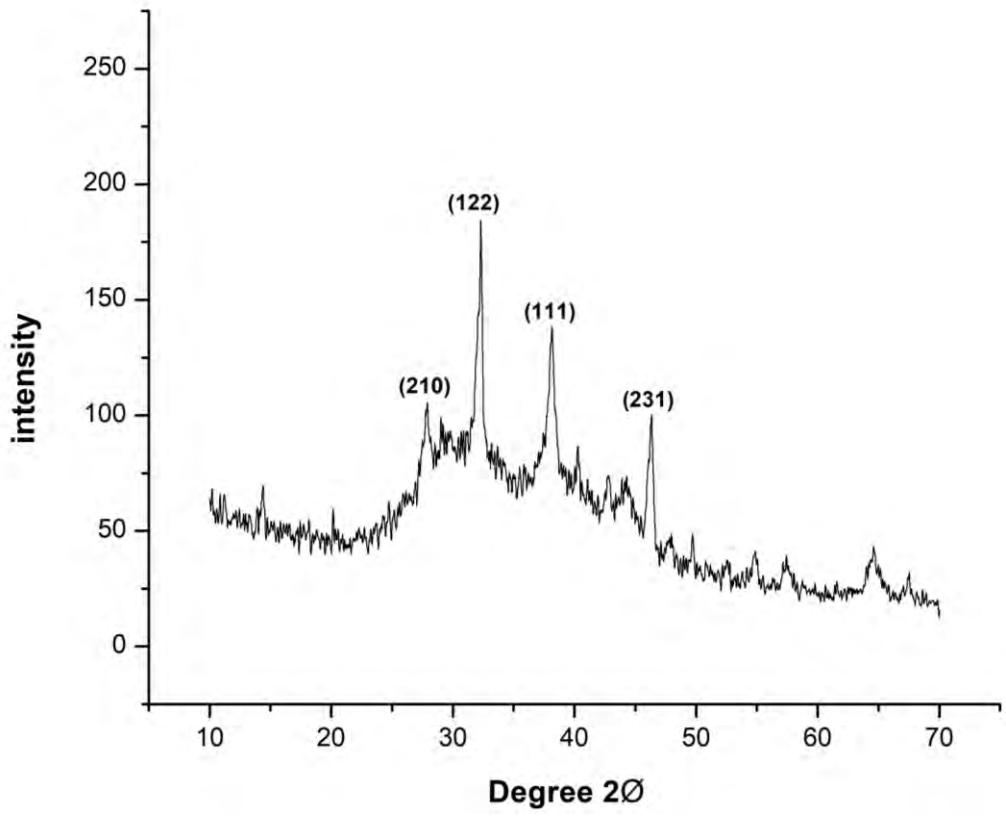


Figure 6: XRD pattern of AgNPs

4.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was done to identify the biomolecule and measure vibrational frequency of bonds in molecules. FTIR was carried out in range of 4000-515cm^{-1} and major peaks 3287 cm^{-1}, 1610 cm^{-1}, 1320 cm^{-1}, 1074 cm^{-1}, 773 cm^{-1} (Figure 7). The observed peak at 3287 cm^{-1} and 1610 cm^{-1} corresponding to O-H and C=C stretching vibration of phenolic compounds (Rezaei *et al*., 2014) Peak at 1320 cm^{-1} corresponding to N-O stretching, 1074 cm^{-1} to C-C and 773 cm^{-1} N-H vibration, respectively (Faisal *et al*., 2020). FTIR results confirmed possibility of proteins, flavanones, amino acids, cellulose, polyphenols, and terpenoids having functional groups of aldehydes, carboxylic acid, alcohols, and ketones in the extract. These molecules have strong ability to bind to metal and play vital role as a capping agent and stabilizing agent in AgNPs formation (Shivakumar *et al*., 2017).

