A DISSERTATION REPORT ON

# "Biological synthesis of silver nanoparticles using *Rivina humilis* leaf extract and their effect on virus control in *Nicotiana tabacum* cv. White Burley"

## Submitted to the

**DEPARTMENT OF BIOSCIENCES**

**INTEGRAL UNIVERSITY**

**Submitted By**


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**M. Sc Microbiology (Semester IV)**


**Under the supervision of**


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22.06.2022

# प्रमाण पत्र

श्री प्रभात कुमार मौर्या, पुत्र श्री बच्चा लाल मौर्या एम.एससी. माइक्रोबायोलॉजी इंटीग्रल यूनिवर्सिटी, लखनऊ ने दिनांक 18.02.2022 से दिनांक 22.06.2022 तक **"Biological synthesis of silver nanoparticle using *Rivina humilis* of extract and their effect on virus control in *Nicotiana tabacum* cv. White burley"** शीर्षक में डा. सुशील कुमार, सीनियर साइंटिस्ट के पर्यवेक्षण (Supervision) में सफलतापूर्वक प्रशिक्षण प्राप्त किया।

# CERTIFICATE

Mr. Prabhat Kumar Maurya, S/o Shri Bachcha Lal Maurya, student of M.Sc. Microbiology, Integral University, Lucknow has successfully completed the training from 18.02.2022 to 22.06.2022 on the project entitled, **"Biological synthesis of silver nanoparticle using *Rivina humilis* of extract and their effect on virus control in *Nicotiana tabacum* cv. White burley"** under the supervision of Dr. Susheel Kumar, Sr.Scientist.

for 22/6/22

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# DECLARATION

I, **Prabhat Kumar Maurya** , hereby declare that the project report **"Biological synthesis of silver nanoparticles using *Rivina humilis* leaf extract and their effect on virus control in *Nicotiana tabacum* cv. White Burley"** submitted to **Department of Biosciences, Integral university Lucknow** in partial fulfillment of the requirements of the Degree of Master of Science in Microbiology, is an independent work carried out by the undersigned during a period of four months, under the guidance and supervision of **Dr. Susheel Kumar Senior Scientist, CSIR-National Botanical Research Institute**, **Lucknow** and to the best of knowledge and belief this report has not forms on the basis of award of any Degree/Diploma/Associate/Fellowship or other similar title to any candidate of any university.

Place:

Date:

(Signature of candidate)

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## CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Mr. Prabhat Kumar Maurya** a student of **M.Sc. Microbiology** (2nd year/4th semester), Integral University Lucknow, has completed his four month dissertation work entitled successfully.

He has completed this work from ***CSIR-NBRI*** (National Botanical Research Institute) LUCKNOW under the guidance of **Dr. Susheel Kumar**. The dissertation was a compulsory part of his M.Sc. Microbiology degree.

I wish his good luck and bright future.

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TO WHOM IT MAY CONCERNS

This is to certify that **Prabhat Kumar Maurya** a student of M.Sc. Microbiology (IV Semester) Integral University has completed his four months dissertation works entitle **"Biological synthesis of silver nanoparticles using *Rivina humilis* leaf extract and their effect on virus control in *Nicotiana tabacum* cv. White Burley"** successfully. He has completed this work from 18-02-2022 to 22-06-2022 under the guidance of **Dr. Susheel Kumar** (Senior Scientist) **Division of Plant Molecular Virology CSIR NBRI Lucknow**. The dissertation was a compulsory part for the award of his M.Sc. Degree in Microbiology

I wish him bright and a great future.

**(Dr Snober S. Mir)**

Associate Prof & Head

Department of Biosciences

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1. Biosynthesis of silver nanoparticles *Rivina humilis* plant extract.
2. To study the effect of silver nanoparticles on virus infected *Nicotiana tabacum* cv. White Burley plant.

# ABBREVIATIONS

# ABBREVIATIONS

| | |
|---|---|
| Ag | Sliver |
| $AgNO_3$ | Sliver Nitrate |
| AgNPs | Sliver nanoparticles |
| Au | Gold |
| AuNPs | Gold nanoparticles |
| Cm | Centimeter |
| Conc. | Concentration |
| Ft | Foot |
| G | Gram |
| H | Hours |
| In | Inches |
| L | Liters |
| M | Meter |
| M | Molarity |
| Mg | Milli Grams |
| Min | Minutes |
| Ml | Milli liters |
| Mm | Millimeters |
| mM | Milli Molar |
| MQ | Milli Q |
| Ng | Nanograms |

| | |
|---|---|
| Nm | Nanometers |
| NPs | Nanoparticles |
| Sec | Seconds |
| Soln. | Solution |
| Spp. | Species |
| Vol. | Volume |
| NP | Nanoparticle |
| Fig. No. | Figure Number |
| $Na_2HPO_4$ | Disodium Phosphate |
| $NaH_2PO_4$ | Monosodium Phosphate |
| °C | Degree centigrade |

# CHAPTER-1

# INTRODUCTION

# INTRODUCTION

The term "Nano" has been derived from the Greek word that means "Nanos"i.e. dwarf. Nanotechnology is a stream of science that deals with the study of Nanomaterials that ranges to approximately 1 to 100 nm that can be manipulated at the atomic or molecular level and be used as well as applied in several fields of sciences. Nanoparticles and nanostructured materials are the fundamental building blocks for various Nano-technological fields.

Although the term nanotechnology is new, the concept of using nanoscale materials can be dated centuries back. Nanoparticles were used by artisans during the ninth century by Mesopotamians for generating luster and glitter effects on the surfaces of the pots (Reiss and Hutten 2010). For the concept of Nanotechnology,the credit goes to the Americanphysicist,Richard Feynman, who has achieved the Nobel Prize for "fundamental work in quantum electrodynamics".He highlighted the concept behind nanotechnology in his lecture "There is plenty of room at the bottom" on December 29$^{th}$, 1959.

Nanoscale size properties are the base of nanoparticles, which has opened a new area of research in recent years for the development of numerous methodologies to synthesize nanoparticles of shape and size. Nanoparticle possesses the different size and shapes like spherical, hexagonal, triangle, rod etc. which are being utilized in different fields of application. Although, the physical, chemical and biological properties of the nanoparticles differ from the properties of both individual atoms or molecules because it depends on the size of the macro material that either possess a constant property or not. The concept of nanotechnology is new therefore it can show adverse and unknown effects.The use of nanoparticles can be associated with risk factors in the ecosystem, because of its mobility, exposure, absorption, reactivity, and toxicity, which havebeen reported several concerns by the scientific and environmental communities.

