GREEN SYNTHESIS OF SILVER NANOPARTICLES USING NEEM LEAVES AND STUDY OF THEIR ANTIMICROBIAL ACTIVITY

A Dissertation submitted in partial fulfillment for the Degree of

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In

NANOSCIENCE AND TECHNOLOGY

Submitted By

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CERTIFICATE

This is to certify that the dissertation entitled **"Green Synthesis of Silver Nanoparticles using Neem Leaves and their Antimicrobial Activity"** submitted by Ms. Aparajita (2K12/NST/04) to Delhi Technological University (Formerly Delhi College of Engineering) in the partial fulfilment of the requirements for the award of the degree of **Master of Technology** in **Nanoscience and Technology (Applied Physics Department)** is a *bonafide* record of the candidate's own work carried out under my supervision. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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DECLARATION

I hereby declare that the Report on **"Green Synthesis of Silver Nanoparticles using Neem Leaves and their antimicrobial activity"** which is being submitted to the Delhi Technological University, in partial fulfillment of the requirements for the award of the degree of **Master of Technology** in **Nanoscience and Technology** in the **Applied Physics Department**, is carried out by me.

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ABSTRACT

In the present work, Silver nanoparticles were synthesized using aqueous extract of Neem (*Azadirachta indica*) leaves. Silver salt was added to aqueous leaf extract, which was used as reducing as well as capping agent. Characterizations of the synthesized nanoparticles have been done using XRD, SEM, optical absorption and emission. The absorbance maxima was observed at 410nm for Neem. The presence of biomolecules responsible for reduction of metal ions in Neem leaves was studied using FTIR. The effect of various parameters like concentrations, reaction pH, mixing ratio of the reactants, temperature and interaction time on the morphology and size of synthesized silver nanoparticles were studied. Silver nanoparticles of spherical shape were obtained. Green synthesis of silver nanoparticle were found to have enhanced antimicrobial property and showed zone of inhibition against isolated bacteria from garden soil sample. Based on the results obtained it can be said that the resources obtained from plants can be efficiently used in the production of silver nanoparticle and it could be utilized in various fields such as biomedical, nanotechnology and so on.

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ABBREVIATIONS

AAS	Atomic absorption spectroscopy
Ag NP	Silver nanoparticle
DNA	Deoxyribonucleic Acid
DOS	Density of States
FTIR	Fourier Transform Infrared
NP	Nanoparticle
OD	Optical density
PL	Photoluminescence
ROS	Reactive Oxygen Species
Rpm	Revolution per minute
SEM	Scanning Electron Microscope
SNPs	Silver Naoparticles
SPR	Surface Plasmon Resonance
UV-VIS	Ultraviolet-Visible
XRD	X-ray diffraction

CHAPTER 1

OVERVIEW AND BACKGROUND RESEARCH

1.1 Introduction

Richard Feynman's famous talk "There is plenty of room at the bottom" laid the foundation to revolutionize the field of nanotechnology [1]. Nanotechnology is an interdisciplinary field which includes physics, chemistry, biology, material science and medicine. The field of nanotechnology is one of the most active research field and is based on synthesis of small sized nanoparticles which has at least one dimension in the range of 1-100nm(10^{-9}) .Nanotechnology is offering wide range of applications in the field of biosensors, bionanotechnology, biomedicine etc. Nanoparticles are being used for the treatment of various diseases like cancer and also in gene therapy and targeted drug delivery.

Silver nanoparticles are one of the promising researches in the nanotechnology industry. Researchers are trying to use economical and environment friendly method for the synthesis of silver nanoparticles, where green synthesis has proved to be a promising process. Green synthesis of metal nanoparticles is gaining importance because of their biocompatibility, low toxicity and eco-friendly nature [2]. The green synthesis methods include synthesis of nanoparticles from bacteria, fungus, yeasts [12], plants [13] and DNA [14]. The biosynthesis method employing plant extracts like *Pelargonium graveolens* , *Medicagosativa, Azadirachta indica, Lemongrass, Aloevera, Cinnamomum Camphora* [15][18], have drawn great attention as an alternative to conventional methods because plants are found in abundance in nature .

Azadirachta indica is common plant also known as Neem, which is found abundantly in India and nearby Indian subcontinents like Nepal, Bangladesh and Sri Lanka etc. It belongs to *Meliaceae* family and is known for its various applications especially its medicinal property [17]. *Azadirachta indica* leaf extract is used in the synthesis of various nanoparticles like gold, zinc oxide, silver etc. The phytochemicals present in Neem are terpenoids and flavones which act as reducing as well as capping agent and help in stabilizing the synthesized nanoparticles. When silver salt is treated with Neem leaf extract, the silver salt is reduced to silver nanoparticles. The synthesized nanoparticles are capped with neem extract and also exhibit enhanced antibacterial activity.

Silver nanoparticles are found to be non-toxic to humans but at low concentration they are effective against microorganism like bacteria, virus and fungi. Antimicrobial capability of SNPs allows them to be suitably employed in numerous household products such as textiles,

food storage containers, home appliances and in medical devices [19]. The most important application of silver and SNPs is as tropical ointments to prevent infection against burns and open wounds.

1.2 Scope of the Research:

The present project aims at the green synthesis of silver nanoparticles and to study their antibacterial activity. The objective of the project can be summarized as:

- Synthesis of silver nanoparticles using Neem leaves (*Azadiracta indica)* .
- Characterization of synthesized nanoparticles using Absorption spectra,Emission spectra, SEM, XRD, FTIR.
- To study the effect of various physico-chemical parameters like reaction time, pH, temperature and reactant concentration on the synthesis of nanoparticles.
- To culture bacteria from garden soil sample.
- To study the antimicrobial effect of the synthesized silver nanoparticles .

1.3Literature Overview

1.3.1 What is nanotechnology?

Nanotechnology is the most active research field and is based on synthesis of small sized nanoparticles which has at least one dimension in the range of 1-100nm (10^{-9}) [22, 28]. Nanoparticles are the fundamental unit of nanotechnology. Nanosciences and nanotechnology aims at creating material at nanoscale with large surface area to volume ratio so as to produce particles with enhanced physico-chemical properties. When the size of the particles changes from bulk to nano level, various changes in properties can be observed like, changes in magnetic susceptibility, improved electrical conductivity [30], reactivity, strength and colour change [29,31]. These enhanced properties can be exploited in a wide range of applications like water purification, for example removal of arsenic from water using nanomaterials, in cosmetic and food industry renewable clean energy and aerospace developments. Nanotechnology will potentially lead to great impacts in terms of energy saving and minimal harm to the environment The nanotechnology sector has a large market worldwide which is expected to grow 1 trillion US dollars by 2015 [21], and 3 trillion US dollars by 2018 [23].