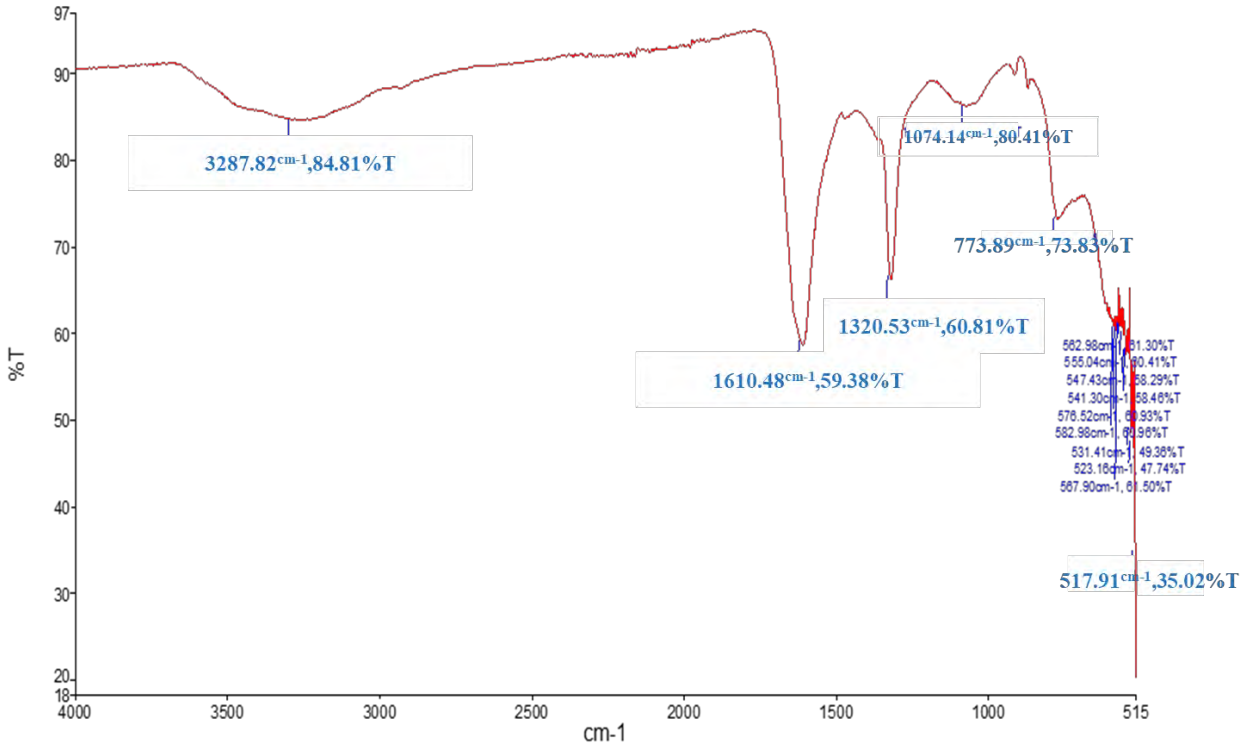


Figure 7: Fourier Transform Infrared Spectroscopy (FTIR)

4.1.4. Dynamic light scattering

Size distribution and surface potential of AgNPs were calculated using Dynamic Light Scattering (DLS) technique. Samples were dispersed in dH_2O followed by sonication to keep PolyDispersity Index (PDI) lower than 0.5 that is highly recommended for analysis. High intensity peaks were observed at 72.5nm and average particle size measured as 71.6nm that were slightly higher than SEM size due to poor dispersion. This was due to the fact that DLS calculated the hydrodynamic size (i.e. size and surface water molecule) (Figure 8a). Zeta potential measured was -21.6mV as shown in figure 8b, the negative potential was due to the use of plant extract and it also indicate stability of particle in aqueous medium. Suspension that reveled ≥15mV potential are considered to be stable thus AgNPs were reflected to be stable and well dispersed (Slavin *et al*., 2017).

