Green synthesis of Silver nanoparticles from *Pedicularis punctata* and their biomedical applications

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Author's Declaration

I**, Nawal Naveed Abbasi D/O Muhammad Naveed Abbasi,** Registration no 02272111020, MPhil Biotechnology scholar, Department of Biotechnology , Faculty of biological sciences, Quaid-i-Azam university Islamabad, Pakistan, hereby declare that the quoted data in thesis entitled **"Green synthesis of Silver nanoparticles from *Pedicularis punctata* and their biomedical applications"** 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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Dedicated to Almighty ALLAH and the Beloved Prophet Muhammad (P.B.U.H),
my Loving Family and teachers.

Certificate of approval

This is to certify that the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam university, Islamabad, Pakistan accepts the dissertation entitled **"Green synthesis of Silver nanoparticles from *Pedicularis punctata* and their biomedical applications"** submitted by Nawal Naveed Abbasi, in the present form as satisfying the dissertation requirement for the Degree of Master of Philosophy in Biotechnology.

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(Nawal Naveed Abbasi)

List of Abbreviations

Abbreviation	Stands for
AgNO3	Silver nitrate
AgNPs	Silver nanoparticles
ALT	Alanine transaminase
AST	Aspartate aminotransferase
DFPAE-AgNPs	dragon fruit peel aqueous extract fabricated silver nanoparticles
DPPH	2, 2-diphenylpicrylhydrazyl
HPLC	High performance liquid chromatography
KPK	Khyber Pakhtunkhwa
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
PEG	Polyethylene glycol
PGA	Propylene Glycol Alginate
PhGs	phenylethanoid glycosides
PVP	Polyvinylpyrrolidone
P.Punctata	*Pedicularis punctata*
RNS	Reactive nitrogen species
ROS	Reactive oxygen specie
SDS	Sodium dodecyl sulfate
SERM	Selective estrogen receptor modulator
TNF	Tumor necrosis factor

Abstract

The massive collapse of ecosystem as a result of urbanization and industrialization is unrestrained. The implications of switching from chemically synthesized materials to utilization of green chemistry are compelling. Recent advancements in nanotechnology have impending applications in food, agriculture, oil and gas, aerospace, healthcare and many other industries. The use of these materials gained importance when it was analyzed that a substance's physiochemical qualities may be influenced by its size. It was then scientists grasped the importance of these materials. Nanoparticles have shown improved characteristics, such as their size, dimension, dispersion, distribution, and shape of particles. AgNPs were synthesized from *Pedicularis punctata* commonly known as Kashmir Lousewort. Various characterization techniques including Ultra visible spectroscopy (UV-VIS), Dynamic light scattering (DLS), Fourier Transformed Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM), X-Ray Diffraction (XRD) were used.UV –Vis spectroscopy showed highest peak t 430nm, which confirmed formation of AgNPs. XRD pattern confirmed crystalline structure of NPs with average size of 28.566nm.FTIR showed capping of AgNPs with phytochemicals present in *P.punctata,* which which act as reducing and capping agent. SEM revealed irregular shape in morphology with particle size approximately 30nm-60nm.HPLC fingerprinting was done for both *P.punctata* extract and silver nanoparticles which revealed the presence of phenyl glycosides mostly known for their anticancer activities. Furthermore, hemolysis activity confirmed, AgNPs were slightly haemolytic with 4.55±0.44% hemolysis which is considered as safe. The brine shrimp lethality assay of AgNPs showed LC50 value of 74±7.38ug/ml which shows they are moderately toxic. Anticancer activity was tested using ROS/RNS, Caspase 3 gene expression, mitochondrial membrane potential and MTT assay. All the activities confirmed silver nanoparticles have high anticancer activities. This could be used for cancer therapy and drug delivery agents. Further studies can help us understand the therapeutic effect of silver nanoparticles on animal models.

1. Introduction

Nanotechnology is one of the most promising technologies of 21st century that has gained importance in life sciences. The reason is their physiochemical properties which have impending applications. Richard Feynman provided the understanding of nanotechnology in 1959. He presented a lecture entitled ―There's Plenty of Room at the Bottom" where he introduced a new idea to reduce materials into nano size. After the discovery of this new field two approaches ―Bottom up" and ―Top down" were introduced to synthesize nanostructures (1). Among different types of nanoparticles are carbon-based nanoparticles, magnetic nanoparticles, metallic nanoparticles, semiconductor nanoparticles, dendrimers and lipid nanoparticles (2) . These are used in various industries like food industry, which improves shelf life of food products and improves mechanical barriers ,cosmetic industry using them for production of sunscreens providing better UV protection and deeper sun protection, fabric industry producing stain-resistant clothing, automobile industry providing lighter and stronger body and improving fuel consumption efficiency, IT industry using it in computing and electronics, providing much faster, miniature, and more portable systems that can easily manage and store a large set of data and healthcare industry mainly using it to explore useful purposes in pharmaceuticals, diagnostic, magnetic resonance imaging (MRI), in drug delivery, and various other medical instruments and techniques (3) .

Metallic silver was traditionally being used as silverwares to store food. Silver nitrate being used in surgery to clean wounds and colloidal silver was used to treat mental illness, stomach ulcers, epilepsy, gastroenteritis, and various infections, like syphilis and gonorrhea (4). Ag nanoparticles have gained enormous interest due to their unique characteristics like optical, electrical ,thermal, and biological applications (5). They are increasingly being used in health care, medical, cosmetic and food industry. They have unique antibacterial characteristics, bind to DNA and efficiently disrupt the cell replication and combat antibiotic resistance. Silver nanoparticles based filters that prevent biofilm colonization are commonly used for water purification (6).Silver nanoparticles possess broad spectrum antimicrobial properties. They act as antibacterial by releasing Ag + ion that can interact with sulphur and phosphorus of the cell wall

causing it to form pits and eventually burst. Various pathogenic bacteria, like *Escherichia coli, Klebsiella pneumonia* and *Staphylococcus aureus;* (ii) some fungi, such as *Candida albicans* and *Aspergillus niger* (iii) viruses, such as Hepatitis B and human immunodeficiency virus (HIV) can easily be killed at very low concentrations .It also possess cancerous properties by interacting thiol group and binding to NADPH dehydrogenase enzyme liberating ROS and interacting with respiratory enzymes thus damaging ATP formation and respiratory cycle of the cell. The anticancer properties of silver nanoparticles are studied against breast cancer , liver cancer, lung cancer, nasopharyngeal carcinoma, glioblastoma, and prostate cancer (7) .

Various methods like physical, chemical and biological have been used to synthesize nanoparticles. Chemical and physical methods include lithography, electrochemical method, and sonochemical and laser ablation (8). These methods have shortcomings due to their requirements which are high pressure and temperature, imbalance in size distribution and use of toxic chemicals. Taking into account the toxicity and environmental concerns associated with the synthesis of metal nanoparticles, various biological routes can be used such as bacteria, fungi, or plants, in order to synthesize nanomaterials (or nanoparticles). Because bacteria are known to create a variety of inorganic elements either intracellular or extracellular, they are powerful bio factories for synthesizing metal nanoparticles like silver and gold.

Plant based synthetic approach can effectively reduce and stabilize the nanoparticles. Plants have intrinsic organic processes to convert inorganic metal ions in the form of nanoparticles. The phytochemicals like terpenoids, alkaloids, saponins, phenols and alcohols carry out reduction processes. The proposed mechanism behind their synthesis includes short incubation period, growth phase and termination period. Despite the fact that biomolecules involved in a process, including concurrent reduction and capping are hypothesized in various studies, there is a severe shortage of proper plant extract characterization in line with the common phytoconstituents, which makes it extremely difficult to understand the relationships between production, morphology, and plant extract components. Green synthesis techniques are conditioned to use biodegradable, well-dispersed and bio soluble particles which are more acceptable for reducing, stabilizing, or capping. Surfactants such as CTAB and SDS, polymers like PVP, PEG, and PGA, and thiols like dodecanethiol and thioglycerol are used as chemicals for capping in the majority of nanoparticle manufacturing techniques (9). These compounds

interact strongly with the metal nanoparticle surfaces and are hence effective stabilizing agents. However, it is important to note that these chemicals are difficult to remove off nanoparticle surfaces, are not biodegradable, and some are harmful to biological systems. Green synthesis concentrates majorly on naturally occurring reducing and stabilizing substances such as carbohydrates, proteins, amino acids, lipids, nucleic acids (DNA) and biological extracts (plant, bacterial, viral, and fungal extracts).

Pedicularis punctata commonly known as Kashmir Lousewort belongs to family Orobanchaceae.600 species of *Pedicularis* have been reported. They are found in Asia, Europe, and North America. The phytochemicals present in Pedicularis sp. are majorly phenols, flavonoids, iridoids, alkaloids and, phenylethanoids. Due to the secondary metabolites of these phytochemicals, they protect against inflammation, act as antioxidant, antibacterial, antifungal, antipyretic, diuretic, antidiabetic, antihemolysis ,anticancer activities, antithrombus, protect liver, relax muscles and repair DNA and neurons (10).

The current study is focused to synthesize Ag nanoparticles using Pedicularis *punctata*. The characterization was done through UV, DLS, XRD, SEM, FTIR, and EDX. This was done to confirm particles size, morphology and functional groups attached that play role in their reduction and capping. The FTIR analysis can depict the functional groups associated with nanoparticles but chromatographic techniques could provide more accurate information on the compounds involved in the process and capping of nanoparticles. For this purpose, HPLC fingerprinting was done to study the phytochemicals present in extract and capping agents of silver nanoparticles. In order to study biomedical applications various assay were done to analyze the anticancer properties of silver nanoparticles along with important phytochemicals that helps in their capping and stabilizing.

1.1. Aims

Nanoparticles are being explored in medicines for their multiple uses. From solving limitations of conventional cancer treatment to overcoming multidrug resistance they are being explored in cancer therapy. In order to overcome the potential side effects of chemically synthesized drugs green synthesized nanoparticles can provide a better alternative. The major purpose of our study is to biosynthesize silver nanoparticles using *Pedicularis punctata*.