Various nanoparticles have been synthesized till now like gold, silver, zinc oxide etc. which has been used in various sectors due to their enhanced properties [3].

1.3.2 Basic Concepts

One nanometer (nm) is equal to one billionth part of a unit scale, or 10^{-9} of a meter. Driven by the motivation of nanomaterials present in nature, scientists have been using various methods for the synthesis of nanoparticles. A DNA double helix has a diameter of 2 nm, whereas bacteria example, Mycoplasma is 200 nm in length. The size of a virus is 25-250 nm. By convention, nanotechnology is concerned with natural and synthetic materials in the range of 1 to 100 nm, as defined by the National Nanotechnology Initiative (US). The lower limit is set by the size of atoms since nanotechnology devices are build by atoms and molecules. The upper limit is more or less arbitrary. These new phenomena make nanotechnology different from devices which were miniaturized version of an equivalent macroscopic device. As the size of the material changes from bulk to nano range their properties changes. The three basic property of a nanomaterial which makes them distinguishable from bulk material are [2]:

- Larger Surface to Volume Ratio
- Quantum Confinement
- Quantized Energy States

According to Dr. K. Eric Drexler, Nanotechnology can be used in [1];

- Nearly free consumer products
- PCs billions of times faster than today
- Safe and affordable space travel
- Virtual end to illness, aging, death
- No more pollution and automatic cleanup of existing pollution
- End of famine and starvation
- Reintroduction of many extinct plants and animals.

Properties of the solids are size dependent. The properties of the bulk materials are mostly retained till their size is reduced to micrometer range. When the size of materials is reduced to nano level, the material does not exhibit its normal characteristics. For example, consider the case of gold nanoparticles, it does not show characteristic yellow color as the size of gold is reduced to nano level. Gold show different colors like orange, red, purple or greenish

depending upon the actual size of the particles. Compared to bulk, the melting point and chemical properties also change when the material is reduced to nano scale. The melting temperature of nano form reduces significantly. Electrical conductivity changes and the material become insulators when dimensions are reduced to nanometers [5]. The primary cause for the drastic change in behavior of nanomaterials compared to their bulk form is that in nano level the number of atoms on the surface is a big fraction of the total number of atoms in the material i.e. large surface to volume ratio.

1.3.3 NANOPARTICLES: Deviation from Bulk Behavior

Nanoparticles are structures consisting of atoms or molecules, with a size range of 1- 100 nm (or 1000 A^0 as 1 nm = 10 A^0). They have large surface area to volume ratio which makes them different from bulk material and changes the property of the material.

Particles of different sizes scatter different wavelengths of light. This fact has been used from ancient times to produce beautiful colors in stained-glass, due to the presence of the nanomaterials in the glass having size comparable to the wavelength of light. What makes nanoparticles very interesting and endows them with their unique properties is that their size is smaller than critical lengths that characterize many physical phenomena. Generally, physical properties are associated with some characteristic length such as scattering length, thermal diffusion length, etc. The electrical resistance of a metal is dependent upon the mean free path or scattering length, the electrons travel between any two successive collisions.

1.3.4 Dimension Effects

When the size of a material is reduced to nanometer range (<100nm), we observe a change in the properties of the material. When the material is confined in nano range in one dimension it is called nanotube (2D). If it is confined in two dimensions it is nanowire (1D) and when it is confined in all the three dimensions it is known as quantum dot (0D). The confinement of a particle in one dimension, two dimensions and three dimensions can be deal as the case of **"particle in a potential box"**.

For a particle in a box of length **a** with potential

$v = 0 \quad 0 < x < a$

$v = \infty \quad x < 0 \quad or \quad x > a$

The solution of the Schrodinger equation yields eigen values.

$$E_n = \frac{[(n]^2 * h^2)}{8 * m * a^2} \qquad \textbf{...(2.1)}$$

And eigenfuctions as

$$\varphi = A \sin\left(\frac{n * \pi}{a}\right) x \qquad \ldots(2.2)$$

1.3.5 Density of States

Density of status *D(E)is defined as the number of states per unit energy per unit volume (E and E+ dE)*. It is an important quantity. It enables to gain understanding of various spectroscopic properties of materials. As the energy of a particle in a one-dimensional box is

$$E_n = \frac{h^2}{8ma^2} n^2$$

$$\frac{dn}{dE} = \frac{8ma^2}{h^2}\frac{1}{2n} = \frac{a}{h}\frac{\sqrt{2m}}{E}$$

or

$$D(E)\left(= \frac{dn}{dE}\right) E^{-\frac{1}{2}} \qquad \textbf{…. (2.3)}$$

This shows how the density of states will vary with change in energy. Density of states is inversely proportional to the square root of energy. We discuss below the variation or density of states for solids of different dimensions.

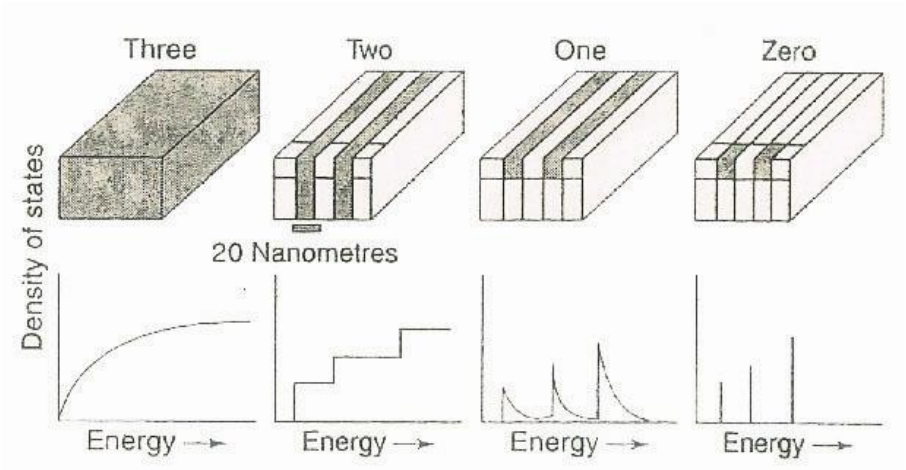


Fig 1.1: Density of state Vs Energy (Graph)

1.3.6 Density of States for a Zero Dimensional (0D) Structure

Neglecting the periodic potential existing in solids, we can imagine a zero dimensional solid in which electron is confined in a three dimensional potential box with extremely small (<100nm) length, breadth and height as a 0-D solid. This will have a discrete energy level given by:

$$D(E) = \Sigma\delta\ (E - \varepsilon_i) \qquad \ldots(2.4)$$

Where the summation is over all *i* quantum states and ε_i are the discrete energy levels and δ is Dirac function.