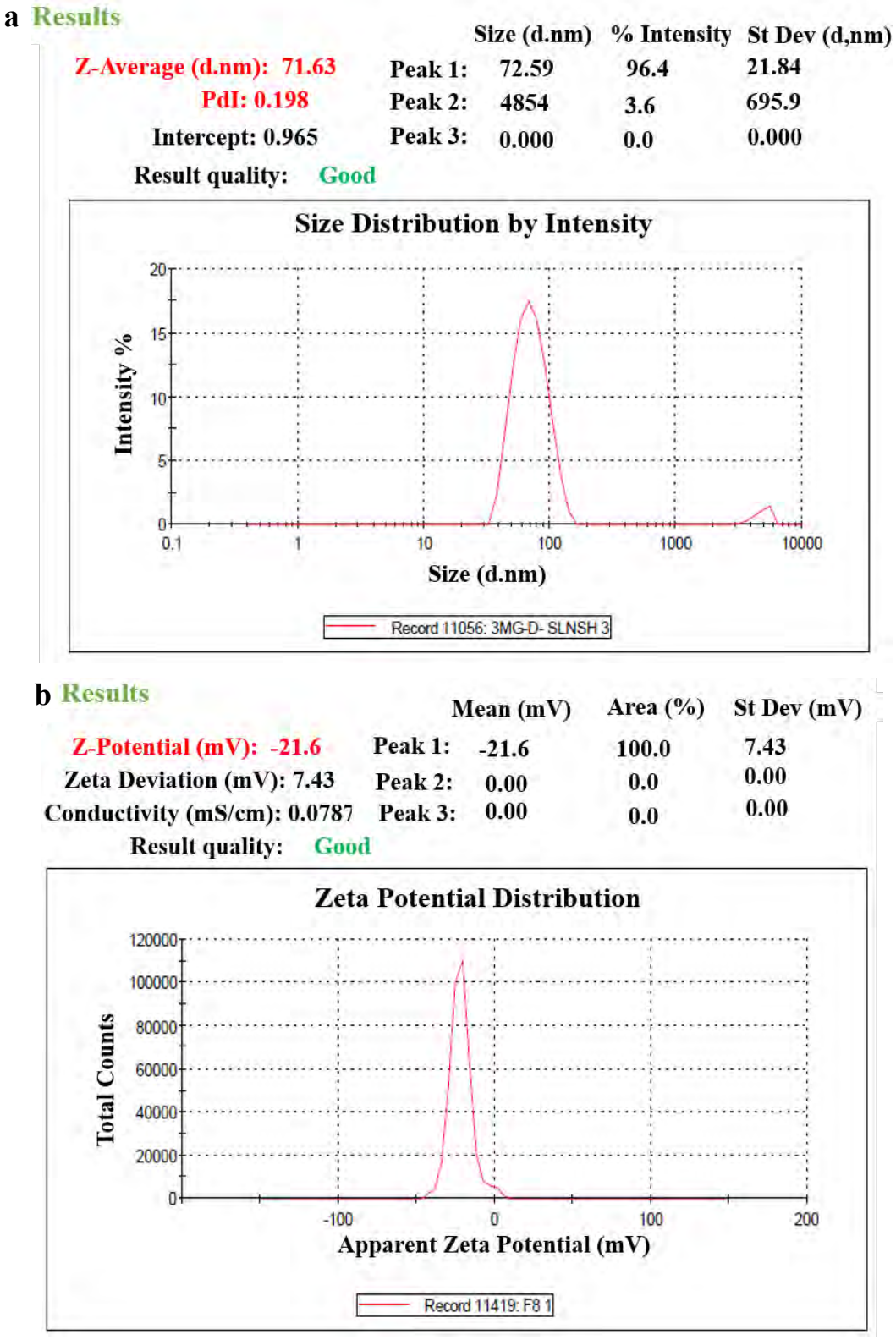


Figure 8: Zeta size (a) and zeta potential of AgNPs (b)

4.1.5. Scanning Electron Microscopy (SEM)

Morphology of biosynthesized AgNPs were analyzed by SEM as shown in Figure 9. Results showed that AgNPs have irregular shape from triangular to rectangular shape with clear boundaries between each particle, which defined the sustainability of nanoparticles with capping agent in leaf extract of *Viola odorata* (Hamedi & Shojaosadati, 2019). AgNPs formation consist of three steps. In the first step, metal ions in reaction mixture reduced by the reducing agent which produced atoms that act as nucleation centers and reduced further ions in solution that lead to clusters of metal in next step. Surface ions are reduced again and again and to reach high level of nucleation which result in formation of larger particles. In last step the particles interact with polymer to prevent further aggregation and stabilized nanoparticles are formed (Hamedi *et al*., 2017). Our results are correlated with the reports of (Khodashenas & Ghorbani, 2019), who studied that morphology of nanoparticles depends upon their interaction with capping and stabilizing agents in solution and their methods of preparations.