Silver and silver nanoparticles (AgNPs) have protective role of in plant protection.There are a number of mechanisms by which AgNPs control growth of pathogens. Silver nanoparticles have the ability to anchor the bacterial cell wall and penetrate it, causing structural changes in the cell death. There are formation of “pits” and accumulation of the nanoparticles on the cell surface.

Another mechanism is the formation of free radicals by the AgNPs. This formation of free radicals makes membrane porous which ultimately leads to cell death (Danilcauk *et al.*, 2006; Kim *et al.*, 2007). Silver is considered the most capable nanomaterial with fungicidal, bactericidal, and viricidal properties. Nano silver exhibits high level of toxicity to the microorganisms and lower to the mammalian cells.

It has been suggested that AgNPs inhibit viral nucleic acid replication, while their antiviral activity depends on the particle size, as well as on the distribution of interacting ligand/receptor molecules (Lü *et al.* 2009; Papp *et al.* 2008). Elbeshehy *et al.* (2015) studied the effect of biosynthesized AgNPs on leaves of fava bean infected with BYMV (bean yellow mosaic virus) which showed severe symptoms, including yellow mosaic, mottling, crinkling, size reduction, and deformation, symptoms that were absent from the non-infected leaves.

As we know that NPs have both positive and negative effects on plant growth and development.Krishnaraj *et al.* (2012) studied the effect of biogenic AgNPs on hydroponically grown *Bacopamonnieri* growth metabolism, showed a significant effect on seed germination, and induced the synthesis of protein and carbohydrate and decreased the total phenol contents and catalase and peroxidase activities.

Synthesis of AgNPs throughthe physical and chemical methods is costly and involves toxic chemicals. In this regard, the use of a biological method for the synthesis of AgNPs provides reliable, nontoxic, clean, eco-friendly, and green experimental protocol. Synthesis of AgNPsusing fungus has highlighted much importance in recent years for their metal tolerance capacity and secretion of extracellular enzymes or proteins in a large amount, which might play asignificant role in the metal reduction process. Since the unique physiochemical properties of AgNPs depends on their sizes and shapes, the size-controlled synthesis of nanoparticles is an emerging area of research.By controlling various conditions of the reaction, nanoparticles of various size and shapes can be achieved.

# CHAPTER-2

# REVIEW OF LITERATURE

# REVIEW OF LITERATURE

**1. *Rivina humilis* spp.**

*Rivina humilis* is a species of floweringplant in the family *Petiveriaceae*.Earlier it was previously placed in the pokeweed family, *Phytolaccaceae*. It can be found in the southern United States, the Caribbean, Central America, and tropical South America. It can be commonly known as pigeon berry, rouge plant,baby peppers, blood berry, and corralito.

**Fig. 1** *Plant morphology of Rivina humilis* Linn.

**1.2 Classificationof *Rivina humilis*Linn.**

| | |
|---|---|
| Domain | Eukaryota |
| Kingdom | Plantae |
| Phylum | Tracheophyta |
| Sub phylum | Angiospermae |
| Class | Equisetopsida C. Agard |
| Order | CaryophyllalesJuss.exBercht. |
| Family | *Petiveriaceae* |
| Genus | *Rivina* |
| Species | *Rivina humilis* |

### 1.3 Morphology

Bloodberry is an upright herb that appears like vine, with a height of 0.4–2 m (1.3–6.6 ft) and theleaves of this evergreen perennialplantare up to 15 cm long and 9 cm wide. Flowers are conoid, 4 to 15 cm long with a stalk of 1 to 5 cm in length and filament of 2 to 8 mm long. Sepals are of 1.5 to 3.5 mm in length. The flowers are of white or pink or sometimes green in color. The fruit is a glossy, bright red berryof 2.5 to 5 mm in diameter.

### 1.3 Habitat

*R. humilis* can be found in forests, hammocks, shell middens, thickets, roadsides, and disturbed areas at elevations from sea level to 1,700 m (5,600 ft). It can survive in less or partial sun and is tolerant of full shade. It is also tolerant of salt spray and saline soils. It is considered invasive in New Caledonia, where it was likely introduced in 1900. It is considered a weed in Queensland and Australia. It is neutralized in Australia and on Cocos Islands.

### 1.4 Uses

Bloodberry is cultivated as an ornamental plant in warm regions throughout the world and is preciously known as a shade-tolerant ground cover. It can also be grown as a houseplant and in greenhouses. The juice made from the berries was used as a dye and ink at one time. The juice of the berries has been tested in male rats and are reported to be safe to consume.

## 2. *Nicotiana tabacum*

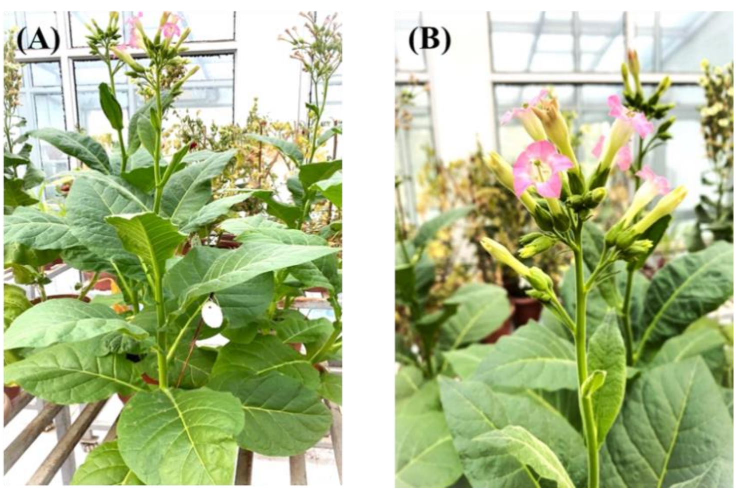



**Fig. 2** *Nicotiana tabacum* (A) whole plant, (B) The flower

Tobacco plant is a perennial plant, and it is erect and glandular herb which is found only in cultivation. *N. tabacum* is a native of America but now it is commercially cultivated throughout the world.It is herb that grows to the height of 1-2 m with large green leaves having trumpet shaped white-pinkish flowers. All parts of herb are sticky, covered with glandular hairs, which exudes a yellow secretion that contains alkaloid known as nicotine.It is commonly known as Fumo, Petume, Etum, Tobacco.The leaf is used, usually after aging and processing in various ways – that are for smoking, chewing, snuffing, and extraction of nicotine.