1.3.7 Density of States for a One-Dimensional (ID) Structure

A particle confined in a one direction is like a particle in a one dimensional potential well. However the potential in two directions is infinitely large but in the third dimension it is zero. The density of states in this case is expressed as

$$D(E) = \frac{dN}{dE} = \sum_{(1<E} (E - (i)\frac{1}{2} \qquad \ldots(2.5)$$

Where ε_i are discrete energy levels. The graphical representation of density of states for 1D structure is shown in above figure 2.1.

1.3.8 Density of States for Two-Dimensional (2D) Structures

For 2D structures which is basically a thin film structure (two directions long, one direction < 100 nm). The density of states is given by

$$\mathbf{D(}E\mathbf{) = 0 \text{ for } E < \varepsilon_I}$$

$$\mathbf{= 1 \text{ for } E > \varepsilon_I} \qquad \ldots(2.6)$$

Where ε_I is the *i*th energy level within 2D quantum well

The graphical representation of density of states for 2D structure, a staircase structure, is shown in Fig. A.

1.3.9 Density of States for Three-Dimensional (3D) Structures

For a 3D structure which is equivalent to a three-dimensional box of length *a*, width *b* and height *c* with potential $V = 0$ inside the box and $V = \infty$ outside the box, the solution of Schrodinger equation shows that the energy states are:

$$E_{n_x, n_y, n_z} = \frac{(h^2)}{8 \cdot m \cdot a^2} (n^2_x + n^2_y + n^2_z)$$

And the density of states in this case

$$D(E) \propto E^{1/2} \qquad \ldots(2.7)$$

1.3.10 Silver Nanoparticles

AgNP were first synthesized in 1951[32]; using chemical method where silver nitrate acted as Ag+ source and sodium citrate were the reducing agent. Since then, silver nanoparticles have been intensely investigated by scientists who altered its shape and size to obtain a novel metal with enhanced conductivity, strength, catalysis[4] ,bactericidal activity, catalytic behavior etc [26]. Silver nanoparticles because of its novel properties and low cost of manufacture have been used in various applications like in microelectronics [24], bacterial disinfectant, in cosmetic, food and textile industry.

Two different approaches for the synthesis of nanoparticles have been used till now. These are Top down approach and Bottom up approach. In case of Top down synthesis, the nanoparticles are synthesized starting from bulk materials eg. Ball milling, Laser ablation, etching etc. whereas in the Bottom up approach, nanomaterials are synthesized by nucleation of smaller atoms, example biological method [27]. Nanomaterials can be produced by 3 methods: physical method, chemical method and biological method. Physical and chemical methods include ball milling, laser ablation, lithography, sol gel method which are very useful in preparing monodispersed nanoparticles[27].The nanoparticles thus formed has shorter lifetime and tends to agglomerate. To prevent the agglomeration of nanoparticles capping agents are used (eg. Citrate, polymer etc.).These capping agents can sometimes alter the desired properties of the formed nanoparticles. Some of the methods for the synthesis of silver nanoparticles are mentioned in the table below:

Table 1.1.Commonly used physical and chemical techniques for Ag NP production

Techniques	Method	Reference
UV photolysis	physical	33
Metal vapour deposition	chemical	34
Thermal decomposition	physical	35
Sonochemical decomposition	chemical and physical	36
Electrochemical techniques	chemical	37
Laser ablation	physical	38
Microwave plasma synthesis	chemical	39
Biosynthesis/Green synthesis	physical	40

However physical and chemical methods of synthesis are very expensive and involve the use of hazardous chemicals. There is a need for cost effective and eco friendly method for the synthesis of nanoparticles. Other method for the synthesis of nanoparticles is the biological method, which include synthesis of nanoparticles using microbes like bacteria, fungi [12], yeast, plant leaf extract [13], enzymes and DNA [14]. This method is also called the green synthesis method because it is environment friendly. This method does not make use of toxic chemicals. Instead it uses plants and microbes which are easily available in the environment and do not have negative impact on the environment. Green synthesis method is cost effective, environment friendly and easy .However this method is comparatively slow and produces polydispersed nanoparticles. Studies are being done to synthesize nanoparticles of uniform morphology using this method.

1.3.11Biological synthesis of silver nanoparticles

The chemical or physical methods for the production of silver nanoparticles are extremely expensive and not eco friendly. Hazardous chemicals and many toxic materials are used in these methods which are harmful to the environment. Because of the use of silver nanoparticles in various fields, it is essential that silver nanoparticles should be available at low cost, thus, there is a need for a cheap and eco friendly method for the production of silver nanoparticles. The problem was solved by the use of biological method for the synthesis of silver nanoparticles. The growing need for a cheaper , non toxic and environment friendly method for the synthesis of nanoparticles have lead to the discovery of biological method of synthesis , which include synthesis using microbes like bacteria, fungi, yeasts, or plant extracts and DNA. This method is better than chemical methods as it can be used in many

medical applications because of its non toxic nature. Biological method of synthesis of nanoparticles is a bottom-up approach and is considered to be a very attractive possibility to have green synthesis. The mechanism of action involved in the synthesis for metal nanoparticles includes oxidation or reduction method. Cells are capable of reacting with metal ions by processes like oxidation, methylation, demythlation etc. In microorganism enzymes act as the reducing agent whereas in case of plants, phytochemicals act as an antioxidant or exhibit reducing properties.