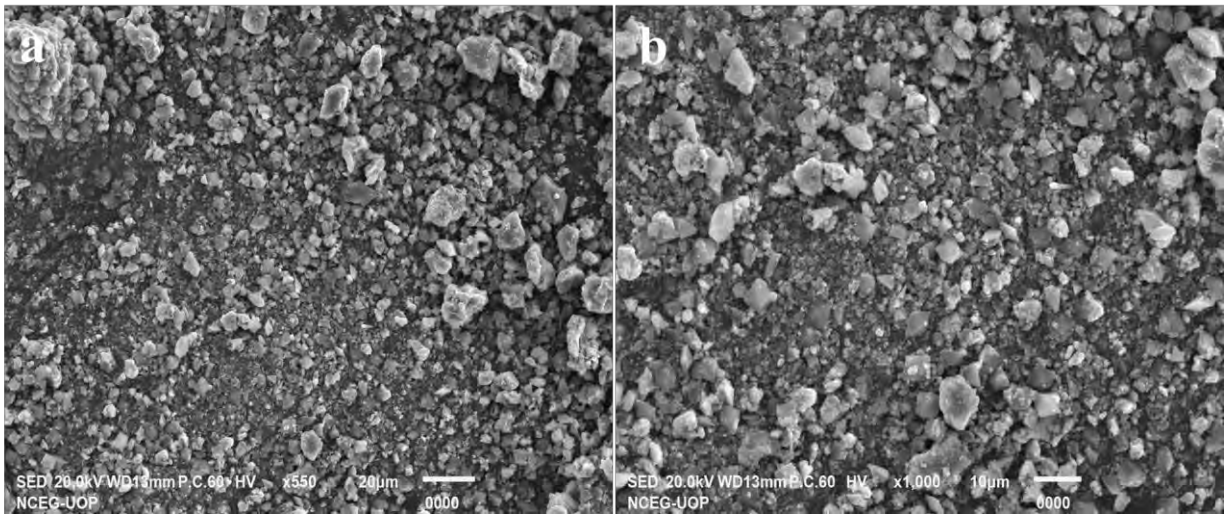
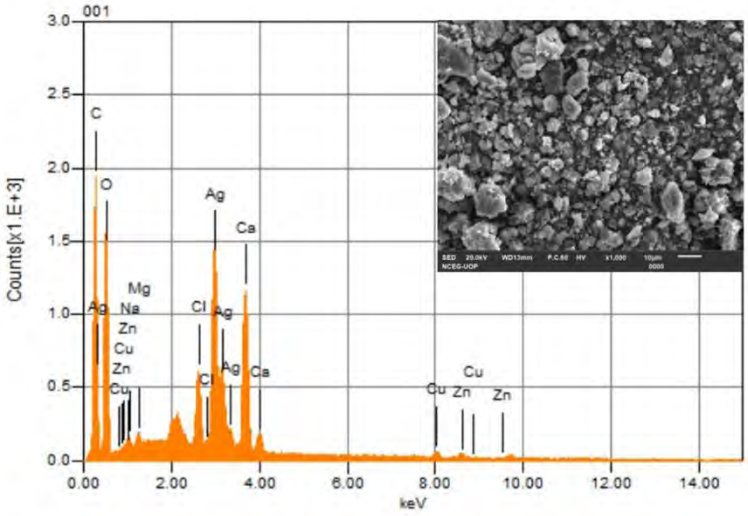


Figure 9: SEM micrograph of AgNPs (a) show micrograph at 20µm (b) micrograph at 10µm

4.1.6 Energy Dispersive X-rays (EDX)

EDX analysis displayed in Figure 10, provided quantitative elemental composition of AgNPs synthesized from leaf extract of *Viola odorata*. Total weight estimated for Ag in EDX graph is about 20% followed by low percentage mass for the rest of metals such as Magnesium, Zinc and Copper. Metallic silver show strong absorption peak at 3Kev and high percentage of oxygen probably because of presence of biomolecules found in leaf extract of *Viola odorata* that are used for reduction of silver ions (Kaviya *et al*., 2011).



Formula	mass%	Atom%	Sigma	Net	K ratio	Line
C	26.63	41.86	0.03	48236	0.0060841	K
O	41.13	48.54	0.14	40224	0.0231847	K
Na	0.37	0.31	0.04	1496	0.0003503	K
Mg	0.49	0.38	0.03	3106	0.0006991	K
Cl	2.22	1.18	0.02	22544	0.0049264	K
Ca	7.74	3.65	0.05	62961	0.0176144	K
Cu	1.62	0.48	0.06	3285	0.0032135	K
Zn	1.15	0.33	0.07	1957	0.0022843	K
Ag	18.64	3.26	0.10	110505	0.0346181	L
Total	100.00	100.00				

Figure 10: EDX analysis of AgNPs from leaf extract of viola odorata

4.2. Biological Applications

4.2.1. Antioxidant capacity of AgNPs

Antioxidant potential of AgNPs synthesized from *Viola odorata* were determined via three different assays, namely, DPPH (2,2-diphenyl-1-picrylhydrazyl assay), TRP (total reducing power) and TAC (total antioxidant capacity). The antioxidant potential of AgNPs were concentration dependent and were observed to increase with increasing concentration of nanoparticles. Free radical scavenging activity was identified by discoloration of DPPH reagent from dark to yellow by accepting hydrogen electron from antioxidant present in sample (Nadeem *et al*. 2019). Highest DPPH value was calculated as 73.55±1.17µg AAE/mg at 200µg/mL that were higher than silver nanoparticles synthesized from *Zingiber officinale* (Faisal *et al*.2020). Similarly, highest value measured for TAC and TRP are 78.37±1.97 and 70.04±0.93µg AAE/mg at 200µg/mL, respectively (Figure 11). TAC depends on the depletion of Mo (VI) to Mo (V) through antioxidant mediators make a phosphate molybdate complex that is recognized by its green color (Prieto *et al*., 2015). TRP depends on reduction of Fe^{+3} to Fe^{+2} ion by the sample in case of any redox potential (Yanan Li *et al*., 2018). Nanoparticle's antioxidant activity is because of the presence of flavonoids, phenolics, polysaccharides and specially phenol that are capped on the surface of AgNPs and contribute to its antioxidant activity (A. K. Mittal *et al*., 2014).