## 2.1 Classification of *Nicotiana tabacum*

| | |
|---|---|
| Domain | Eukaryota |
| Kingdom | *Plantae* – Plants |
| Division | *Magnoliophyta* - Flowering plants |
| Class | *Magnoliopsida* – Dicotyledons |
| Order | *Solanales* |
| Family | *Solanaceae* |
| Genus | Nicotiana |
| Species | *Nicotiana tabacum* |

## 3.NANOPARTICLE

Nanoparticles were developed for drug delivery and vaccine purpose which has changed the medicinal scenario in 1960. The term "nanotechnology" first time used as scientific field by NarioTanigushi in 1974in his paper was “Nanotechnology”. The first paper published in 1980 by K. Eric. Drexhr of Space Systems Laboratory, Massachusetts Institute of Technology was titled as "In approach to the development of general capabilities for molecular manipulation". In 2000, the United States National Nanotechnology initiative was founded to coordinate Federal nanotechnology research and development and is evaluated by the President's Council of Advisors on Science and Technology. Nanoparticles possess unique optical, electronic, magnetic, and catalytic properties than the bulk material due to their high surface area to volume ratio (Paulose *et al.*, 2011; Vijayakumar*et al.*,2013). They can be metallic, mineral, polymer-based or a combination of materials (Rana and Kalaichelvan, 2013; Sahayaraj and Rajesh, 2011).

## 3.1PROPERTIES OF NANOPARTICLES

The particle shows unique properties at size reduced below 100 nm from bulk material based on Quantum mechanics (Bhusan, 2007). The properties of nano materials include enhanced diffusivity, reduced density, higher electrical resistance, increased strength, lower thermal conductivity etc. in comparison with bulk materials. Amongst the nano and bulk scale, the properties exhibited by them are entirely different and this feature characterizes them. There is a lot of research to investigate these properties and to understand their fundamentals. The reasons ofexisting physical properties of nanomaterials are generally related to different origins such as greater surface to volume ratio, high surface energy, quantum confinement, high reactivity, reduction in imperfections etc. (Roduner, 2006). They exhibit following properties of nanoparticles.

### 3.1.1 SIZE EFFECTS AND SURFACE EFFECTS

Depending upon various particle size and materials used to prepare nanoparticles surface properties of nanoparticles are changed i.e. solubility, transparency, color, absorption or emission wavelength, conductivity, melting point and catalytic behavior. If surface properties are not in controlled manner, a nanoparticle immediately converts into larger particles due surface effects, so that agglomeration occurs, and size dependent properties also lost. Thus, these two properties of nanoparticles are needed in order to maintain disparity of nanoparticles and their technological applications (Borm, 2006).

### 3.1.2 OPTICAL PROPERTIES

Nanocrystalline systems have gaining attention due to their novel optical properties, which differ extremely from bulk crystals. Nanoparticles are remarkably efficient in absorbing and scattering light, unlike many dyes and pigments, have a colour that depends upon the size and the shape of the particle. Due to conduction of electrons on the metal surface which undergo a collective oscillation when excited by light at specific wavelengths results in strong interaction of the nanoparticles known as a surface piasmon resonance (SPR). This oscillation results in unusually strong scattering and absorption properties i.e. the origin of the colour of nanomaterials. As the size of particles is reduced to nanoscalelevel, nanoparticles absorb the light with a specific wavelength due to Surface Plasma Resonance (Mulvaney, 1996; Park and Kim, 2008). The optical spectrum shifts are used for quantitative and qualitative analysis of particle size, distribution and concentration as well as the effect of particle shape.The gold and silver nanoparticles show the color phenomena with splendid tinting strength, color saturation and transparency, bulk Silver appears whitish in color, but nano-sized gold appears brown in color. As seen in the Raman Effect phenomena, decreasing size of the particle will increase the Kubo gap and therefore the energy emitted by the photons will change frequency and hence their color change.

### 3.1.3. STRUCTURAL PROPERTIES

The chemical reactivity of the NP, surface is much higher than that of its bulk counterpart. This is an important property related to reactivity, solubility, sintering performance etc. Property has also associated with the mass and heat transfer between the particles and their surroundings. Moreover, the crystal structure of the particles may change with the nanosized range particle size in many cases. This is imputing to the compressive force exerted on the particles because of the surface tension of the particle itself. The crystal structure critical particle size and the size effect differ with the materials.

### 3.1.4 THERMAL PROPERTIES

Melting begins at the surface. As the particle size decreases, surface to bulk atom ratio increases dramatically i.e. the melting point of the material decreases from that of the bulk material to nonmaterial because their migration at lower temperature is easy. This reduction of melting point is regarded as one of the unique features of the NPs related with aggregation and grain growth of the nanoparticles or expansion of sintering performance of ceramic materials. Hence, melting point of NPs differs from their corresponding bulk materials due to their free size and surface. Several examples could be found to demonstrate the melting point depression as function of the particle size. A variety of nanoparticles like Au, Ag, CdS etc. have been investigated for their thermal stability and melting. Melting of nanoparticles is determined either by X-ray diffraction or by electron diffraction.

### 3.1.5 MECHANICAL PROPERTIES

Mechanical properties of nanomaterials are higher than their bulk material and the hardness of crystalline materials increases with the decreasing crystalline size counterpart by one or two orders of magnitude (Nihara, 1991). This remarkable improvement in mechanical strength is attributed to reduction in the probability of defects at nanoscale.

### 3.1.6 MAGNETIC PROPERTIES

The electromagnetic forces become predominant properties as bulk material reduce to nanosized. At nanoscale, mass of material or particle is so small, that the electromagnetic forces go beyond the gravitational force i.e. gravity becomes negligible. Because of this, nanoparticles are used as raw materials for several electronic devices. The electromagnetic properties of nanoparticles play immense role for the improvement of the product performance. The minimum particle size requires to keep the ferroelectric property differs depending on the kind and composition of the materials. For the magnetic property, bulk ferromagnetic materials have spontaneously magnetized domains. Ferromagnetic fine particles are exceedingly small in size i.e. less than about 1.0 μm, have a single magnetic domain structure and show super-paramagnetic property. In this case, even if the individual particles are ferromagnetic with the single magnetic domain structure, the particles collectively act as paramagnet. In superparamagnetic particles, spins are oriented in one direction and switch coherently in the opposite direction, but the magnetization disappears by the thermal fluctuation, when the external magnetic field is taken away. The time for disappearing of magnetization depends upon the particle size, this magnetization of the material responds with the external magnetic field such as found in paramagnetic material if they are small enough. But this magnetization decreases gradually as the particle size becomes larger. Gold which is a stable substance as bulk shows unique catalytic characteristics as nanoparticles (Matsui, 2005).