Synthesized nanoparticles are aimed to get identified using characterization techniques. A comparison of anticancer activities between *P.punctata* extract and silver nanoparticles are also studied and they are also explored for their biocompatibility.

1.2. Objectives

The study has the following objectives:

- Green synthesis of Silver nanoparticles using *Pedicularis Punctata.*
- Characterization of *P.punctata* based biosynthesized silver nanoparticles using UV-spectrophotometer, XRD, SEM and DLS to confirm their synthesis, crystal structure, morphology and size respectively .EDS was done to identify the elemental composition of silver and FTIR to study the functional groups attached with silver nanoparticles.
- HPLC fingerprint analysis of *P.punctata* extract and silver nanoparticles to study the specificity and composition of these phytochemicals and their medicinal value.
- Evaluation of anticancer activities on HepG2 by studying mitochondrial membrane potential, caspapse3 gene expression and caspase 3/7 gene activity, viability and ROS/RNS production
- To Study biosafety of silver nanoparticles using a commonly used brine shrimp lethality assay and RBCs hemolysis.

2. Literature review

2.1. Nanotechnology

Nanotechnology, which deals with the synthesizing, manipulating, and designing particles and modifies their structures from around 1-100 nm, is one of the most significant fields in current science. This is done by utilizing the unique properties of nanoparticles relative to material in bulk. The development of other technologies, many of which have received far greater attention, is intertwined with nanotechnology. Thus, there is an increased pressure to commercialize this technology. With the emergence of diversity in their production environmental scientists are also challenged by toxicological potential of these nanoparticles (11). Biogenic method for reduction of metal precursors has more advantages for biological applications where the safety of NPs is crucial, environmentally benign, less expensive, and free of chemical impurities. Biogenic reduction is a "Bottom Up" strategy which utilizes extracts of a natural substance for stabilization, growth termination, and capping qualities. Additionally, the size and morphology of nanoparticles are also influenced by the concentrations of various reducing agents (12).

2.2. Nanoparticles

Nanoparticles are synthesized by manipulating the matter between 1 and 100nm in size having an extraordinary capability to work at molecular level to produce enormous structures with novel molecular organization. Some unique properties of nanoparticles are also dependent on large surface area, their electronic activity, are mobile in free state, have quantum effect and interaction to environment (13).The composition of nanoparticles mainly depends upon its applications. The surface coating is critical in their synthesis since many nanoparticles lose their unique properties as soon as they are aggregated and precipitated from suspension. Thus, coating material must facilitate dispersion. Consequently, a nanoparticle can be divided into two or three layers: a core, which may or may not be present, a surface, which is frequently coated, and a shell material. Frequently, nanoparticles are only identified by their core since it is crucial for its applications .The small size of nanoparticles suggest that they should form stable dispersions as they are significantly stable to Brownian motion (14). This, however, would disregard the nanoparticles' high surface energy. The nanoparticles will aggregate and precipitate out of

solution after any collision between two particles. In order to prevent two particles from coming too near to one another, it is important to stabilize the dispersion of the nanoparticles. This can either be done by providing charge and some solvent molecules tightly bound to surface. Like charges cause repulsion and thus prevent aggregation. Another method in which a moderately lengthy molecule is linked to the surface of the particle. The lengthy chain will have a strong affinity for the solvent. Therefore, the relative interactions between the polymer chain and the solvent are a barrier to aggregation. Solvent between the two particles and around the chains must be removed in order for the particles to coalesce (15).

2.3. Classification of nanoparticles

2.3.1. Based on origin

On the basis of origin nanoparticles are divided into natural, incidental and engineered. Natural nanoparticles originate in nature like minerals, sea, dust storms, volcanic ash, forest fires, and space. These are the byproduct of anthropogenic activities like power production, motor exhaust and cigarette smoke. On the other hand, engineered nanoparticles are produced by wide range of chemicals, equipment and technologies.

2.3.2. Based on dimensions

Nanoparticles are classified into four types based on dimensions. Zero-dimensional nanoparticles have all of their dimensions at nano-scale. Quantum dots (QD) are considered among the most commonly used as they have the highest level of quantum captivity. One-dimensional nanoparticles have one of their dimensions outside of the nano size range and two of them within this range. These particles serve as one of the main building elements for the creation of nanowires, nanotubes, and nanorods etc. 2D nanoparticles have their two dimensions outside nanometric size and are used in thin films, Nano sheets, or Nano coatings. One well-known example of these nanoparticles is carbon nanotubes (CNT). They consist of a 2D sheet of rolled graphite having tubular structure. Three-dimensional nanoparticles show their nanoscale feature internally but none of them are at nanoscale. Fullerene and dendrimers are examples of 3D nanoparticles (16).

2.3.3. Based on chemical composition

Nanomaterials are divided into four types; (1) inorganic-based nanomaterials which include metals like gold, aluminum, iron, copper oxide, silver, platinum etc. (2) Carbon-based nanomaterials like graphene, fullerene, and carbon fiber. (3) Organic-based nanomaterials like dendrimers, liposomes, and micelles and (4) Composite-based nanomaterials which are mixture of various nanomaterials and these showing complications in their structures to form metal-organic framework. The most often utilized nanoparticles among the many are titanium dioxide, carbon nanotubes, silica, copper, clay, and aluminium oxide in the construction industry (17). Silver, titanium dioxide, copper oxide, magnesium oxide nanoparticles , carbon nanotubes, etc. are a few of them utilized in package components that interact with active packaging and extends the shelf life of products(18). Nanoparticles used in drug delivery system are liposomes, micelles, polymeric nanoparticles (19) .These organic NPs are attractive delivery system for molecules, especially for drug delivery and biomedical applications since they can load molecules either via conjugation on the surface or in the core, or by physical encapsulation. The inorganic metal oxide nanoparticles like titanium dioxide (TiO2), copper oxide (CuO), calcium oxide (CaO), iron oxide (MgO) etc are used in antimicrobial applications .Inorganic nanoparticles gained more attention compared to organic materials being non-toxic, hydrophilic, biocompatible, and extremely stable. (16)

2.4. Approaches for nanoparticles synthesis

Two different fundamental principles for synthesis of nanoparticles have been investigated. Top-down approach involves converting bulk material to nanometer scale while in Bottom-up approach smaller molecules are clustered in macro scale range without effecting their original properties. It‘s interesting to note that altering chemical concentrations and reaction circumstances can change the morphological characteristics of nanoparticles, such as their size and form (e.g., temperature and pH).

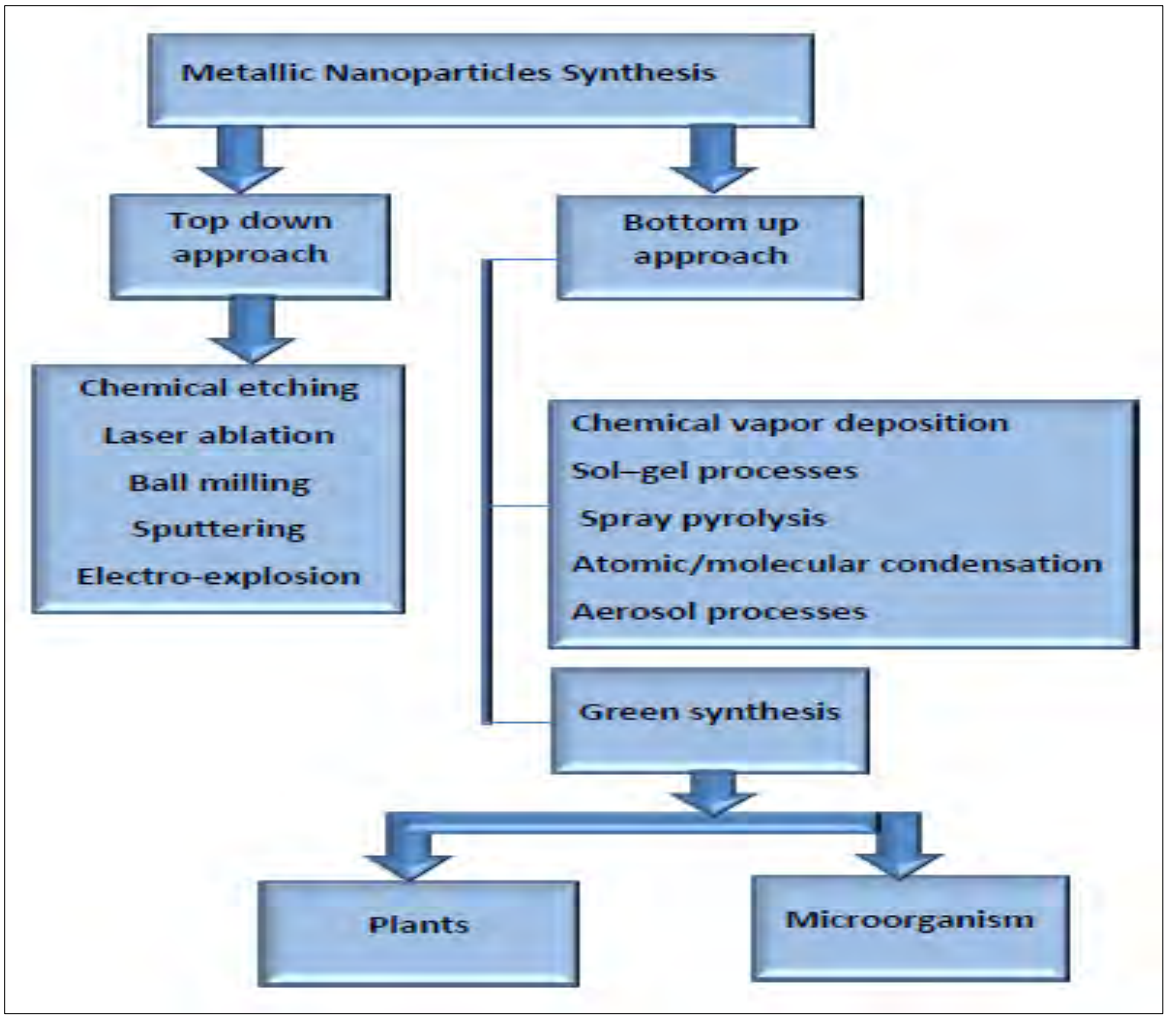


Figure 1:Approaches for nanoparticles synthesis

2.5. Methods for production of silver nanoparticles

2.5.1. Physical methods

Physical method involves the harness of mechanical pressure like laser ablation, evaporation condensation and high energy ball milling. Laser ablation and evaporation-condensation are commonly used methods for synthesizing silver nanoparticles. These methods are preferred over other chemical method which take place without solvent contaminants and the uniform distribution of nanoparticles. The efficiency of this method depends on various factors like its wavelength when it strikes a metallic target, the length of time laser pulses strike, ablation time, and the composition of the effective liquid medium (20).