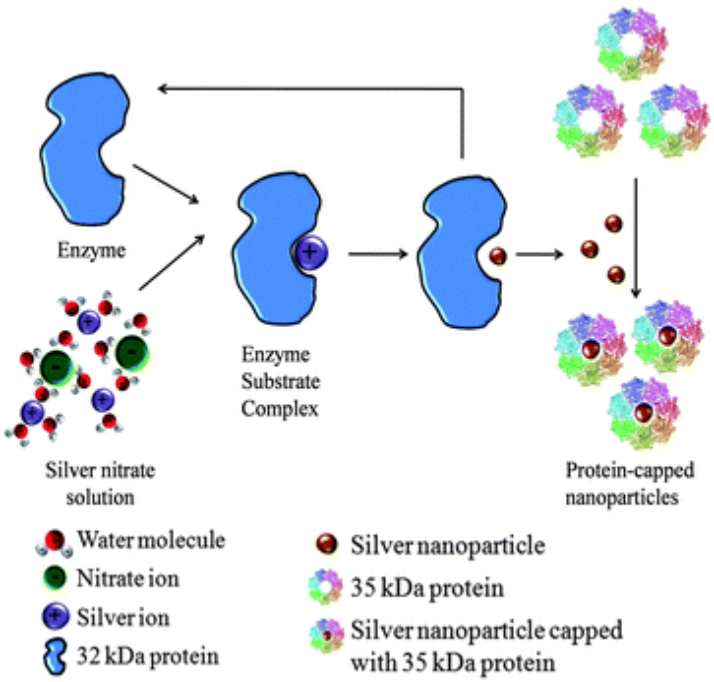


Fig 1.2 Mechanism of silver nanoparticles synthesis

Table 1.2 : Silver Synthesizing microbes and the particle size [41]

Serial number	Organism	Particle size (nm)
1	*P. stutzeri* AG259	200
2	*Bacillus megaterium*	46.9
3	*Plectonema boryanum*	1 to 200
4	*Enterobacter cloacae*	50 to 100
5	*Escherichia coli*	5 to 25
6	*B. licheniformis*	50
7	*Lactobacillus fermentum*	11.2
8	*Klebsiella pneumonia*	50
9	*Proteus mirabilis*	10 to 20
10	*Brevibacterium casei*	50

However, the use of microorganism in the synthesis of nanoparticles is a very slow process. This has led to the use of plant extract in the synthesis of nanoparticles. The advantage of using plant products for the synthesis of silver nanoparticles is that they are easily available in the environment, are non toxic and do not cause harm to the environment. It is a fast process as compared to the use of microorganism and plants have a broad range of metabolites (eg. phytochemicals) that help in the synthesis of nanoparticles. For the synthesis of nanoparticles three main components are required, which are reducing agent that help in the reduction of silver salt, solvent medium and stabilizing and capping agent.

Phytochemicals, present in plants, are responsible for the reduction process. The phytochemicals are those which are responsible for the colour in plant for example the red colour of tomato is due to the presence of phytochemicals. Terpenoids, flavanoids,carboxylic acid, ketones, aldehydes are few main phytochemicals present in plants. Flavones and quinines are water soluble phytochemicals which enhance the reduction reaction. It has been investigated that silver nanoparticles are formed by the reaction with an anthaquinone: emodin, benzoquinone: cyperoquinone, dietchequinone, and remirin present in xerophytic and mesophytic plants respectively. Although different plants have different phytochemicals present in it, the mechanism of nanoparticle synthesis involves reduction of metal salts by the phytochemicals. .

Table 1.3: Silver Synthesizing plants and the particle size [41]

Serial number	Organism	Particle size (nm)
1	*Medicago sativa*	2 to 20
2	*Azadirachta indica*	50
3	*Aloe vera*	15 to 20
4	*Cinnamomum camphora* leaf	55 to 80
5	*Carica papaya* fruit	15
6	*Cinnamomum zeylanicum* bark	50 to 100
7	*Jatropha curcas*	10 to 20
8	*Desmodium triflorum*	5 to 20
9	*Coriandrum sativum* leaf	26
10	*Piper betle* leaf	3 to 37

1.3.12 Synthesis of silver nanoparticle using neem leaf

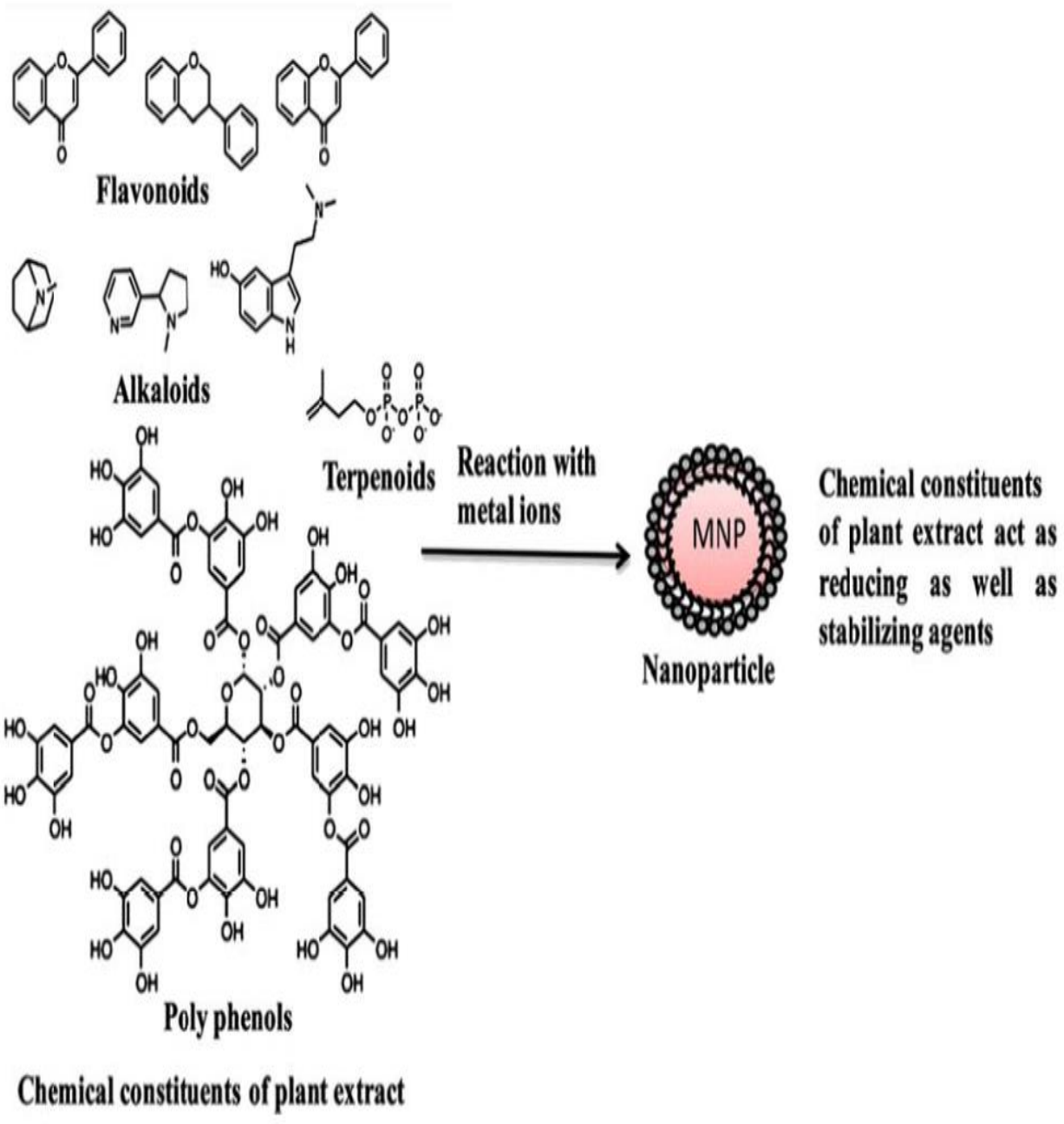
Azadirachta indica is common plant also known as Neem, which is found abundantly in India and nearby Indian subcontinents like Nepal, Bangladesh, Sri Lanka etc. It belongs to *Meliaceae* family and is known for its various applications especially its medicinal property.