## 4.SILVER NANOPARTICLES

Silver is a shimmering, soft, white transition metal possessing high electrical and thermal conductivity.Before realization that microbes are agents for infections, Silver has been known for their medical and therapeutic benefits. The most natural question to ask as soon as starting to deal with silver nanoparticles (AgNPs) is: why are AgNPs nanoparticles so interesting? Why even bother to work with such nanosized structure as their handling and synthesis is very much difficult than bulk atoms. The answer itself given by nature and there is anecdotal evidence of its use as far back as ancient Egypt and Rome, Nanoparticle having unique property of surface-to-volume ratio because of these property silver nanoparticles have been used in wide range of application (Li el at., 2001; Reidy *et al.*, 2013). This might for example be in the catalytic industry; some nanoparticles have efficiently work as good catalysts. Additionally, some nanoparticles also used in the medical and therapeutic agent as they show a high surface to volume ratio. Recently, nanoparticles received great attention in field of Biology and Biochemistry. Silver nanoparticles are generally ranging from 1 to 100 nm in size and that is size as that of human proteins. Silver nanoparticles contain about 10000-15000 silver atoms (Oberdorster *et al.*, 2009). 'Silver' is mainly composed of a large percentage of silver oxide due to their large surface ratio to bulk silver atoms.As the size of silver particles decreases to nanometer range, at this state, drastic change in physical properties of material as well as material may be sintered at lower temperature. Currently, AgNPs also incorporate into a wide range of medical devices, such as bone cement, surgical instruments, surgical masks, etc. In the recent past, researchers have received enormous interest in silver nanoparticles (AgNPs) due to their extraordinary defence mechanism against wide range of microorganisms and also drug resistance strains of microorganisms (Sharma *et al.*, 2009).Additionally, different concentrations and particle size, silver has historically has been used for treatment of wounds (Qin, 2005; Atiyeh *et al.*, 2007; Lansdown, 2006). In fact, AgNPs are now replacing silver sulfadiazine as an effective agent in the treatment of wounds.Moreover, Samsung has created, and marketed material called silver Nano, which includes AgNPs on the surfaces of household appliances. The silver nanoparticles exhibit important physicochemical properties, such as pH-dependent partitioning to solid and dissolved particulate matter the electromagnetic properties such as particles of smaller size not becomes not only become transparent but also emit special light by plasma absorption in presence of UV light. Silver nanoparticles exhibits great biological activities compared to the regular metal (Lok *et al.* 2006; Pal *et al.* 2007). Due to their properties they have received great attention in biomedical imagining using SERS.

In fact, the surface plasmon resonance and large effective scattering cross-section of individual AgNPs make them ideal for molecular labelling (Schultz *et al.*, 2000).Thus, many targeted silver oxide nanoprobes are currently being developed (Mody *et al.*, 2010). AgNPs are gaining attraction day by day because of their unique properties and find uses and applications in diverse fields including medical, food, textile engineering, consumer, electronics, biotechnology, nanobiotechnology, bioengineering sciences, optics, and water treatment and agriculture. Moreover, Silver has a strong antimicrobial potential, which has been used since the ancient times as an antimicrobial agent. As an antimicrobial agent used in air sanitizer sprays, pillows, respirators, socks, wet wipes, detergents, soaps, shampoos, toothpastes, washing machines, and many other consumer products; as bone cement: and in many wound dressings. Silver ions are used in the formulation of dental resin composites; in coatings of medical devicesas a bactericidal coating in water filters.

**4.1SYNTHESIS OF SILVER NANOPARTICLES**

Recent years, there has been an extraordinary growth in synthesis of silver nanoparticles due to wide range of applications. These Silver nanoparticles successfully used in diagnosis and treatment of cancer (Popescu *et al.*,2010; Baruwati *et al.*, 2009). Nanotechnology discovers the idea that the assembly can be hierarchical and controlled in specific ways.Fabrication of nanoparticles also requires understanding the fundamentals of chemistry and physics at nano level as well as how to commercialize them. Broadly speaking, there are two approaches to making products with nanoscale characters and attributes: top down and bottom-up.In top-down approach, suitable bulk material collapse into fine particles by size reduction is achieved by various physical and chemical treatments such as lithographic techniques e.g. grinding, milling, sputtering and thermal/laser ablation.

Top-down production methods introduce imperfections in the surface structure of the product, and this is a major limitation because the surface chemistry and the other physical properties of nanoparticles are highly dependent on the surface structure (Tbakkar el al., 2010). In the bottom-up approach, nanoparticles can be synthesized using chemical and biological methods by self-assemble of atoms to new nuclei which grow into a particle of nanoscale i.e. the nanoparticles are built from smaller entities, for example by joining atoms, molecules and smaller particles (Thakkar *et al.*, 2010).

## 4.2BIOLOGICAL METHODS OF SYNTHESIS

Various chemical and physical methods are in used for the synthesis of nanoparticles. These methods have certain limitation such as requires toxic, flammable agents, long time for synthesis and difficulty in purification. Moreover, the agent cannot be easily deposited off Nowadays looking towards global warming and climate change issues, it is becoming a liability of every researcher to accentuate on an alternative synthetic route which should be cost effective and ecofriendly. Therefore, green synthesis of nanoparticles has been an emerging research area now a day. Green synthesis method does not require high temperature, pressure, energy and toxic chemicals. Keeping in view of the aesthetic sense, the green synthesis is best known procedure itself and proving its potential at the top.Biological synthesis of silver nanoparticles highlight because complicated reaction could be completed within a fraction of minutes i.e. rapid Additionally, biological synthesis is of great attention as its nontoxic, easy, controlling desire shape and size characteristics, inexpensive. Natural Sources are used for green synthesis of silver nanoparticles, such as bacteria, fungi, yeast, algae, plant extracts, biomass, etc. These natural sources have antioxidant or reducing properties normally responsible for the reduction of metal compounds in their respective nanoparticles. The current prospective on biological system has created a commercial importance due to their enzymatic reactions, photochemical characteristics and herbal nature.