2.5.2. Chemical methods

The chemical method for synthesizing silver NPs occurs through chemical reduction with organic and inorganic reducing agents. Silver ion is reduced by some of the reducers such as sodium citrate, ascorbate and sodium borohydride. Various stabilizers are being used as protective agents to avoid agglomeration. Poly vinyl alcohol, poly vinylpyrrolidone, surfactants and polymethylmethacrylate act as effective protective agents for stabilizing NPs.

2.5.3. Biological methods

Biological methods for production of nanoparticles are much cheap, cost effective and less toxic. Their size can be monitored by changing various conditions.

Table 1:Biogenic silver nanoparticles synthesis from bacteria,fungi (21)**,algae** (8) **and plants** (21)**.**

Bacteria	Fungi	Algae	Plant
Bacillus licheniformis	*Cladosporium cladosporioides*	*Spirulina platensis*	*Moringa oleifera*
Pseudomonas stutzeri AG259	*Penicillium fellutanum*	*Chaetoceros calcitrans*,	*Zingiber officinale*
Proteus mirabilis PTCC 1710	*Fusarium oxysporum*	*Chlorella salina*,	*Cymbopogon citratus*
Klebsiella pneumonia	*Aspergillus fumigatus*	*Isochrysis galbana*	*Scabiosa atropurpurea subsp. maritima*
Enterobacter cloacae	*Verticillium*	*Tetraselmis gracilis*	*Coccinia indica*

2.6.Factors affecting silver nanoparticles synthesis

The factors affecting silver nanoparticles synthesis are discussed below:

2.6.1.Plant species:

Different plant species have different abilities to synthesize AgNPs, some of them are more efficient than others.This is due to phytochemicals present in them which reduce nanoparticles.

2.6.2.Effect of concentration:

The concentration of the silver ion can affect the rate and efficiency of AgNP synthesis.Various studies report 10^{-3} M as most acceptable concentration which shows much better surface plasmon resonance.

2.6.3. pH:

The pH during the synthesis reaction of mixture can affect the stability and properties of the AgNPs synthesized. Studies on silver nanoparticles synthesis favors the basic medium for better production.

2.6.4.Temperature:

The temperature of the reaction can affect the rate of AgNPs synthesis. With elevation in the temperature rate of reaction also increases causing more collisions.

2.6.5.Contact time:

Reaction time also influence the formation of silver nanoparticles. By increasing the time of contact Plasmon band formation also increases as a large concentration of Ag+ are converted to Ag^0.However with increasing this contact time further it may lead to aggregation causing increase in particle size (22).The rate of agitation also plays very significant role as it can change the rate of nucleation and growth of AgNPs.

2.6.6.Light exposure:

Light exposure can affect the rate of AgNP synthesis, as some plant extracts contain phytochemicals that are photo-sensitive.

2.7. Applications of Silver nanoparticles

2.7.1. Antibacterial properties

The antibacterial effect of AgNPs on various pathogens have been reported in literature. It has been noted that they show stronger effect on gram negative bacteria rather than on gram positive bacteria which might be due to difference of cell wall thickness. Moreover, other factors like charge, size and concentration also contribute towards the enhanced antibacterial effects. Various hypothesis suggests that silver nanoparticles exhibit antibacterial activity by destructing bacterial membrane and leaking cellular content, the generation of reactive oxygen species, disrupting DNA structure and preventing DNA replication or destabilizing proteins and inactivating enzymes. The outer membrane of Gram-negative bacteria possesses water-filled channel called as porin that allows AgNPs to enter bacterial cells. The main function of porin is to passively move hydrophilic molecules of different size and charge across the membrane. According to a hypothesis, the strength of silver nanoparticles to make attachment with the cell wall of bacterial is caused by the electrostatic forces between positive charge of silver ions and the negative charge of surface of the cell membrane due to the presence of carboxyl, phosphate, and amino groups. This electrostatic interaction allows silver nanoparticles to penetrate the cell membrane, changing its structural makeup and increasing its permeability. The disintegration of the membrane then follows the dissipation of the proton motive force (PMF) causing membrane destruction (23). Silver ions produce ROS and cellular oxidative stress by interacting with disulfide bonds of enzymes which are responsible for cellular metabolism and thiol groups. AgNPs are being used in dentistry, pathogens like *Streptococcus mutants, Escherichia coli and Staphylococcus aureus* have the ability to colonize acrylic materials responsible for dental infections. AgNPs can be incorporated to acrylic resin that inhibits the growth of such bacteria (24). Silver nanoparticles are also explored for their capacity to restrain the growth of multidrug-resistant strains.

In a recent study using AgNPs along with other drugs including kanamycin, ampicillin, and chloramphenicol, observation was made that the combination of AgNPs + chloramphenicol reduced the growth of S. aureus, E. coli, and S. typhimurium by up to 50% (25). The same strains' growth was simultaneously inhibited by treatment with AgNPs + kanamycin, by nearly 95% effectiveness. Their combined effect was more efficient. AgNPs change the membrane potential and integrity, increasing permeability and improving antibiotic resistance. Another study explains the synergistic effect of AgNPs and antibiotics, and in this experiment the ampicillin (Amp) was used as a reducing and capping agent. Amp-AgNPs were tested for their

antibacterial abilities against drug-sensitive and drug-resistant Gram-positive and Gram-negative bacteria. According to their MIC values, the Amp-AgNPs were in every instance more efficacious compared to ampicillin or chemically produced AgNPs (26).

2.7.2. Antifungal properties

AgNPs exhibit antifungal properties by inhibiting fungal spore germination, disrupting cell wall and producing reactive oxygen species. Silver nanoparticles engage with thiol-containing proteins present in the cell wall forming complexes with electron donors resulting in the deactivation of membrane-bound enzymes and proteins (27).The antifungal activity of silver nanoparticles was reported against Candida specie. A comparison between the antifungal and the antibacterial activity of the silver NPs shows lower activity (28). Another study finds significant activity of environmental friendly silver nanoparticles on C. albicans and C. tropicalis using ribose and SDS as stabilizers. (29). A very recent study on antifungal activity of green synthesized AgNPs against *C. albican.*, *C. glabrata*, *C. parapsilosis*, *C. tropicales*, and *C. krusei* reports inhibition zone ranging from 14-22 mm (30).

2.7.3. Anticancer properties

Silver nanoparticles exhibit anticancer properties causing ROS to be produced and cell damage. They can promote the apoptosis by accelerating or slowing down the expression of important genes and regulating essential signaling pathways. Additionally, AgNPs can lessen distant metastasis by preventing the migration and angiogenesis of tumor cells. AgNPs causes glutathione to oxidize and raises the level of lipid peroxidation in cellular membranes, causing cytoplasmic constituents to leak from wrecked cells. The activation of p53, caspase-3 and p-Er K1/2 cause apoptosis and regulate cell division. The mode of action of AgNPs on the breast cancer cell line was studied. Various morphological changes on cytoplasm ,vacuole and chromatin inhibitory action on metalloproteinase (MMPs) were observed (31).Cancer cells also show more permeation and retention (EPR) effect leading to more nano silver and generating cytotoxic silver ions . The Vascular endothelial growth factor (vegf) also promotes angiogenesis (32).

2.7.4. Antidiabetic properties

AgNPs have beneficial effect on diabetes by improving insulin sensitivity and glucose metabolism. The antidiabetic ability of AgNPs is tested against digesting enzymes for carbohydrates including glucosidase and amylase. These carbohydrate digestive enzymes are liable for the breaking down the oligosaccharides and disaccharides into monosaccharides. Their inhibition helps to treat non-insulin diabetes which reduces the rate at which blood glucose is released. The enzymatic activity level is reduced with increasing concentration of AgNPs (33).It is mandatory to do more research inorder to fully understand the antidiabetic properties of AgNPs.

2.7.5. In drug delivery

With the advancement in medical field, chemotherapy is still challenging due to various side effects. The unique interaction of silver nanoparticles with biomolecules provides their role in drug delivery. Ag nanoparticles provide alternative due to their controlled size and increased reactivity. Controlling the release of biologically active silver can be regulated by coating with ligands and increasing half-life in circulatory system. It has been reported that silver nanoparticles on contacting biological fluids adsorb the proteinaceous coat on their surface, providing stability and establishes the biocompatibility of nanoparticles and controls how the nanoparticle-corona combination interacts with biological macromolecules. According to reports, chemically synthesized AgNPs cause intracellular proteins to exhibit structural abnormalities as a result of a fragile surface that easily oxidizes under physiological settings, manifesting many harmful effects like oxidative stress. When AgNPs are internalized into cells, it may cause losing the protective surfaces they had, weakly retain their functionalities, and form a protein corona on the periphery of the cell that can completely obscures their surface and causes an intracellular aggregation, impairing their distinctive properties. The functionalization of nanoparticles help regulate the medicines for sale are made available that would otherwise have a limited bioavailability profile (34). The bio-conjugated AgNPs offer several demands for overcoming complex clinical complexities that are normally time-consuming to handle by the conventional methods. The commonly used peptides for drug delivery of AgNPs are cell-penetrating TAT (trans-activating transcriptional activator and TAT-like peptides) (35), RGD (arginylglycylaspartic acid) for adhesion of cells to the extracellular matrix (36), and cell-penetrating pep-I peptide (37).