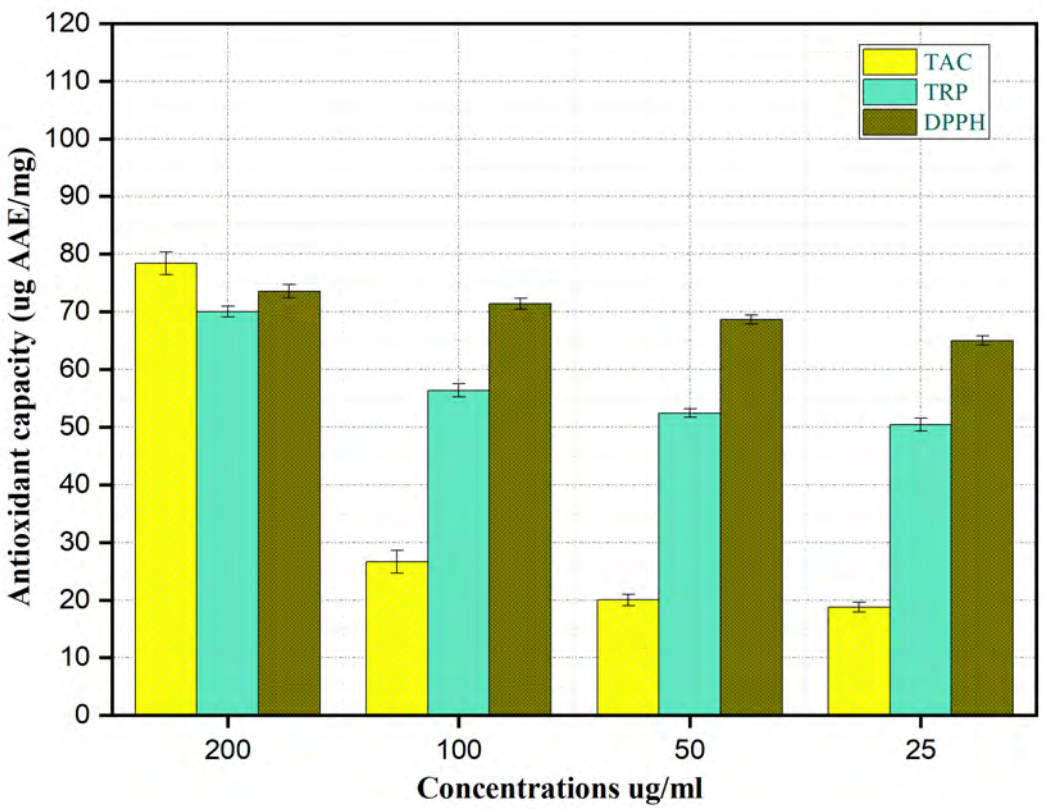


Figure 11: Antioxidant activity of AgNPs

4.2.2. Antibacterial activity

Antibacterial activity of green AgNPs was investigated through disc diffusion method against *Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, *and Klebseilla pneumonia (*Figure 12). Zone of inhibition for gram negative *(Escherichia coli, Pseudomonas aeruginosa and Klebseilla pneumonia) was higher in comparison with gram positive* (*Staphylococcus epidermidis* and *Bacillus subtilis*), that may be due to change in composition of cell wall. A thick peptidoglycan layer (20-80nm) constitutes the cell wall of Gram positive bacteria which in turn is comprised of linear polysaccharide chains crosslinked by short peptides, providing a more hard structure that makes silver nanoparticle penetration more difficult, whereas Gram negative bacteria have a thinner peptidoglycan layer (Shrivastava *et al*., 2007). The cell wall's toughness and significant cross-linking not only provides fewer anchoring sites for silver nanoparticles, but they also make them harder to penetrate (Baron *et al*., 1996). These

NPs perforate the cell membrane of the microbes via tiny pores, causing mineral imbalances and intracellular protein and enzyme leaks, consequently arresting cell growth and cell necrosis (Reidy *et al*., 2013). *Klebsiella pneumoniae* had the biggest zone of inhibition, measuring 12±0.34mm, followed by *Bacillus subtilis* (11.6 ± 0.24mm), and *Escherichia coli* (11.5±0.24mm). The antibacterial capability of AgNPs is primarily determined by morphological characteristics such as form, size, and surface area; however, electrostatic interactions between positively charged NPs and negatively charged bacterial cells are vital in determining bactericidal activity.