Kingdom:	Plantae
Division:	Magnoliophyta
Order:	Sapindales
Family:	Meliaceae
Genus:	*Azadirachta*
Species:	***A. indica***

Neem is an evergreen plant and its stem, leaves, roots are used for various medicinal purpose in different households [19]. Neem is eaten as vegetables. It is common medicinal plant which shows antibacterial, antihelminthic and antiviral properties. *Azadirachta indica* leaf extract is used in the synthesis of various nanoparticles like gold, zinc oxide, silver etc. The phytochemical present in is terpenoids and flavones. These phytochemicals act as reducing as well as capping agent and help in stabilizing the synthesized nanoparticles. When silver salt

is treated with Neem leaf extract, the silver salt is reduced to silver nanoparticles. The synthesized nanoparticles is capped with neem extract and also exhibit enhanced antibacterial activity.

In case of synthesis of silver nanoparticles, salt like silver nitrate ($AgNO_3$) is used. The phytochemicals present in Neem leaves reduce silver nitrate to atoms which then nucleate to form a nanoparticle. These atoms have a tendency to form large particles due to agglomeration. To prevent this capping and a stabilizing agent is used. In case of Neem leaves, the leaf extract act as the capping as well as the stabilizing agent. The size and shape of the silver ion depends upon various physic-chemical parameters like temperature, concentration of silver salt to reducing agent, pH etc. By varying different parameters, the size and shape of the particles can be controlled. Therefore, the two main components important for the synthesis of any metal nanoparticle is the reducing agent and the metal salt.



1.3 Mechanism of action of neem leaf extract with silver salt to form nanoparticles

1.3.13 Importance of Silver nanoparticles

1) It is used for purification and quality management of air, biosensing, imaging, drug delivery system.

2) Biologically synthesized silver nanoparticles have many applications like coatings for solar energy absorption and intercalation material for electrical batteries, as optical receptors, as catalysts in chemical reactions, for biolabelling, and as antimicrobials.

3) Though silver nanoparticles are cytotoxic but they have tremendous applications in the field of high sensitivity bimolecular detection and diagnostics, antimicrobials and therapeutics, catalysis and micro-electronics.

4) It has some potential application like diagnostic biomedical optical imaging, biological implants (like heart valves) and medical application like wound dressings, contraceptive devices, surgical instruments and bone prostheses.

Hence, the aim of present study is to synthesize and characterize silver nanoparticles by using aqueous Neem leaf extract and to see the antimicrobial activity.

1.3.14 Action of silver nanoparticles on microbes

The exact mechanism employed by silver nanoparticles against microbes is yet not understood clearly. However various theories on the antimicrobial activity of silver nanoparticles has been given by various scientist. Silver nanoparticles have the unique ability to attach and penetrate the cell wall of bacteria. Formation of 'pits' on the surface of the cell causes accumulation of silver nanoparticles. Once these silver nanoparticles enter the cell wall of bacteria it causes many structural changes in the cell membrane of these organisms like change in the permeability of the cell membrane leading to the death of the cell as there is accumulation of the nanoparticles on the cell surface. Another mechanism by which silver nanoparticles kill the bacteria is the formation of free radicals by them. The formation of free radicals by the silver nanoparticles was suggested by spin resonance spectroscopy, according to which when these free radicals come in contact with the cell membrane it damage the cell and caused a change in its permeability by making the membrane porous leading to cell death. Another theory suggested that release of silver ions by the nanoparticles interacted with the thiol group present in many vital enzymes in the cells and thereby inactivate them. When the silver ions come in contact with the bacterial cell, it enters the cell and damages it by inhibiting various functions inside the bacterial cell. Silver ions inhibits the respiratory enzymes in the cell and produce reactive oxygen species (ROS) which is harmful to the cell. Silver acts as soft acid. According to the rules of chemistry, base reacts with acid, in case of silver, a soft acid with react with soft base. Sulphur and phosphorous which are present in the

cell are soft bases. The hereditary unit of any organism is deoxyribonucleic acid (DNA). DNA is made up of nucleotides and has sugar phosphate backbone. Nanoparticles being a soft acid will react with sulfur and phosphorous and lead to inhibition of DNA replication in bacteria and cell cycle will stop and the microbe is killed. Another theory suggests that the nanoparticles also alter the signal transduction pathway in the microorganism. Signal transduction in bacteria involves the phosphorylation of protein substrate except in gram negative bacteria where there is dephosphorylation in tyrosine residue. The nanoparticles causes a change in the tyrosine of bacterial peptides. The peptide substrates present on tyrosine residues was dephosphorylated by the silver nanoparticles, which stops the signal transduction pathway transduction inhibition and the growth of bacteria is ceased. Many theories related to the interaction of silver nanoparticles with the bacterial cell is known.