2.8. Nanotoxicology

Nanotoxicology research aims to understand the potential hazards of nanomaterials, as well as to develop strategies for safe design, production, and use of these materials. The miniature size and large surface to volume ratio of silver nanoparticles exhibit their high reactivity and tendency to cross some biological barriers. In addition, they can pose serious environmental and health risks. Various studies report the health risk of inhaling nanoparticles cause respiratory problems, in humans may cause gray discoloration of the skin (argyria) and can also cause mutation in DNA. AgNPs caused morphological changes in A549 cells causing shrinkage of cell, cell extensions, a very restricted spreading, and ultimately cell death (38).Hydrocarbon-coated AgNPs caused cellular changes in alveolar macrophage cell line in rat model (39) .Another study reports shrinkage of cells due to uncoated particles in macrophage cell line ,whereas PVP-coated particles caused cell elongation (40). Oral exposure to AgNPs in BALB /C mice showed a significant increase of ALT, AST, and hepatotoxicity (41). AgNPs penetrate porcine skin where it is localized on the surface and in the higher stratum corneum layers, causing irritation. (42).Oral administration of PVP-AgNPs with size 30 nm in an obese mice indicated the development of fatty liver disease (43). Negative effects were found in a study on the effects of AgNPs size ranging 20–100 nm treating adult and immature Drosophila fly in terms of survival, lifespan, size of ovary, and egg-laying capacity (44). Ag-NPs may have a concentration-dependent impact on health and the environment. A study conducted on embryos of zebra fish that had been exposed to Ag-NP had phenotypic flaws, changed physiological processes, including bradycardia, axial curvatures, and body part degeneration. Nanoparticle clusters were visible throughout the larvae's epidermis, supporting the skin abnormalities brought on by the Ag-NP. The slimy coating is a result of nanoparticle penetration via the embryos damaged skin. Additionally, exposure to AgNPs caused blood to accumulate in various areas of the body, leading to edema and necrosis (45). The harmful impact of green synthesized *Stenocereus queretaroensis* based AgNPs in short-term, did not have any negative effects in the in vitro and in vivo models, However, more research is needed to understand long-term exposure (46). The combination of green synthesis and nanotoxicology can be an effective way to produce safe and sustainable nanoparticles.

2.9. *Pedicularis punctate*

Pedicualris plant, commonly called Kashmiri lousewort is found in Asia, North America and Europe. Approximately 600 species of pedicularis are reported, some of them being *Pedicularis longiflora Rudolph, P. bicornuta Klotzsch, P. oederi Vahl, P. cheilanthifolia, and P. pectinata*. *Pedicularis punctata* commonly known as Kashmiri lousewort is a perennial herb located at an altitude of 2700-4500 m in the Himalayas, from Pakistan to Kashmir. Its flowers are pinkish red in color, a white spot in throat, with a long slender flower tube. Flowers have a bifid beak that curves before abruptly enlarging into a base like a knee. With pinnately lobed bracts, the flowers are produced in somewhat dense short spikes. The shape of the leaves ranges from oblong to elliptic, with rather broad, ovate, coarsely serrated lobes. Typically, the plant contains numerous 10–25 cm tall stems. Plant flowers mostly from July to September. The phytochemicals reported in Pedicularis plants are majorly polyphenols, flavonoids, iridoids, lignin and alkaloids (10). Various medicinal plant species of this genus have been demonstrated to be helpful in treating a wide range of conditions, like fever, cold, cough, debility, fatigue, urinary tract infections, leucorrhoea, improving blood flow, nausea, digestive and reproductive issues, measles, severe hepatitis, rheumatism, paralysis, malignant sores, and sterility.

Figure 2.*Pedicularis punctata*

Various studies on Pedicularis specie report anti-inflammatory, anti-bacterial, anti-fungal, antipyretic, anticancer, antihemolytic, antioxidant, immunomodulatory, hepatoprotective, analgesic, diuretic, and muscle-relaxant action (10).Pedicularis plant show antioxidant potential.

Antioxidant properties of phG in Pedicularis is reported (47). The anti-inflammatory properties of Pedicularis plants are a result of the immunomodulatory actions of flavonoids, iridoids, glycosides, monoterpenoids, phenolics, and triterpenoids.

Phenylethanoid glycosides belong to group of water-soluble compounds mostly found in ayurvedic medicines. PhGs provide neural protection, treat heart conditions, such as congestive heart failure, atrial fibrillation, and supraventricular anti-inflammation. PhGs possess poor bioavailability due to their poor absorption through the intestinal epithelial membrane and breakdown by enzymes in the GIT due to hydrophilic nature. The metabolic studies of PhGs suggest they can act as prodrugs, can easily be broken down in vivo (48).

Table 2. Taxonomy of *Pedicularis punctata*

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Laminales
Family	Orobanchacae
Genus	Pedicularis
Species	*Pedicularis punctata*

3.Materials and Methods

3.1. Plant extract Preparation

Pedicularis punctata was collected from mountains of district Shangla, KPK at an altitude of 7000ft. Whole plant was washed properly with water and then kept for drying in shade for 4 weeks. 40g of dried leaves were grinded in a grinder to form powder.500ml of distilled water was added and boiled for about three hour at 70°C.Extract was then cooled at room temperature and filtered twice through Whatman filter paper and then supernant was kept in refrigerator and stored at 4°C.

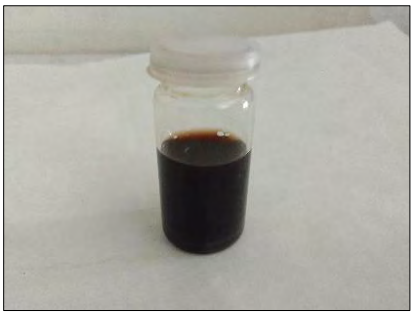


Figure 3:*Pedicularis punctata* plant extract

3.2. Silver nanoparticles salt synthesis

1 mM AgNO3 was prepared by weighing out 33.8mg of AgNO3 in 200ml of distilled water. After dissolving the solution was constantly stirred for 30 minutes at room temperature.

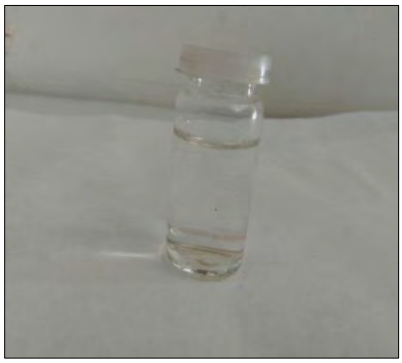


Figure 4 :Silver nitrate salt

3.3. Silver nanoparticles synthesis

1mM AgNO3 solution was prepared and added to plant extract drop wise in 1:5 ratio. The solution was constantly stirred at 70°C for 3 hours. The color change was observed reducing silver into silver nanoparticles. The solution was placed in dark overnight. The Ag nanoparticles were obtained by centrifugation at 3000 rpm in three rounds. The supernant was discarded each time and pellet washed with distilled water. The final pellet containing Ag nanoparticles was poured into petri plate and dried in incubator at 40°C.The powder obtained was grinded in pestle and mortar to obtain fine particles. The obtained Ag nanoparticles were then used to study characterization and biological applications.

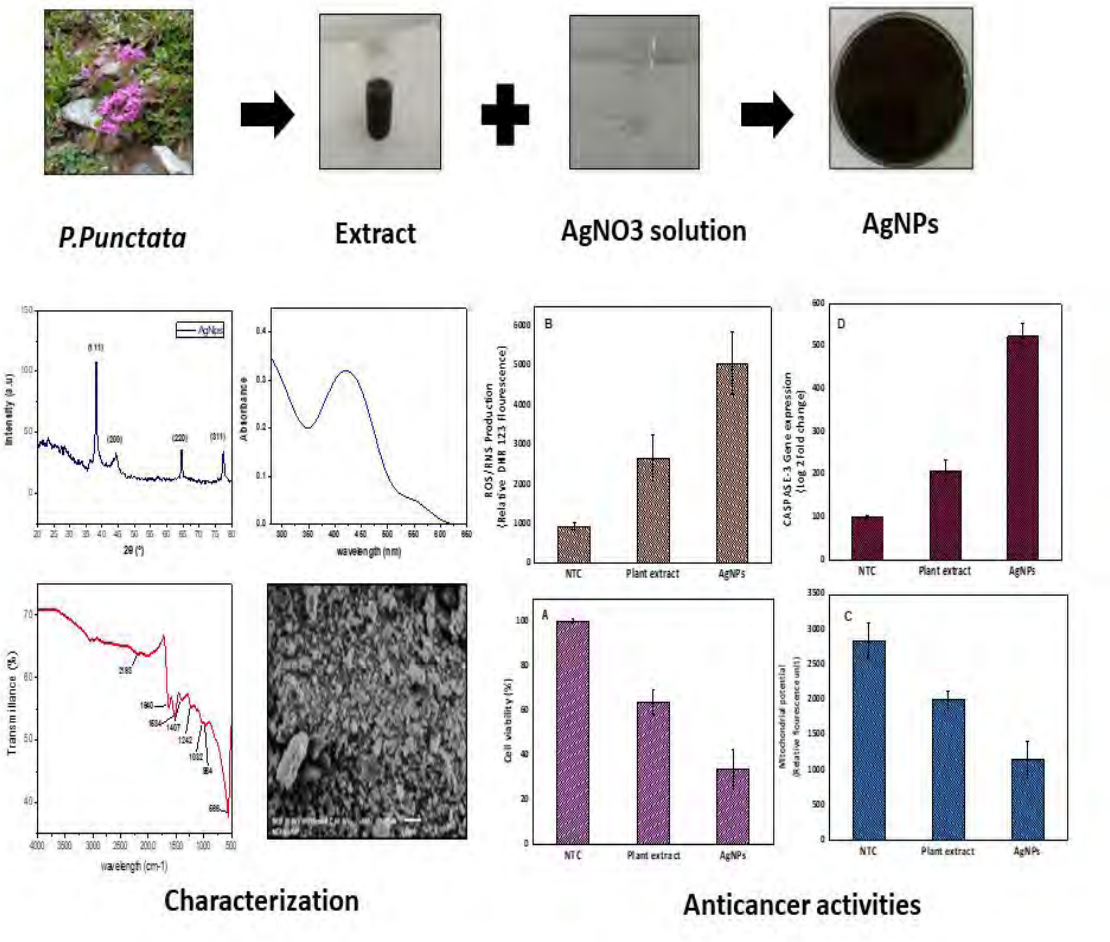


Figure 5:Graphical representation for synthesis, characterization and biological applications of AgNPs from *Pedicularis punctata* plant extract

3.4. Characterization

The characterization of AgNPs was done through various techniques like UV Vis spectroscopy, XRD, FTIR, DLS, EDX and SEM.