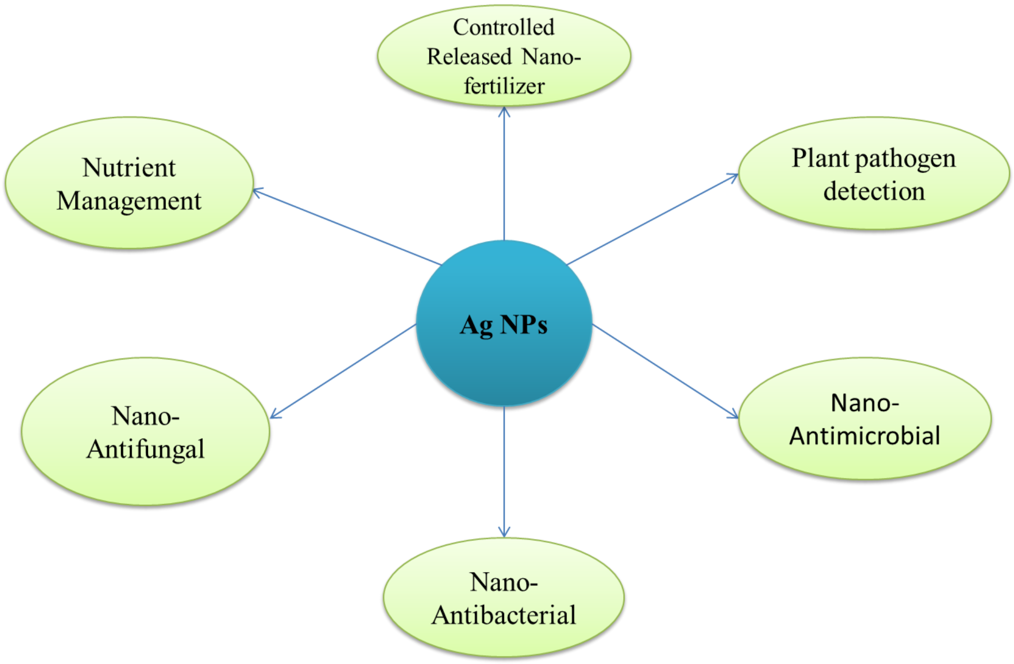
## 4.3 UV-VISIBLE SPECTROPHOTOMETER

UV Visible Spectroscopy can be used to study unique optical properties of nanoparticles. UV-visible spectroscopy confirms the formation of metal nanoparticles by measuring surface Plasmon resonance (SPR) and evaluating the collective oscillations of conduction band electrons in response to electromagnetic waves.This is due to tie metallic nanoparticles are also known to demonstrate different characteristic colors. The SRP occurs only in the case of nanoparticles and not in the case of bulk metallic particles (Papavassiliou, 1979). This used to know the information about the size, structure, stability, and aggregation of the nanoparticles (Patra and Baek, 2014). Absorption spectra of silver nanoparticles have specific peak between 400nm to 450nm where broadening of peak indicate that the particles are polydisperse (Mohanpuria *et al.*, 2008; Shankar *et al.*,2003).

**4.4HIGH RESOLUTION TRANSMISSION MICROSCOPY (TEM/ HRTEM)**

Thetechnique of high resolution transmission microscopy (HRTEM) has been developed by Max Knoll and Ernst Ruska in Germany long back in 1931. TEM is an important and commonly used technique for the characterization of nanomaterials, used to obtain quantitative measures of particle and/or grain size, size distribution, shape and crystalline structure of silver nanoparticles.HRTEM is a microscopic technique involves: (i) irradiation of ultra-thin specimen by high energy electron beam which is diffracted by the lattices of a crystalline or semi crystalline material and propagated along different directions, (ii) Imaging and angular distribution analysis of the forward-scattered electrons (unlike SEM where backscattered electrons are detected), and (iii) energy analysis of the emitted X-ray's. Advantages of TEM include high quality, complete and powerful magnification of element and compound structures. Its limitations are laborious sample preparation, artifacts from sample preparation and large and expensive (Zhang *et al.*, 2016; Reidy *et al.*, 2013; Williams and Carter, 2009; Lin *et al.*, 2014).

## Sliver Nanoparticle application in plant protections



# CHAPTER-3

# MATERIALS AND METHODS

<u>**MATERIALS AND METHODS**</u>

## Source of virus cultures/infected tomato

Virus cultures used in this study were obtained from naturally infected tomato plant and further maintained in the insect proof glasshouse of virus laboratory at CSIR-NBRI, Lucknow.

## Raising and maintenance of experimental tests species

Potting medium for growing experimental species was prepared by mixing coarse sand, soil and farmyard manure (1:1:2) (Gowdy 2002). Earthen pots were sterilized by formalin while soil mixture by autoclaving for 1 h at 121°C and 15 psi. All seedlings were raised in shallow-earthen pots of 12 inches (diameter) and transplanted into pots of 6.0-inch diameter at 3-4 leaf stage. Plants belonging to families *Leguminosae* and *Cucurbitaceae* like beans, cucumber, gourd etc. were raised directly from seeds in pots. The seedlings of the *Solanaceae* family: tobacco, tomato, eggplant and chilli were raised in shallow earthen pots and transplanted in pots at 4-5 leaf stage. All test plants were maintained in an insect-proof, light and temperature-controlled glasshouse. The virus cultures were also maintained by mechanical inoculations to healthy *Nicotiana tabacum* cv. White Burley and *Lycopersicon esculentum*at 3-4 leaf stage, after every 35-45 days interval.

Host plants (indicator or systemic) used during study.

| **Family** | **Host plants** |
|---|---|
| *Amarantheceae* | *Gampharenaglobosa* |
| *Chenopodiaceae* | *C. amaranticolor*, *C. murale* |
| *Cucurbitaceae* | *Cucurbita maxima, C. sativus*, *Luffa cylindrical* |
| *Fabaceae* | *Vigna unguiculata, Phaseolus vulgaris* |
| *Solanaceae* | *Capsicum annuum, Daturainnoxia,Lycopersiconesculentum, Nicotianatabacum*cv. White Burley, *N. tabacum* cv. Petit Havana, *Nicotiana tabacum* Samsun NN, *N. rustica,N. benthamiana*, *N. glutinosa*, *Petunia hybrid, Solanummelongena, Hyoscyamusmuticus* |

## Virus Transmission (Mechanical transmission) by sap

Neo-formed infected leaf tissue was macerated in 0.1M potassium phosphate buffer, pH 6.8 (supplemented with 0.5% β-mercaptoethanol, 0.1% thioglycolic acid, 0.01M EDTA, 0.1% Na2SO3 and 0.01M DIECA additives) in a ratio of 1:10 (w/v) for each unit weight of tissue using a sterile pestle and mortar. The homogenate obtained, after squeezing through double layered muslin cloth was rubbed onto carborundum pre-dusted leaves of various recipient host species as described earlier by Noordam (1973).