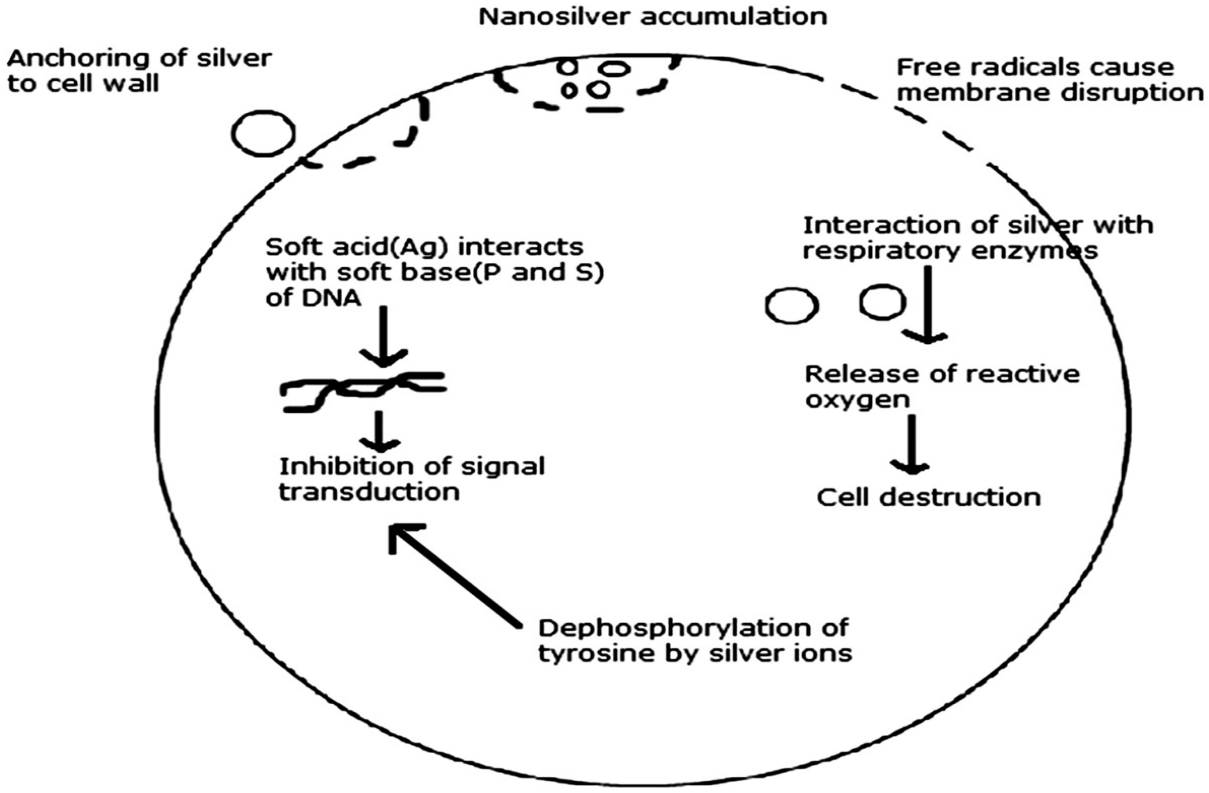


Fig1.4: Various modes of action of silver nanoparticles on bacteria

1.3.15 Protein inactivation

The antibacterial property of silver has been known since ages when our ancestors used utensils made up of silver but the mechanism involved in inhibition of microbes by silver has been understood recently. There are various mechanisms by which silver inhibits the growth

of microorganism. Silver reacts with the thiol (-SH) group of enzymes present in the cell to form a stable structure of S-Ag, thus deactivating the enzyme. The thiol containing compound in the protoplast of the bacteria is responsible for ion transport and energy generation. However, when silver binds the thiol group ion transport and other activities are stopped. The basic mechanism is that silver forms disulphide bond during a catalytic reaction (R-S-S-R) between hydrogen bond in the thiol group and oxygen in the cell. In this process ,water is released and two thiol groups come together via a disulfide bond [25]. This leads to a change in the structure of the enzyme and alter its function.