3.4.1. UV Visible spectroscopy

UV visible spectroscopy analyzes how much silver nanoparticles absorbs light. This is done by measuring intensity of light that passes through silver nanoparticles with respect to the reference sample or blank. The absorption spectra of AgNPs in the UV-visible region provides information about the size, shape, and their surface chemistry. Sample preparation involved dissolving 1mg of Ag nanoparticles in 1ml distilled water. The analysis was done by (HALO DB-20S UV-VIS Double Beam, Australia) at absorbance range of 200-800nm.

3.4.2. X rays Diffraction (XRD)

XRD is used to ascertain nanoparticle size and shape by analyzing the changes in positions of diffraction peaks. It confirms the crystalline nature of nanoparticles. Diffraction data was observed through CuKa radiation (wavelength,1.5406: voltage; 40kV and current 30mA). The calculation of crystalline silver nanoparticles was done using Sherrer equation.

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

D is the size of the particle

K is Scherer's constant (K=0.94)

λ is the X-ray wavelength (1.54178Å)

β is full width at half maximum (FWHM) of the diffraction peak

θ diffraction angle

3.4.3. Fourier Transform Infrared Spectroscopy

FTIR uses infrared (IR) light for excitation of the vibrational modes in the chemical bonds of sample, and the resulting absorption or emission spectra can be used for identification and measuring the different functional groups present. It identifies and characterizes unknown materials, detect contaminants, and identify decomposition and oxidation of material. FTIR is

also a non-destructive technique, making it useful for analyzing nanoparticles that are difficult to handle or that are sensitive to damage.

3.4.4. Scanning Electron microscope (SEM)

SEM functions to examine the surface morphology of single nanoparticles with size ranging below 100nm. When imaging nanoparticles with SEM, the sample is typically prepared by dispersing the particles in a liquid, such as water or ethanol, and then depositing them onto a substrate, such as a carbon-coated grid. The sample is then dried, and the grid is placed in the SEM chamber, where it is coated with a very thin layer of gold or another metal to make it conductive.

3.4.5. Energy Dispersive X-ray spectroscopy (EDX)

EDX is generally attached to SEM and works for elemental analysis of materials. Spatially resolved chemical data provided by EDX can be used to examine the direct assessment of nanoparticles (NP) size and morphology by a high-resolution, beam of electrons-based imaging techniques.

3.4.6. Dynamic light scattering (DLS)

DLS is based on the principle that small particles will scatter light differently than larger particles due to their different refractive indices. Dynamic light scattering measures zeta potential and particle size distribution using Zeta sizer Nano –ZS (Malvern Instrument UK). It measures size of small particles in solution when they bombard solvent molecules surrounding them.1mg silver nanoparticles was dissolved in 1ml of deionized water. The Z average measures the intensity weighted mean hydrodynamic size of the all the particles assessed by dynamic light scattering.

3.5. HPLC fingerprinting

HPLC analysis works on the principle of distribution of compounds between mobile and stationary phase. The HPLC analysis confirms the phytochemicals present in plant species HPLC fingerprint was established by choosing a chromatographic column with Agilent ZORBAX

Eclipse XDB-C_{18} .Methanol and 0.2% formic acid act as mobile phase. The rate of flow was 1mL/min and temperature of the column was 30°C with wavelength for detection as 254nm. In order to evaluate the similarity of *P.puncatata* extract and silver nanoparticles, Similarity Evaluation System of Chromatographic Fingerprint of Traditional Chinese Medicine was followed and chromatographic peaks were assessed on basis of fingerprint identification, and the outcomes were examined.

3.6. Cytotoxic assays

3.6.1. Brine shrimp assay:

In order to check the cytotoxic effect of AgNPs, Brine shrimp assay was performed. The cytotoxicity was studied against test organism *Artemia salina* (brine shrimp). For that objective, 96 well plate was used. In the first step, *A.salina* eggs were incubated for 24-48 hours in a plastic tray to hatch them. In the tray, eggs were supplied with 38 g L^{-1} of sterile sea water. In addition, 6 mg L^{-1} of dried yeast and sufficient oxygen was also supplied. Eventually, ten fully grown nauplii (phototropic) were obtained. 25–200 ug mL^{-1} of AgNPs were added into the well to raise the final volume equal to 300uL. 1% DMSO added in sea water is taken as negative control. While on the contrary, for positive control doxorubicin was taken. Table curve 2D v5.01 was used measure the Lethal concentration (LC50) of AgNPs with less than and equal to 50% mortality.

3.6.2. Hemolysis activity:

With written consent, blood samples from six healthy males and six females belonging to the age group of 28-35 years old were taken. Afterwards, hemo-compatibility between AgNPs and RBCs was assessed. Ethical standards were followed because this experiment involved human participant. After taking into consideration aforementioned conditions procedures on human subjects were executed. EDTA vacutainers were used to collect blood samples. This was done to avoid blood from clotting. After extraction was performed, in an Eppendorf tube erythrocyte and 100 uLAgNPs were added and incubated for 1 hour at 35 degrees Celsius. Later, It was then centrifuged for 10 minutes at 10,000 rpm. In due course, 96 well plate was used and 100 uL of supernatant was added in it. To estimate the release of hemoglobin, Bio Tek ELX800 Absorbance Microplate Reader (Bio Tek Instruments, France) was used. The data was collected

at 540 nm. For control, Triton X-100 as positive and DMSO as negative were taken. Following formula was used to state the results in % hemolysis;

$$\text{Hemolysis \%} = \frac{\text{Sample absorbance} - \text{Negative control}}{\text{Positive control absorbance} - \text{Negative control absorbance}} \times 100$$

3.7. Anticancer activities on HepG2 cells

3.7.1.MTT assay:

MTT assay was performed on human liver cancer cells, HepG2 (ATCC HB-8065; American Type Culture Collection, USA). This is done to check viability of HepG2 cell. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) dye was used for this purpose. Dulbecco's Modified Eagle Medium was utilized in order to cultivate HepG2 cells. Cells are plated in 96 well, 200 mg uL-1 AgNPs were added. That plate was pre-set with HepG2 cells with >90% viability; 200 mL per well; 1 x 104 cells per well and placed for 24 h. 10 mL MTT dye (5 mg mL^{-1}) was also added. It was then incubated for 3 hours. As a result of which, Insoluble formazan was formed. In order to dissolve insoluble formazan10% acidified sodium dodecyl sulfate was used. After incubating cells overnight, they were placed in microplate reader and absorbance was measured at 570nm. However, for control non-treated cells (NTC) were taken. Following equation used to represent cell viability in percentage;

$$\text{Viability \%} = \frac{\text{Sample absorbance - control absorbance}}{\text{NTC absorbance - media absorbance}} \times 100$$

3.7.2. Production of intracellular reactive oxygen and nitrogen species (ROS/RNS)

Using Dihydro rhodamine-123 (DHR-123) fluorescent dye the production level of intracellular ROS/RNS was measured as reported by Nazir et al (49). Pre-seeded HepG2 cells were set down on a 96-well plate, then they were rinsed twice with phosphate buffer saline (PBS). It was then suspended in the PBS that contain about 0.4 mM fluorescent DHR-123, and then the mixture was placed in dark for up to 10 min at 30 °C. In the end, fluorescence signals were measured (LEM ¼ 535 nm, lex ¼ 505 nm) using a Versa Fluor fluorometer (Bio-Rad, France).

3.7.3. Measurement of Mitochondrial membrane potential (MMP)

AgNPs resulted in the depletion of MMP which was detected by the method already reported by (50). HepG2 cells were placed for 40 min at 37 °C in a culture medium supplied with 25nM of 3,30-dihexyloxacarbocyanine iodide. Relative fluorescence units (RFU) were marked out as the measure of mitochondrial membrane potential.

3.7.4. Expression of the caspase-3 gene and caspase-3/7 activity

In order to determine the expression of caspase-3 gene in HepG2 cells, firstly, RNA extraction was accomplished using Gene JET RNA Purification Kit (Thermo Scientific) and the quantification was done by Quant-iT RNA Assay Kit (Invitrogen). The first strand of the cDNA synthesis kit was taken for performing reverse transcription of the RNA using the DyNAmo Color Flash SYBR Green qPCR Kit, and Piko Real quantitative PCR was carried out. The Caspase-3 primers used were: 50 -CACGCCATGTCATCATCAAC-30 (reverse primer) and 50 -TGTTTGTGTGCTTCTGAGCC-30 (forward primer) (amplicon size: 210 bp). Pikoreal, a bioinformatic tool was used to examine the data. By following the manufacturer's guide, the Apo-ONE Homogeneous Caspase-3/7 Assay kit was taken for measuring the activity of in vitro caspase-3/7. All data were performed three times.

4.Results and discussion

Pedicularis punctata was utilized for the first time to synthesize AgNPs. After mixing both silver nitrate solution and *P.punctata* extract, the change in colour was observed from brown to greenish grey. The mixture was placed in dark overnight and silver nanoparticles were obtained after centrifugation and then subsequently dried in incubator. They were covered with aluminium foil due to their sensitivity and stored for further analysis.