## Host range and symptomatology

Five or more young seedlings of each test species at 4-5 leaf stage were assessed for susceptibility to the virus through mechanical inoculation. The plants after inoculation were observed and symptoms were recorded up to six weeks, from the day of inoculation.

## Preparation of plant extract

The material required for the plant extract preparation includes beakers, funnel, filter paper, muslin cloth, magnetic stirrer and magnetic bead.

**METHOD:**

1. Fresh *Rumina humilis* was cultivated and collected from the fields of CSIR-National Botanical Research Institute,Lucknow.
2. The leaves of the plant then shade dried properly for about 2-3 weeks.
3. The dried leaves were then crushed with liquid nitrogen making a fine powder.
4. 50g of the powder was then dissolved in 500ml of MQ water on magnetic stirrer.
5. The dissolved sample was then filtered using filter paper [1M] after passing through double layered muslin cloth.

## Preparation of AgNO3 solution

The material required for the preparation of AgNO3 solutionincludesconical flasks, foil paper, silver nitrate powder, MQ water, measuring cylinder, pipette.

**METHOD:**

1. 18.0 mg of $AgNO_3$ [powder] was dissolved in 100ml MQ water in an amber bottle to make 1 mM solution.
2. The solution was then divided into 5 sets in 5 different titration flasks of 250ml.
3. Each flask containing 5ml of filtered plant extract and 45ml of fresh $AgNO_3$ solution.
4. Flasks were then marked-A, B, C, D, E.

## Challenge inoculation

The material required for the challenge inoculationincludesmortar pestle, cotton, inoculation buffer, leaf (infected with desired virus), distilled water, and tags.

1. 50 fresh and healthy plants of Nicotiana tabacum cv. White burley were collected and arranged in 10 rows containing 5 pots in each row.
2. The rows were tagged as follows:

I. Row 1 - Control
II. Row 2 - Control 0 min + virus
III. Row 3 - 15 min + virus
IV. Row 4 - 15 min + virus - 7 days
V. Row 5 - 30 min + virus
VI. Row 6 - 30 min + virus - 7 days
VII. Row 7 - 45 min + virus
VIII. Row 8 - 45 min + virus - 7 day
IX. Row 9 - 60 min + virus
X. Row 10 - 60 min + virus - 7 days

**Method:**

1. Carborundum was sprayed on all 50 plants.
2. The solution of 1mM $AgNO_3$ was sprayed on all the 45 plants from row 2 to row 10 according to the time mentioned on flasks. Row 2 was sprayed with the solution 0 min, row 3 and 4 with 15 min.
3. Row 1 is kept without any spray and virus as it is the control row.

4. It has given only mechanical stress with clean cotton.
5. Row 3rd, 5th ,7th, and 9th were inoculated with a virus.
6. The remaining rows 4th, 6th, 8th, and 10th were inoculated after 7 days with the virus.

# CHAPTER-4

# RESULT AND DISCUSSION

## Result and Discussion

Plant extract filtered with the muslin cloth gave dark green color as shown in left side beaker, the right-side beaker is containing the sample that was filtered with 20 µm Whatman filter paper 125mm showing clear brown color solution (Fig. 3a).


***Figure 3 (a) Beaker (on left hand) showing filtered plant extract with the muslin cloth (b) Beaker (on right hand) showing filtered plant extract with 20 µm Whatman filter paper.***

1. Flask A (0 min) was at once kept on ice after mixing P.E and $AgNO_3$ solution.
2. The rest four flasks were kept under sunlight (at 38˚C) and kept immediately on ice according to the time mentioned on flasks. flask B after 15 min, flask C after 30 min, flask D after 45 min, flask E after 60min.

Mixture of *R.humilis* leaf extract and silver nitrate solution is equally distributed in 5 different flasks, each flask is marked with time – 0 min, 15 min, 30 min, 45 min and 60 min.

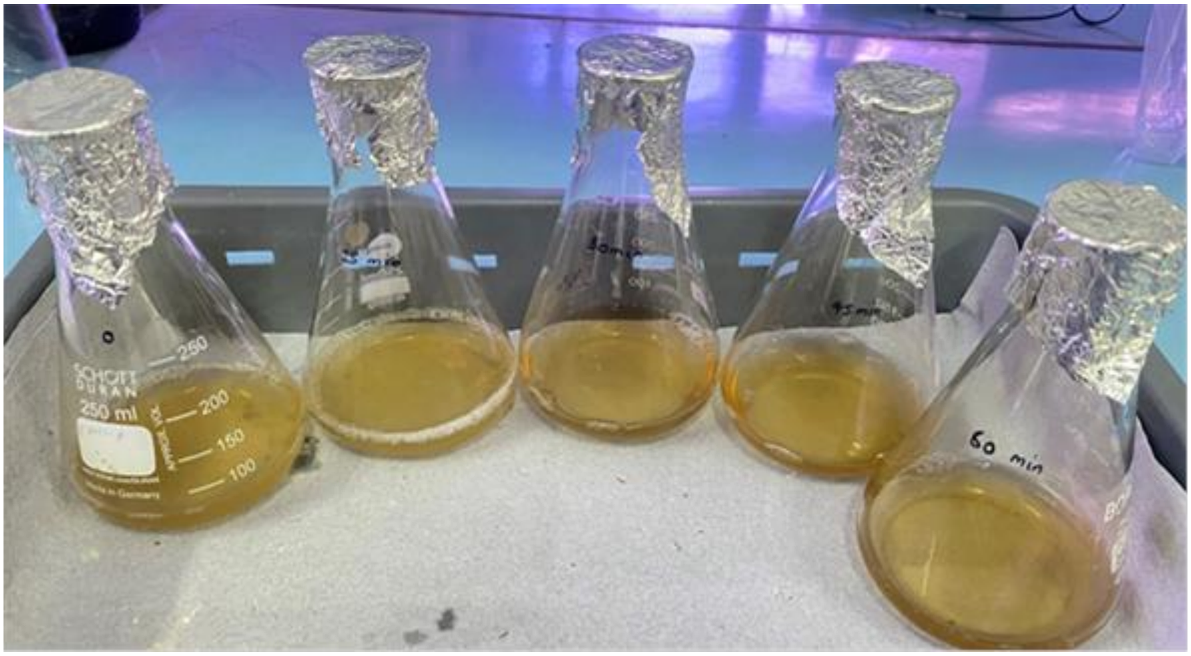



**Fig. 4** Flasks containing 10% filtered leaf extract (5 ml) in 45 ml of fresh $AgNO_3$ solution.Where, Flask A - 0 min, Flask B - 15 min, Flask C - 30 min, Flask D - 45 min and Flask E - 60 min.