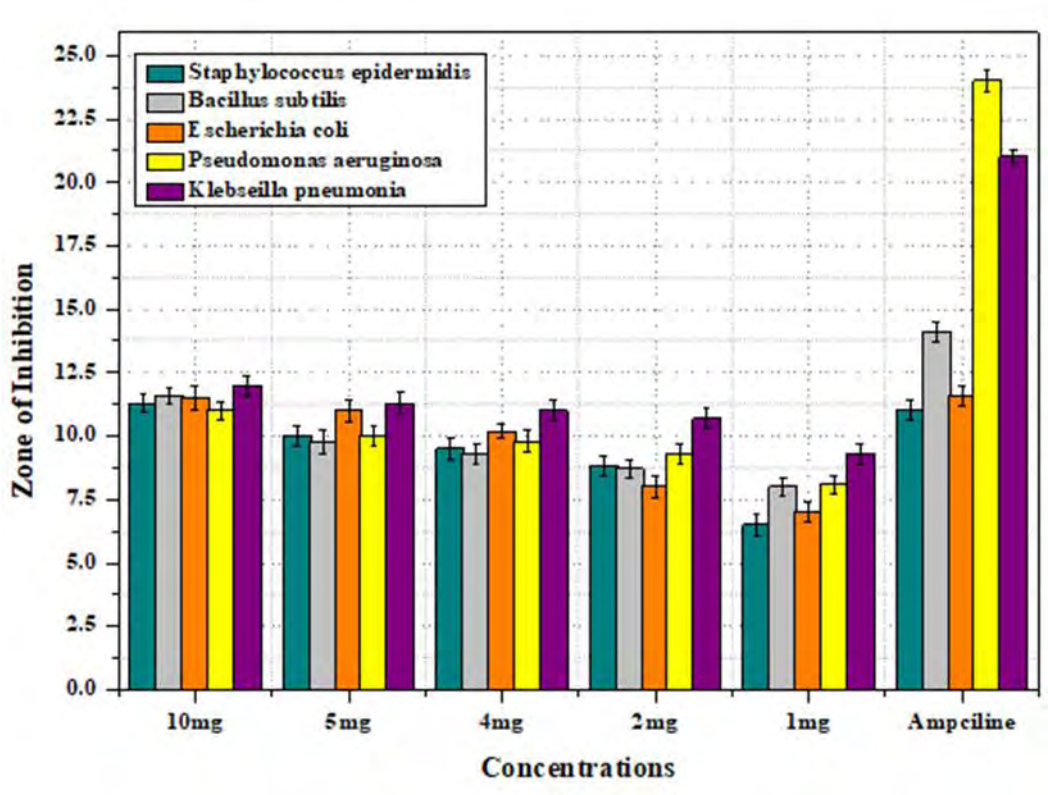


Figure 12: Antibacterial activity of AgNPs against various bacterial strains

4.2.3. Antifungal Activity

Antifungal potential of green synthesized silver nanoparticle was evaluated using agar well diffusion methods. Since huge data available on antibacterial activity of nanoparticles through restricted details is available and shows its effectiveness against

fungal spores. In present work, antifungal potential of AgNPs were evaluated against *Aspergilus niger* and *Candida albicans* (Figure 13). Correspondingly, positive control was provided through Nilstat whereas DMSO was administered as negative control. Studies revealed that the most common method to induce cellular toxicity is by free radical generation and membrane disruption by these nanoparticles. While the interactions between ROS and fungal hyphae or spores is a common factor leading towards fungal growth suppression (Jan *et al*., 2020). *A. niger* showed maximum zone of inhibition 25±0.44mm as compared to *C. albicans* (16±0.39mm). Due to substantial antibacterial and antifungal activity, AgNPs might be utilized as a potent antimicrobial substance not only in pure form but in combination also. They can also be brought under consideration as the antibiotics carriers.