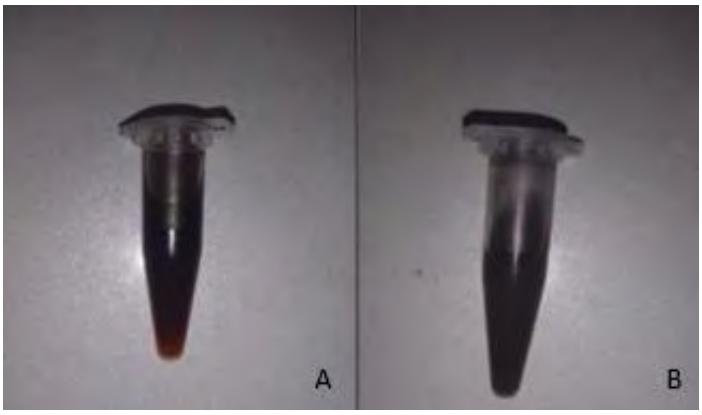


Figure 6:Colour change shows synthesis of silver nanoparticles(A)Brown colour extract (B) Greenish grey colour showing AgNPs synthesis

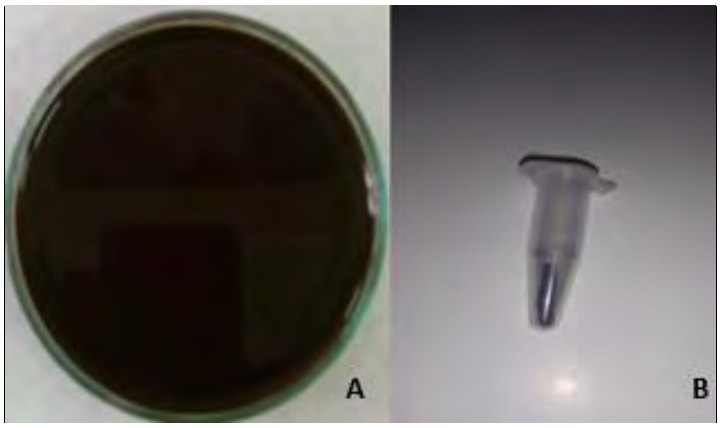


Figure 7:(A) Silver nanoparticles pellet (B) Powdered silver nanoparticles after incubation

4.1. Characterization

Characterization was carried out by UV, XRD, DLS, SEM, EDX and FTIR.

4.1.1. UV-Vis spectroscopy

UV vis spectroscopy was done to test the change in optical properties as a result of silver nanoparticles synthesis. The reduction of Ag^{+} caused colour change of extract. It was done against water as shown in figure7. UV vis spectroscopy shows peak between 400-460nm.The highest peak was observed at 430nm which confirms the silver nanoparticles synthesis. Our results were corresponding to earlier reports (51).

Figure 8:UV -Vis Spectroscopy of AgNPs

4.1.2.X-rays Diffraction (XRD)

Silver nanoparticles were analyzed using XRD at an angle of 2θ between 20° and 80°,which confirms the face-centered cubic structure comparing with standards (JCPDS, No. 04-0783).The major four peaks observed in XRD which were 38.1786°,44.457°,64.5584°and 77.4389° which indicate (111), (200), (220) and (311) lattice planes respectively .The results are in correspondence with previous reports (52). The average particle size of biosynthesized AgNPs

was calculated according to Debye-Scherrer equation. It was 28.566nm from FWHM of peaks. Same results were reported in previous studies (53).

Figure 9: XRD pattern of AgNPs

4.1.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR of silver nanoparticles was done to identify biomolecules in *Pedicularis punctata* extract responsible for reduction, capping and stabilization. It was done at range of 500-4000cm^{-1}. The major peaks were 566,984,1032,1242,1407,1534,1640 and 2193. The observed peak at 566cm^{-1}corresponds to alkyl halide. The changes in peaks at position in between 800-1500 belongs to aldehydes and ketones which are majorly the part of flavonoids, phenolic and lipid containing oils. Band between 1100-1350cm^{-1} belongs to =C-O stretching of carboxyl group of lipids, peak at 1242 correlates to C-N stretching of aromatic amines, l,407 C-C stretch of

aromatics,1534 and 1640 N-H bend of primary amines and 2193 corresponds to vibrations of aromatic amines with a C-N stretch .Our result match with previous reports (54). Hence the phenolic compounds majorly phenylethanoid glycosides are involved in the reduction of silver nanoparticles which is also confirmed by the HPLC analysis.

Figure 10: FTIR spectra of AgNPs

4.1.4.SEM

Morphology of silver nanoparticles was analyzed by SEM as shown in figure. Results show AgNPs with irregular shape from triangular to rectangular and smaller one showing spherical shape with clear boundaries between each particle. The average particle size calculated was 30-60nm. Larger particles may have been formed by agglomeration of smaller ones which might be to reduce surface energy. Our results are correlated with previous reports which also reports (55).Moreover the morphology of nanoparticles also depends on interaction of capping and stabilizing agents in extract .

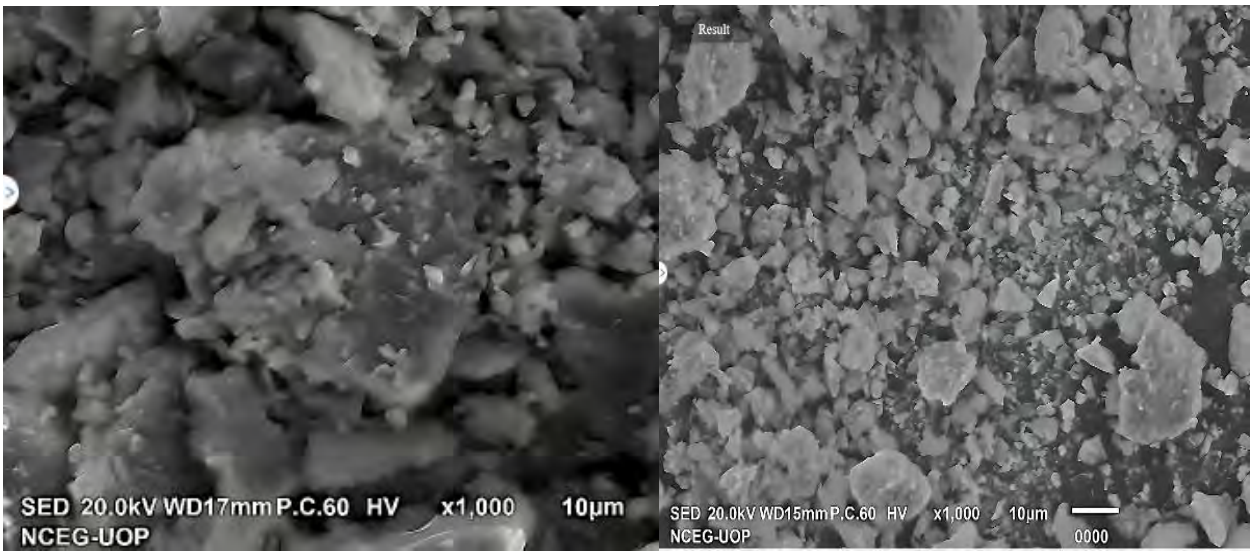
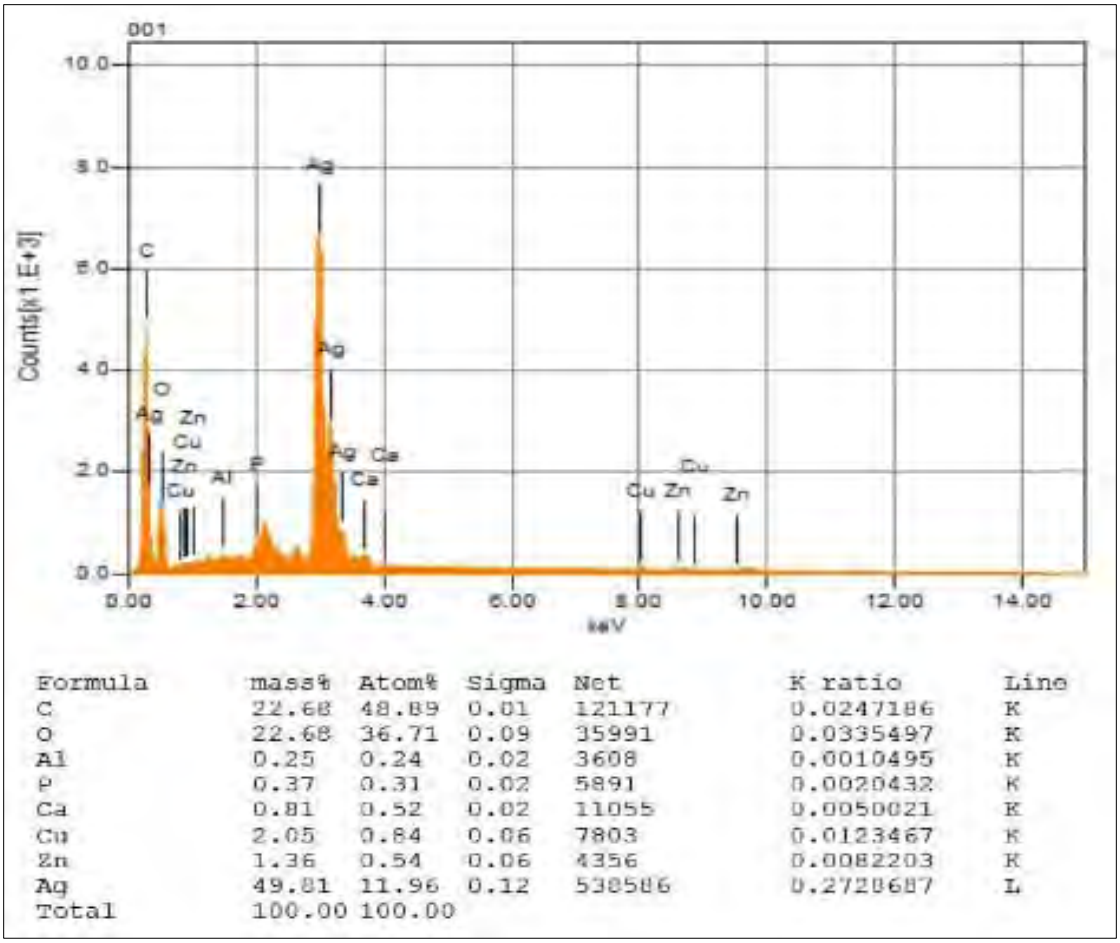


Figure 11: SEM analysis

4.1.5.EDX

EDX analysis shows elemental composition of AgNPs from *P.punctata*. EDX spectrum shows strong signal at 3KeV establishing Ag nanoparticles. Ag shows highest peak at 49.01%, C and O 22.68%, while other elements like Al, Cu, Zn, P, are also present in small amount. The presence of copper might be due to grid while the presence of the other elements maybe as a result of capping compounds on the nanoparticles' surface. Our results corresponds to already published data (56).