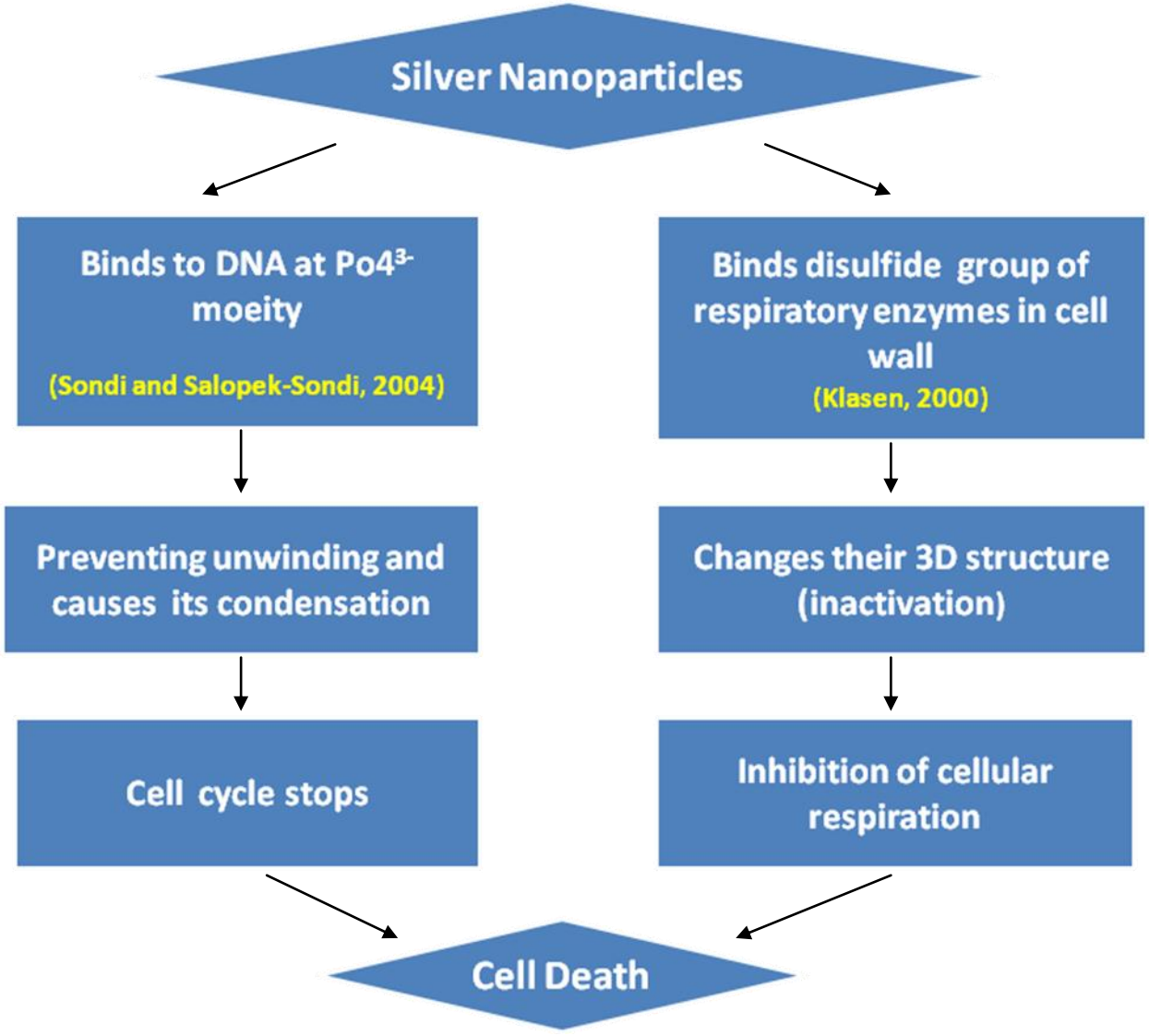


Fig 1.5 Flowchart of silver nanoparticles leading to bacterial cell death

It was observed that the expression of many respiratory enzymes like succinyl co A, fructose biphosphote, aldolase,etc decreased when they were treated with 900ppm of silver solution

[25]. Succinyl co A is the enzyme used in Kreb cycle which converts succinyl-CoA to succinate. Similarly, fructose biphosphate and aldolase are important enzymes in glycolysis which are responsible in the conversion of fructose-1,6-bisphosphate into glyceraldehydes 3-phosphate. Alteration in the structure of these enzymes due to the interaction with silver ions leads to the inhibition of the related pathways and ultimately cell death. Similarly, many other enzyme activities were significantly reduced due to the disulphide formation and hence change in their three- dimensional structure was observed. It was also studied that silver ions bind to the 30S ribosomal subunit, one of the important factor in protein synthesis in prokaryotes. This lead to the deactivation of the ribosomal complex and protein synthesis was stopped. In all the above mentioned cases, the enzymes involved had a major role in energy (ATP) production and their decreased expression lead to cell death.

1.3.16 DNA association

Besides the above mentioned mechanism, a novel mechanism of the antibacterial activity of silver was also proposed. DNA is a double helical structure made up of purines and pyrimidines which pairs using weak hydrogen bonds. When silver enters into the cell it breaks the hydrogen bond between the two antiparallel strands by intercalating between the purines and pyrimidines. This leads to denaturation of DNA molecule.

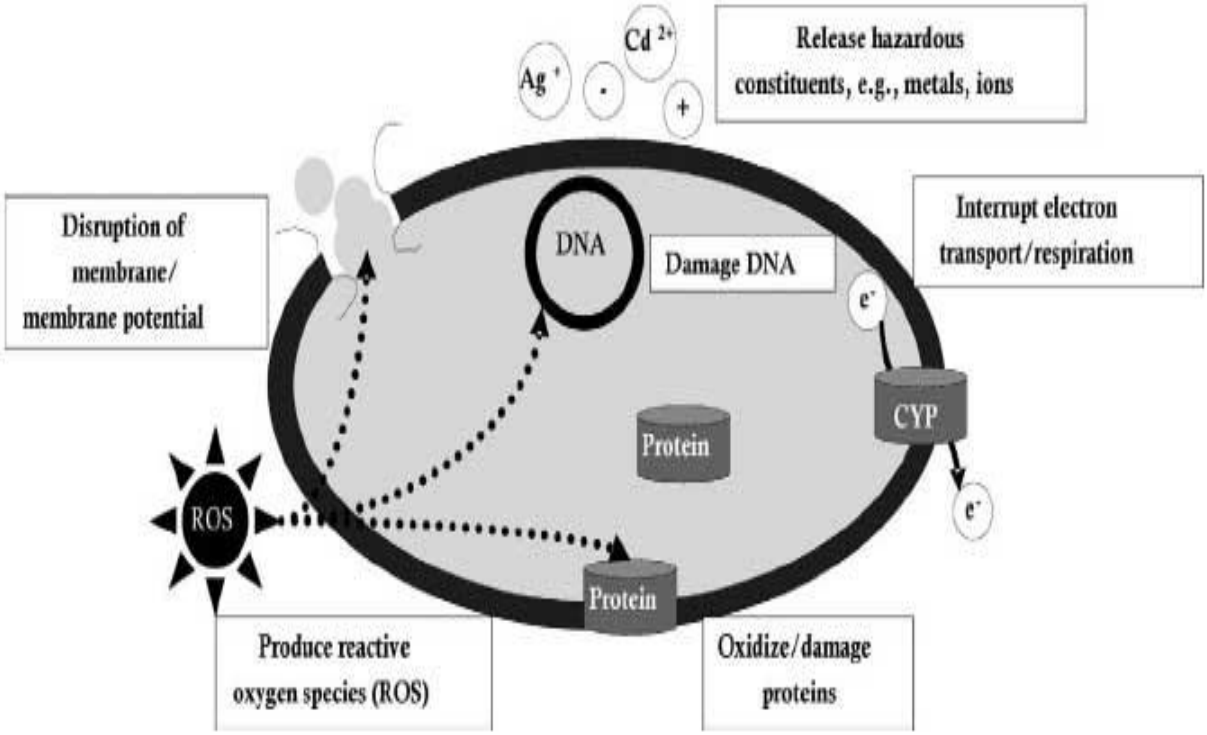


Fig1.6: Mechanism of Antimicrobial action of nanoparti

CHAPTER 2

MATERIALS AND METHODS

2.1 CHARACTERIZATION INSTRUMENT

The synthesized materials were characterized using UV-Vis-NIR spectroscopy, Photoluminescence, X-Ray diffraction (XRD),Scanning electron microscope (SEM), Fourier transform Infra red Spectroscopy (FTIR) . Structural and phase analysis are done by using XRD Bruker D8 Advanced (Cu $K\alpha = 1.54A^0$), SEM was carried out by using (SEM, Hitachi S–3700). An FTIR spectrum was recorded with a single beam Thermoscientific Nicolet 380.