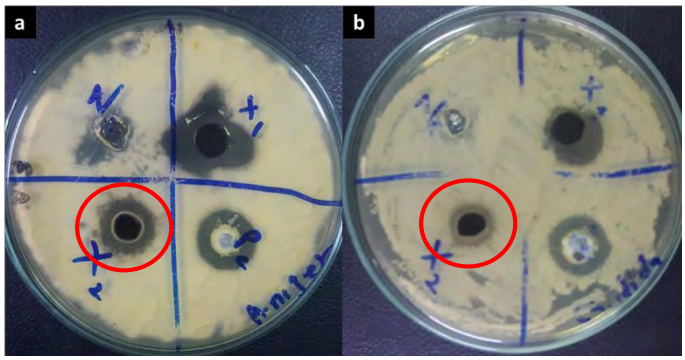


Figure 13: Antifungal activity of Green synthesized AgNPs against *A niger* (a) and *C albicans* (b)

4.3. Hemolysis Activity

With the advancement of nanotechnology in medicine there is a question about the toxicity of nanoparticles and in this regard, it is highly recommended to study toxicity of nanoparticles on blood. Biocompatibility assay of nanoparticles was evaluated by percent hemolysis when mixed with RBCs as nanoparticles damaged the membrane of cell and caused death (Chen *et al*., 2015). According to "American Society for Testing and Materials Designation" nanomaterial with hemolysis rate >5% are hemolytic, NPs with 2–5% hemolysis rate are slightly hemolytic while the NPs having <2% hemolysis activity are non-hemolytic (ASTM, 2017). Our study revealed that green AgNPs are biocompatible

and non-hemolytic at concentration of 50µg/ml, slightly hemolytic at 100µg/ml and hemolytic at 200µg/ml, respectively. Results are shown in Table 4. These results show that Green AgNPs at concentration of 100µg show <5% hemolysis which lie under biocompatible range of standard according to ISO/TR 7406 and therefore recommended for in vivo biomedical applications.

Table 4: Hemolysis Activity

S/No	Concentration µg/ml	% Hemolysis
1	200	6.9±0.52
2	100	3.1±0.31
3	50	1.2±0.15
4	25	0

4.4. Brine Shrimp Activity

For marine ecotoxicity testing, *Artemia salina*, one of the most beneficial test organisms was used. *A. salina* has gained much importance as numerous marine larvae utilize it as live food source for nourishment, thus making it the most suitable and minimal laborious live food accessible for aquaculture. Nanoparticles will have a significant impact on *Artemia salina* due to their excessive inter-linkage with environment via nonselective filter feeding (Arulvasu *et al*., 2014). In current work, cytotoxicity of brine shrimp was found against different concentration of *Viola odorata* mediated AgNPs ranges (200, 100, 50, 25 µg/ml) and percent cytotoxicity were found to be (60±1.12%, 50±0.52%, 40±1.18%, 20±0.39%), respectively (Figure 14). Lethality were found to be directly propositional to concentration of nanoparticles at 200µg/ml it shows maximum mortality rate 60% that is less then as previously reported by (Faisal *et al*., 2020).

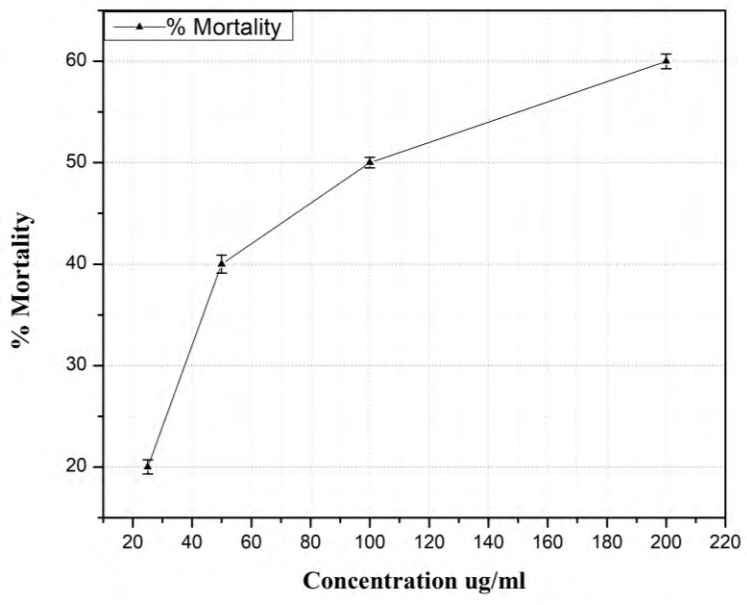


Figure 14: Graphical representation of brine shrimp cytotoxicity

4.5. Cytotoxicity against Liver Cancer cells (HepG2)

Suforhodamine B assay was used to investigate direct influence of AgNPs on cell viability of HepG2 cell line. Cells were treated with 50µg/ml of AgNPs for 24 hrs in 96 well plate and results (Figure 15). Our results suggested that AgNPs show higher cytotoxicity as compared to positive control (Doxorubicin). In non-treated cells (NTCs), percent viability was detected around 100 ± 3.3% of viable cells, that descended to 35.3 ± 5.1% in AgNPs presence and for positive control cell viability were 40.5±3.9%. While lowering the sample values, the results commence against the liver cancer cells were worthy to consider (Shah *et al*., 2020). Morphological changes were also observed using light microscope in cell lines upon the treatment of AgNPs. The non-treated cells are rectangular and clear while upon treatment with AgNPs they loss their rectangular shape and become fibrous and elongated. Different morphological changes were observed in different cell lines (Najim *et al*., 2014). Metastatic processes may affect due to morphological changes in cells such as substrate attachment, migration and invasion (Brandhagen *et al*., 2013). Our results match with previous report (Siddiquah *et al*., 2018).