Formula	mass%	Atom%	Sigma	Net	K ratio	Line
C	22.68	48.89	0.01	121177	0.0247186	K
O	22.68	36.71	0.09	35991	0.0335497	K
Al	0.25	0.24	0.02	3608	0.0010495	K
P	0.37	0.31	0.02	5891	0.0020432	K
Ca	0.81	0.52	0.02	11055	0.0050021	K
Cu	2.05	0.84	0.06	7803	0.0123467	K
Zn	1.36	0.54	0.06	4356	0.0082203	K
Ag	49.81	11.96	0.12	538586	0.2720687	L
Total	100.00	100.00				

Figure 12: EDX spectrum of AgNPs

4.1.6. Dynamic light scattering

Silver nanoparticles made via green synthesis were tested for stability and surface charge using zeta potential. The average nanoparticles size measured was 171.6nm. The larger size of nanoparticles shows aggregation of some particles. Moreover, DLS also calculates the hydrodynamic size i.e, size and surface water molecules. Our results match with following literature (57). PdI of AgNPs was estimated to be 0.308. A PDI of 0.3 and less than this is considered acceptable and indicates homogeneity of particles (58) .The average zeta potential was estimated to be -2.09 mV which shows they are stable .Our results of zeta potential corresponds to already reported data (59).

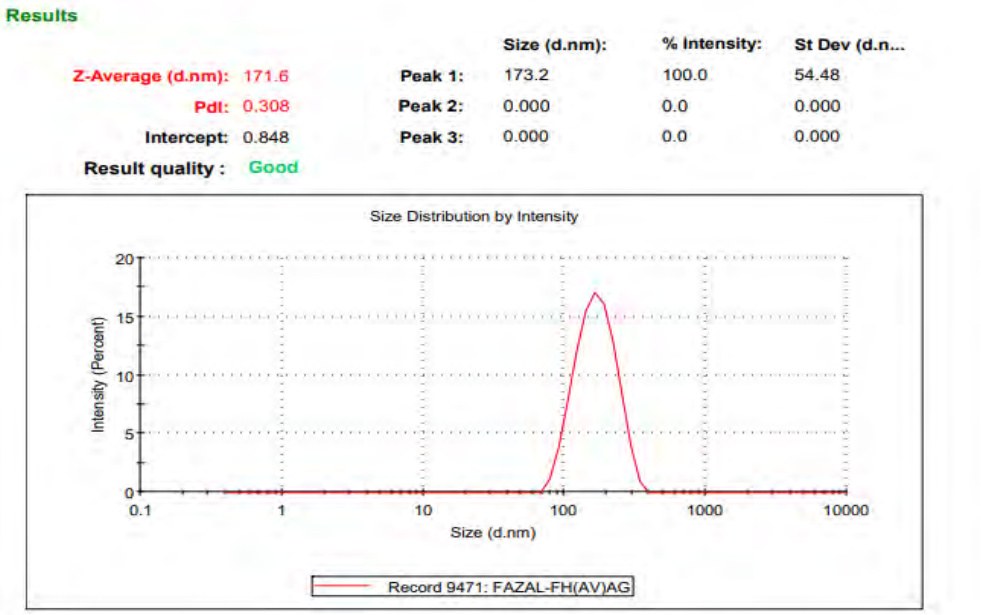


Figure 13: Zeta size of AgNPs

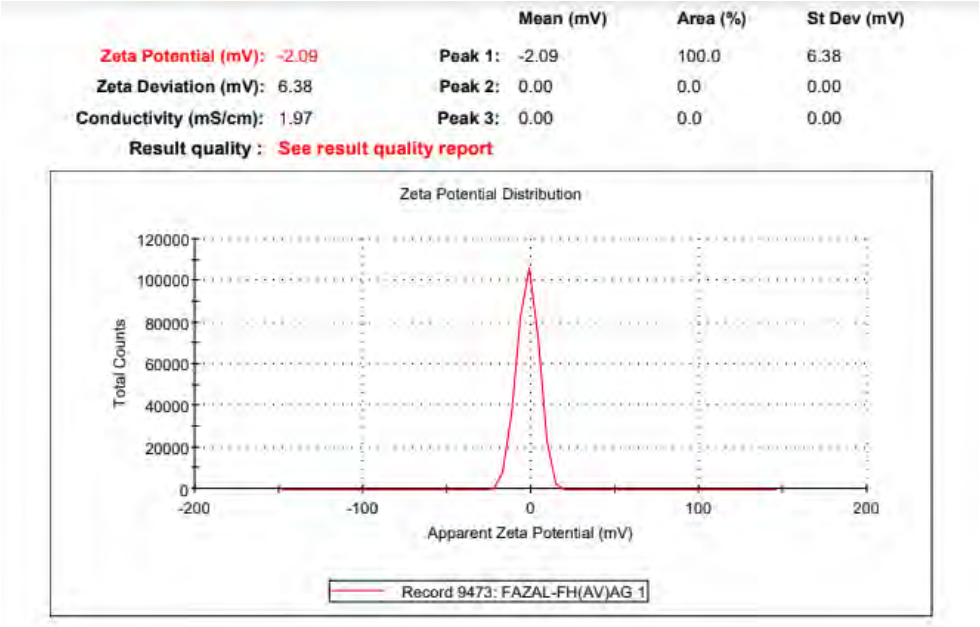


Figure 14:Zeta potential of AgNPs

4.2. HPLC fingerprinting

HPLC analysis was done to quantify phytochemicals in *P.Punctata* extract and AgNPs. Phenylethanoid glycosides are water soluble compounds. They are characterized by hydroxyphenylethyl moiety with glucopyranose linked through a glycosidic bond. HPLC analysis of *P.punctata* plant extract reported presence of phenylethanoid glycosides. HPLC fingerprinting revealed five chromatographic peaks, echinacoside (1), forsythoside B (2), verbacoside (3), isoverbacoside (4) and martynoside (5). Similar compounds were observed in AgNPs which indicate that these compounds might be present in nanoparticles capping. An interesting observation was made during the AgNPs analysis where verbacoside was not quantified and disappeared which might be due to its isomerization or bioconversion. The changes in composition and disappearance of phytochemicals also indicate specificity of capping agents. Echinacosides and forsythoside B were in higher amount in plant extract but isoverbacosides and martynoside were higher in AgNPs. Further studies should be carried out to determine the precise method of the synthesis and capping of nanoparticles. Moreover, phenyl glycosides have wide range of biological applications i.e. protect against various pathogens ,prevent inflammation ,prevent liver damage ,provide immunity and inhibit tyrosinase activity. (60). The anticancer and viability assays depict the useful functions of *P.punctata.*Following are the major phytochemicals isolated;

4.2.1. Echinacosides

Echinacosides (ECH) were isolated from *P.punctata* which exhibit anticancer activities in HepG -2 cells. ECH was also reported to be the major active ingredient in Cistanches *Herba*, which are effective in treating metastatic tumors. The goal of study was to comprehend how ECH affected liver cancer cells which caused aggression. It showed that ECH when tested in dose dependent manner on Huh7 and HepG2 cells caused inhibition of the proliferation, invasion and migration (61). The activity of Echinacoside on breast cancer cells was reported whereby it prevented breast cancer cells from proliferating, migrating, or invading while increasing their rate of apoptosis. This resulted in the downregulation of the expression of miR-4306 and miR-4508. (62).

Figure 15: HPLC fingerprinting of *P.punctata* and silver nanoparticles

4.2.2.Martynosides

HPLC fingerprinting of *P.punctata* confirms the presence of Martynosides.They belong to phenylpropanoid glycosides which exhibit anticancer, cytotoxic and antimetastatic activities. The anticancer activity was reported where the potential of MAR in activation of the estrogen

receptor isoforms in Hela cells was investigated. In osteoblasts they caused nodule mineralization. Their antiproliferative action on endometrial cells shows that martynoside can act as a significant natural SERM (63). Another study reports the MAR activity on ex vivo bone marrow cells by downregulating TNF signaling pathway (64) which provides the key role of MAR in anticancer activity.

4.2.3. Verbacosides and isoverbacosides

Verbacosides have antioxidant properties, lower NF- activation, and prevent nuclear translocation, which may help to control inflammatory responses. They were isolated from *P.punctata* extract showing anticancer activities. A similar study reported the anti-tumor activity of verbacosides from *Pedicularis striata* Pall. Verbacosides caused inhibition of telomerase activity in human gastric carcinoma cells MKN45 (65).Verbacosides and isoverbacosides isolated from *Castilleja tenuiflora* root cultures demonstrated anti-inflammatory activity decreasing the proinflammatory molecules (66). In a recent study, VERB and 5-Fluorouracil (5-Fu) were found to have synergistic effects against colorectal cancer cells. (67).

4.2.4. Forsythoside B

Forsythoside B possess antioxidant, antisepsis properties, and neuroprotective effects. It could also cause inhibition of TNF-alpha, IL-6, IκB and regulate NF-κB. According to a study, Forsythoside B reduced the concentration of TNF-alpha, IL-6, and HMGB1 in lipopolysaccharide stimulated RAW264.7 cells, caused inhibition of the IKK pathway, and altered nuclear factor (NF)- B in rats with sepsis brought on by caecal ligation and puncture (CLP). (68).This compound might be performing same anticancer activities in HepG2 cells also.