All four flasks except flask A- 0 min are kept under sunlight at (38℃) in this image.


**Fig. 5** Synthesising AgNPs in presence of sunlight (38 ℃).

Flasks 0min to 60 min showing variation in colour from yellow to dark brown. This variation of colouraccording to time shows the synthesis of nanoparticles.


**Fig. 6** Synthesized AgNPs at various time points (15, 30, 45 and 60 min).

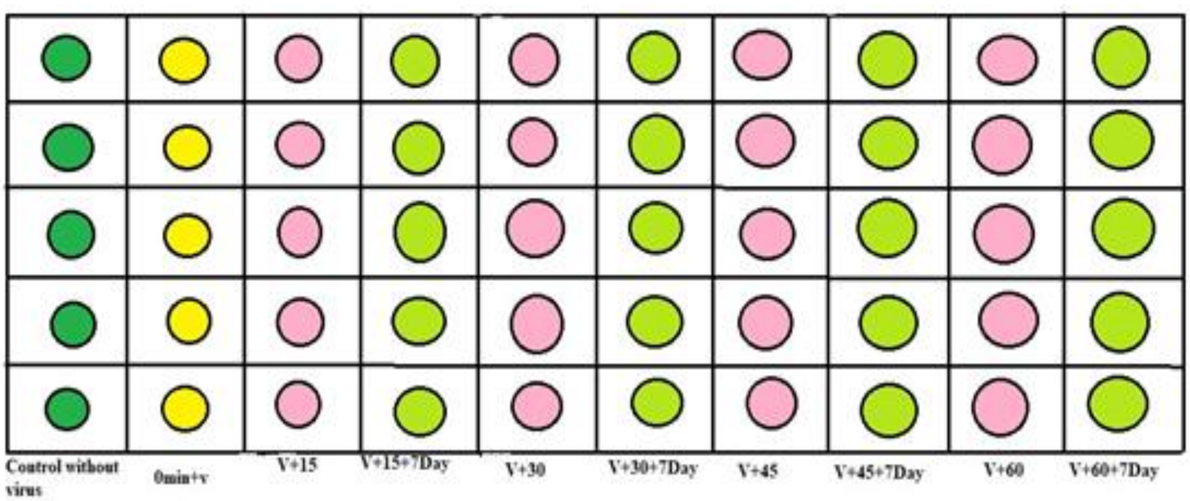



**Fig. 7** The layout of treatment procedure for the challenge of AgNPs treated plants by virus along with their control.

**Fig. 8**Image showing plants after treatment with AgNPs.

Formation of AgNPs by reduction of silver nitrate during exposure to Rivina humilis L.E can be easily monitored from the change in color due to the reaction mixture. AgNPs bear a characteristic yellow brown color due to excitation of surface plasmon vibrations. The change in color of reaction mixture after 1 hr is presented in fig;6. which indicated the successful formation of AgNPs. This formation AgNPs is the indication of silver ions in the medium have been converted into elemental silver having the size of nanometric range. It shows the change in color of the reaction medium as an effect of presence of any type of reducing substance.

When *Rivina humilis* extract was mixed in the aqueous solution of the silver nitrate, it started to change from pale yellow color to yellowish brown as shown in fig:6, this change in colour clearly indicates the formation of silver nanoparticles in the reaction mixture.

One of the main advantages of reduction of silver ions into nanoparticles by using *Rivina humilis* leaf extract was that; reduction occurs very fast as within 60 min reaction becomes stable.

## OBSERVATION:

After 10 days of inoculationof row 3, 5, 7, 9, row 3,5,7 was observed with mild symptoms.

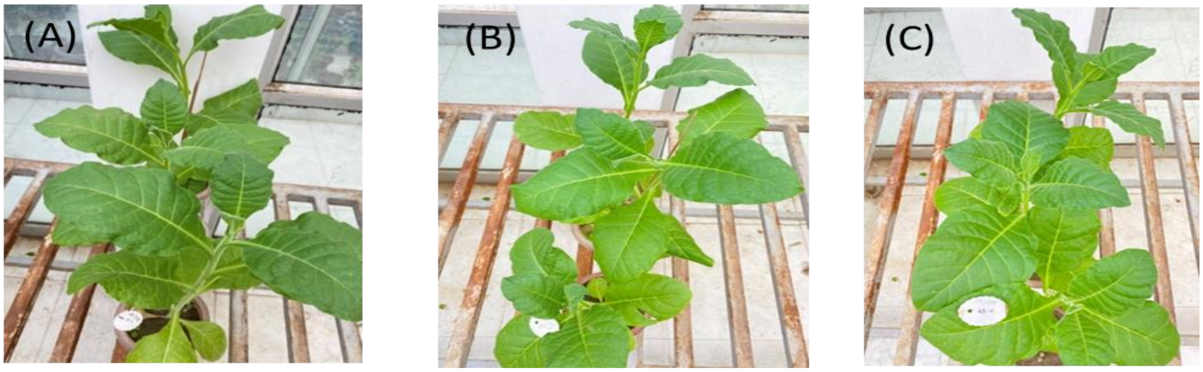



**Fig 9:** (a-c) Plant treated with AgNPs for showing response against the virus challenge**.**

No symptoms were observed on rows – 4, 6, 8 & 10 which were inoculated after 7 days of treatment with NPs.