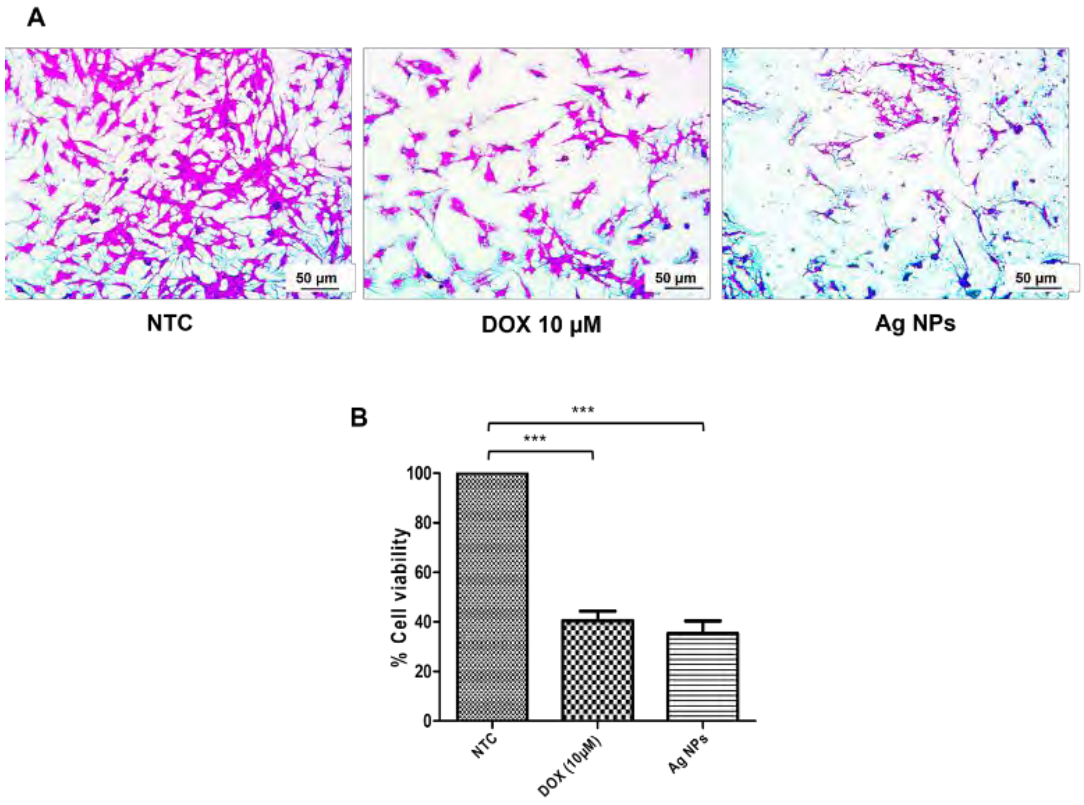


Figure 15: Cytotoxic effects of AgNPs on HepG2 cells upon 24 hours treatment with 50 µg/ml concentration. Untreated cells, DMSO (solvent) and Doxorubicin were included as controls.

Chapter 5

Conclusions

A comprehensive study has been presented by green synthesizing silver nanoparticles from a plant of high medicinal value, *Viola odorata* with a special focus on biological uses of AgNPs. Crystalline AgNPs were produced using green approach, which is aseptic, environmentally friendly and is a nontoxic method for synthesizing NPs. Crystalline nature of AgNPs was confirmed by XRD with average size of 11nm and SEM morphology showed irregular shape of synthesized AgNPs. AgNPs show effective antibacterial and antifungal activities, however, they are more effective against gram negative bacteria because of their thin cell wall. A cooperative analysis of AgNPs was also done against liver cancer cell (HepG2) which show that AgNPs have higher toxicity effect in comparison with DOX drug. AgNPs was also found to be compatible with human RBCs so they can be used in cosmetics and medicinal application. However, it is recommended to conduct further study on silver nanoparticles for exploiting their biomedical applications in both *in vitro* and *in vivo* levels.

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