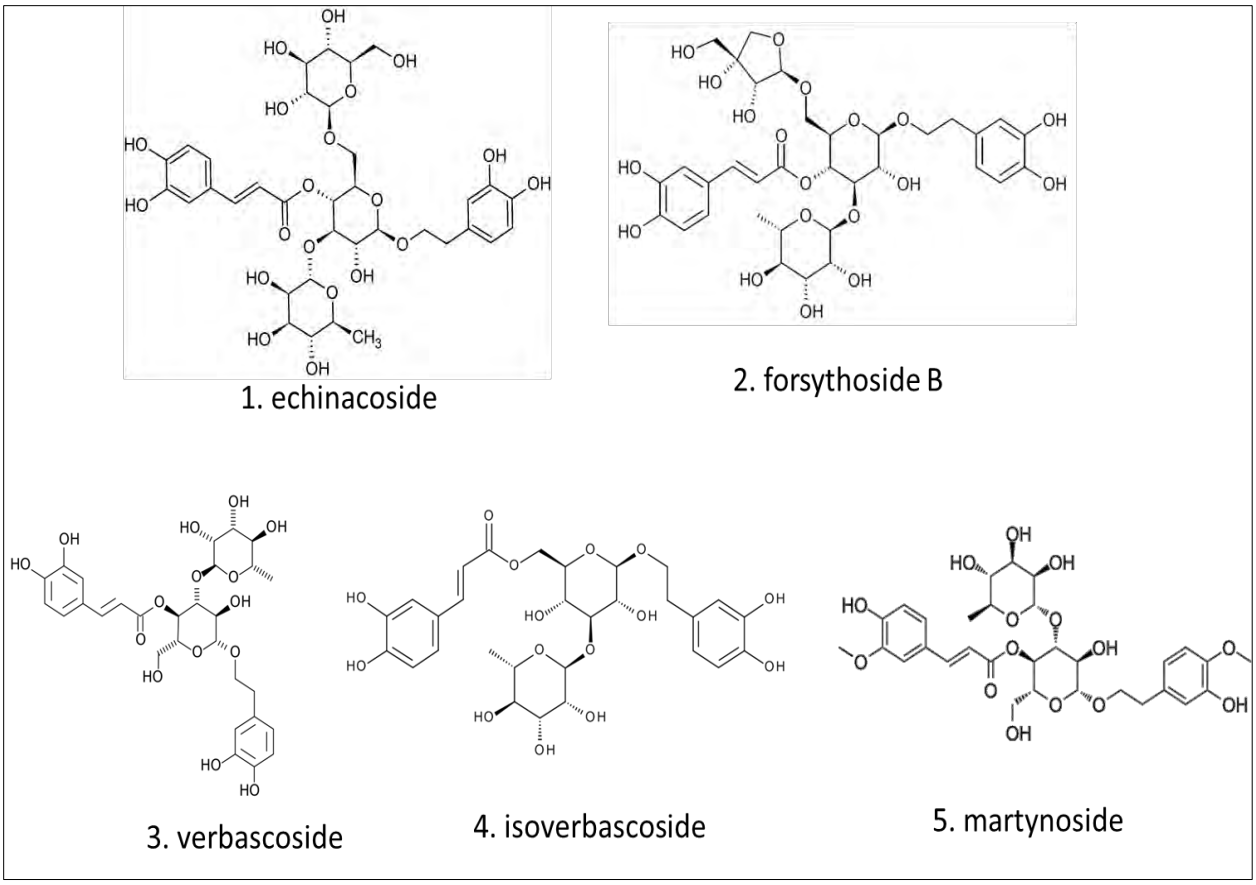


Figure 16 :Structure of Phytochemicals isolated from *P.punctata* extract

4.3. Cytotoxic assays

4.3. 1. Brine shrimp assay:

Brine shrimp lethality assay was carried out to determine the lethality of green synthesized AgNPs and plant bioactive compounds. This provides information on the environmental and aquatic biosafety. The LC50 value for AgNPs was 17.74±7.38ug/ml which shows they are moderately toxic. A comparison between plant extract and silver nanoparticles is shown in figure16, which shows plant extract is nontoxic. A study was reported where effect of Silver Nanoparticles synthesized from Red Sandal Mouthwash was explored using Brine Shrimp Lethality Assay and reports that it is moderately toxic which are consistent with our data **(69)**.

Figure 17. *P.punctata* extract and AgNPs cytotoxicity towards Brine shrimp

4.3.2. Hemolysis activity

It is recommended to study the toxicity of nanoparticles on blood. Hemolysis activity was done to detect the lysis of RBCs when mixed with nanoparticles. According to –American Society for Testing and Materials Designation" nanomaterials with hemolysis rate >5% are considered hemolytic, value between 2-5% are slightly hemolytic and <2% are non-hemolytic. The comaprison of plant extract with AgNPs show that AgNPs are more haemolytic. AgNPs were slightly haemolytic with 4.55±0.44% while *p.punctata* extract was 2.51±0.44% showing nonhemolysis upto 200ug/ml.Hence silver nanoparticles were compatible for use since 5% hemolysis is acceptable for use as drug (70)

Figure 18: Hemolysis activity of *P.punctata* extract and AgNPs

4.4. Anticancer activities on HepG2 cells

4.4.1.MTT assay:

The MTT assay is one of the frequently used techniques for assessing anticancer activity indicating cell viability, proliferation and cytotoxicity. MTT assay was carried out to test the viability of HepG2 cells after treating with AgNPs. Fig.18 shows loss of cell viability by AgNPs by 34.0±8.7%, plant extract by 63.7±5.8% and 100 % cell viability by NTC. A study reporting the effect of AgNPs on HepG2 cells shows cytotoxic effects where AgNPs showed 44 times more inhibitory effect on cancer cells (71). A study was done on silver nanoparticles made from aqueous extract of fruit peel. The IC50 value of the DFPAE-AgNPs on a HepG2 cell line was reported as 37.98 ± 0.21 μg mL -1 (72).

Figure 19: Cell viability of HepG2 cell lines treated with *P.punctata* extract and AgNPs and non-treated cells

4.4.2. Intracellular ROS/RNS production

With increase in loss of cell viability, production of ROS /RNS is also increased. The increase in ROS production directs the use of AgNPs to fight cancer. The abnormal level of ROS causes disruption of cellular components like DNA damage, lipid peroxidation and apoptosis of cell. The agents responsible for ROS triggering can be utilized as therapeutic agent for killing cancer cells. ROS production was reported to be 5056.0±790.04 RFU for silver nanoparticles and plant extract producing 2670±594.23 RFU. Plant extract have ability to scavange some free radicals while silver nanoparticles synthesized from the plant has higher level of ROS generation .A study reports increased ROS generation for green silver nanoparticle tested against MCF-7 breast cancer cells **(73)** .

Figure 20: ROS/RNS production of HepG2 cell lines treated with *P.punctata* extract and AgNPs and non-treated cells

4.4.3. Caspase 3 gene expression and Caspase 3/7 gene activity

Caspases are the enzymes which cause programmed cell death as a result of damage to outer mitochondrial membrane acting as primary effectors of apoptosis. In addition, they also have non-apoptotic roles causing tumor relapse and tumor angiogenesis **(74)** . Caspase 3 gene expression was calculated to be 523.20 ± 29.20 for AgNPs and 208.31 ± 23.48 for plant extract. With increase in Caspase 3/7 gene expression increase in its activity was also observed for AgNPs compared to plant extract. Increase in caspase-3 activities result in apoptosis and are considered as useful for cancer therapy. Our results are similar to previous reported results **(75)**. Another study reported the HeLa cells had increased caspase-3/9 activity. **(76)**.With increased caspase 3 activity silver nanoparticles can be used for targeted drug delivery .

Figure 21:CASPASE 3 gene expression of HepG2 cell lines treated with *P.punctata* based AgNPs, *P.punctata* extract , and non-treated cells

Figure 22:CASPASE 3/7 activity of HepG2 cell lines treated with *P.punctata* based AgNPs, *P.punctata* extract , and non-treated cells

4.4.4. Mitochondrial Membrane potential

The mitochondrial membrane potential of cells determines the apoptosis **(77)**. It also controls the respiratory rates , ATP synthesis and ROS generation. Mitochondrial membrane potential of silver nanoparticles was measured for HepG2 cells . AgNPs resulted in maximum loss of flourescence 1155.33±261.01 RFU. A comparison with NTC shows that silver nanoparticles decrease the mitochondrial membrane potential by thrice .The phytochemicals in *P.punctata* are also reported to play a role in anticancerous activities decreasing the membrane potential which is evident from figure22.The values of MMP reported for *P.punctata* extract are 2008 ±112.95 RFU compared to NTC 2844.33±242.84 RFU.The morphological changes such as substrate attachment,migration and invasion are also effected. Our results are correlated with previous reports **(78)**.

Figure 23:Mitochondrial membrane potential (MMP) of HepG2 cell lines treated with *P.punctata* based AgNPs, *P.punctata* plant extract, and non treated cells

5.Conclusions

Pedicularis punctata based silver nanoparticles provides environment friendly, simple, and efficient routes for the production of nanoparticles. The irregular shape nanoparticles were analyzed with size range between 30-60nm. FTIR analysis shows the functional groups involved in *P.punctata* extract which are mostly aromatic compounds of phenolics. HPLC analysis detected phenylethanoid glycosides which have anticancer activities. Their activity was further confirmed by Caspase gene activity, ROS production, mitochondrial potential and viability test. Biosafety was also tested on brine shrimp and human RBCs.AgNPs show enhanced anticancer activity on HepG2 cells. Phenylethanoid glycosides have poor bioavailability thus silver nanoparticles can be alternative source for increasing their bioavailability. Nanoparticles enhance solubility of phytochemicals, increase their absorption, prevent them from degrading too quickly in the body, and lengthen their circulation time. To further investigate, more research is required for bioavailability of silver nanoparticles capped by phenylethanoid glycoside. Multidisciplinary investigations taking in account long term exposure, variable routes of exposure, and the dosing of AgNps should be conducted in humans in order to ascertain the human toxicity threshold.

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