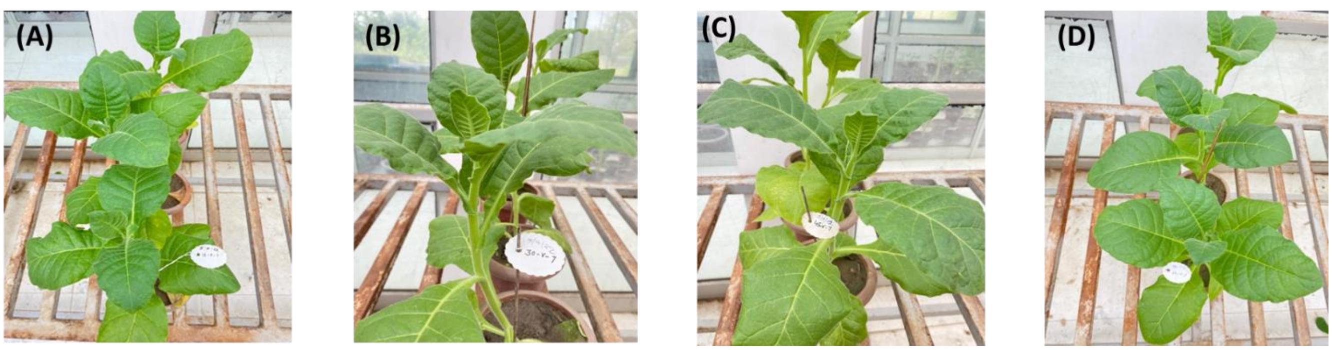



**Fig 10**: (A-D) Plant treated with AgNPs for showing response against the virus challenge after 7 days.

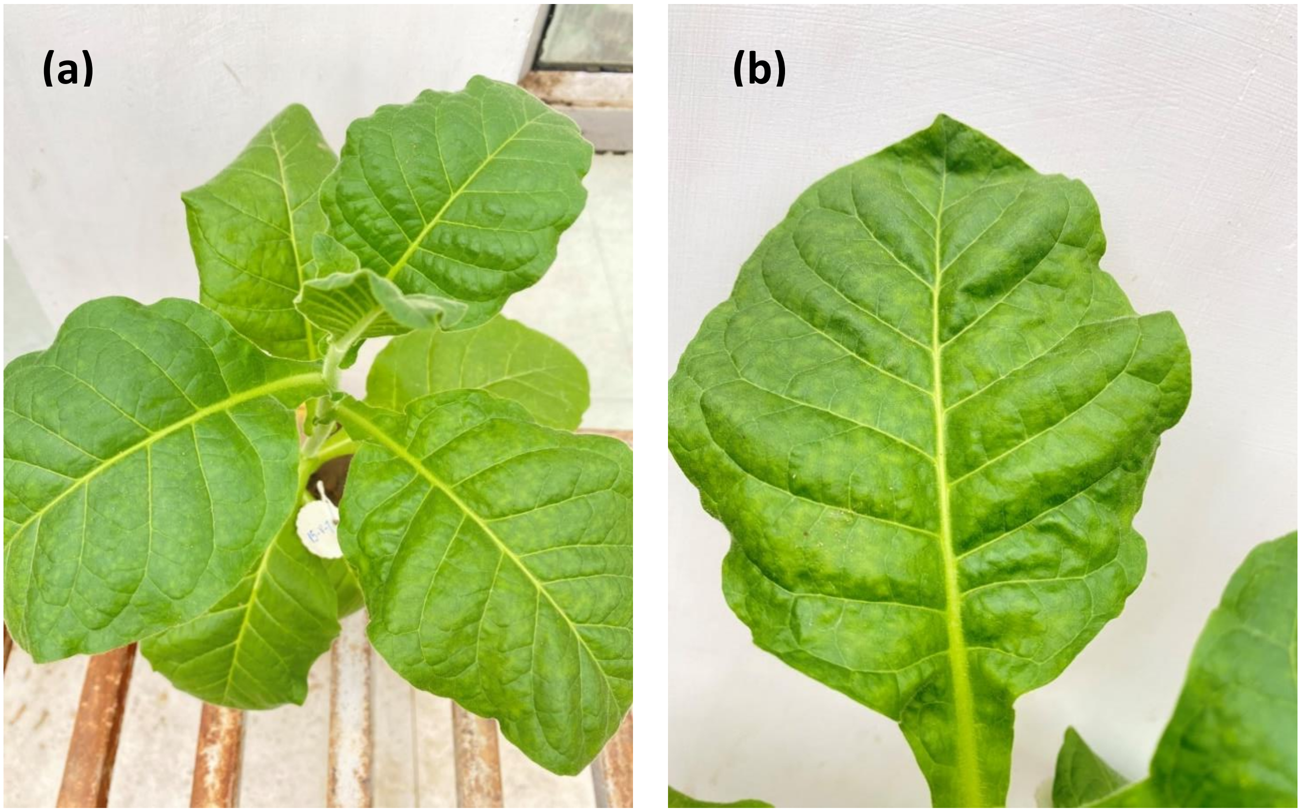



**Fig. 11 :** Plant showing severe symptom (a) Plant (b) Single leaf.

As described above when tomato virus was mechanically inoculated to several host plants, positive response was shown by tobacco and tomato plant species only as shown in image below.

**Fig. 12:** N. tabacum is showing yellow spotted symptoms, these spotted symptoms were spread all over the inoculated leaf after 2 weeks.

**Fig.13:** Tomato plant inoculated by tomato virus showing shoe string symptoms spread all over the plant.

# CHAPTER-5

# CONCLUSION

# CONCLUSION

Theabove review concludes that silver nanoparticles which have properties ofanti-microbial anti-fungal and anti-viral when synthesized using naturally occurring biomaterials (green/eco-friendly materials) result in toxicity free products without disturbing the environmentand also it is an economical approach for a wide variety of treatment which could also change the fate of pharmaceutical field in upcoming days.

Silver nanoparticles can be obtained by physical, chemical, and biological synthesis methods. The synthesis of silver nanoparticles using *Rivina humilis* plant extract is beneficial as it is cost effective, energy efficient, low cost and supplemented to that it is protecting human health and environment leading to lesser waste and safer products. Green synthesized silvernanoparticles have significant aspects of nanotechnology through unmatched applications and synthesis of nanoparticles using plants can be beneficial over other biological entities, which can overcome the time consuming process of employing microbes and maintaining their culture which can lose their potential during biosynthesis of nanoparticles.

Thus the present thesis showed the importance of plant mediated synthesis of silver nanoparticles by conferring the various literatures reported recently. With the vast plant diversity much more plant species are in way to be exploited and reported in future era towards rapid green synthesis of metallic nanoparticles.

# CHAPTER-6

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