2.2.X-RAY DIFFRACTION

X-ray diffraction is a versatile analytical technique used to determine the lattice structure and atomic spacing of any material. It is used in determining various crystal forms, also called "phase". It is based on interference between X-rays and the sample. The result is XRD pattern showing different phases that are present i.e. the peak position, phase concentration i.e. the peak height and the amorphous content. The image is obtained only when the Bragg's Law is satisfied which is given by the formula:

($n\lambda = 2d\sin\theta$)

Where, λ= wavelength of the incident light

d= interatomic spacing

θ= diffraction angle

Cathode ray tube is used to produce X-rays which is directed towards the sample. The sample is scanned through a range of 2θ and all the diffraction directions of the lattice can be attained due to the random orientation of the powdered material.

XRD technique is useful in identification of unknown crystalline materials like minerals, inorganic compounds etc. This has proved useful in identifying unknown samples for studies in various fields like biology, geology, polymer etc. This method is also advantageous in determining the dimension of the unit cell and in determining the purity test of the samples etc. Every metal corresponds to a specific peak and these peaks can be used to determine to know about the material present in any sample.

Identification is achieved by comparing the result obtained from an unknown sample with an internationally recognized database containing reference patterns called JCPDS.

Theory

A crystal lattice is a regular 3-dimensional distribution of atoms in space. These are arranged so that they form a series of parallel planes separated from one another by a distance d, which varies according to the nature of the material. For any crystal, planes exist in a number of different orientations, each with its own specific d-spacing.

When a monochromatic X-ray beam with wavelength λ is incident on lattice planes in a crystal at an angle θ, diffraction occurs only when the distance travelled by the rays reflected from successive planes differ by a complete number n of wavelength. By varying the angle θ, the Bragg's law ($n\lambda = 2d\sin\theta$) (Figure F) conditions are satisfied by different d-spacing in polycrystalline materials. Plotting the angular positions and intensities of the resultant diffraction peaks produces a pattern which is characteristic of the sample. Where a mixture of different phases is present, the diffractogram is formed by addition of the individual patterns.

Utility of XRD for Nanomaterials

Many structural properties of the nanostructures such as crystalline phase, particle size and structure evolution in Bragg planes, macroscopic stress/strain etc. can be revealed with the help of XRD. But here we shall mention few aspects directly relevant to our work.

Crystal Identification

A new method has been attempted-to-prepare nanomaterials. Therefore, it is important to identify these materials with the help of XRD data; we can check the amorphous or crystalline nature of the newly formed material. We can determine if the prepared material is crystalline and its basic lattice structure (e.g. cubic or hexagonal, etc.) by indexing its lattice plans. One can also determine the changes in various lattice parameters of a particular bulk and nanosized material.

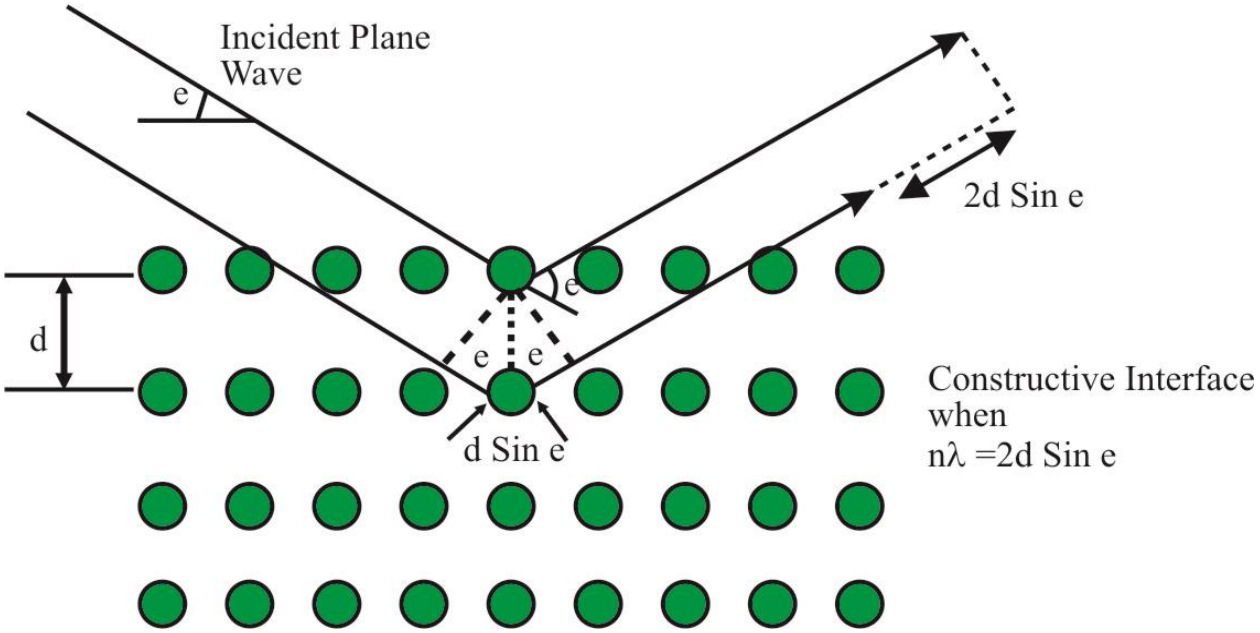


Figure 2.1: Diagram showing Bragg diffraction from a set of planes crystallite Size

Crystallite Size

A perfect crystal would extend in all directions to infinity, so we can say that no crystal is perfect due to its finite size. This deviation from perfect crystallanity leads to a broadening of the diffraction peaks. However, above a certain size (100-500nm) this type of broadening is negligible. Scherrer (1918) first observed that small crystallite size could give rise to line broadening. He derived a well-known equation for relating the crystallite size to the broadening, which is called Debye-Scherrer Formula is given by:

$$Dv = \frac{k\lambda}{\beta cos\theta} \qquad \ldots. (2.1)$$

Where, Dv = Volume weighted crystallite size. D is the "average" dimension of the crystallites normal to the reflecting planes. We call it "average" because the x-ray beam irradiates a large number of crystallites, so that the value of D obtained represents the mean value of the actual size distribution present, λ = wavelength of the radiation, k = Scherrer constant. K varies from 0.89 to 1, but for most cases, is close to 1, β = the integral breadth of a reflection (in radians 2θ) located at 2θ commonly considered as the full width at half maxima (FWHM) in radians for a certain peak position 2θ. Value of Scherer constant used is 0.89.

Crystallite size is a measure of the size of a coherently diffracting domain. Due to the presence of polycrystalline aggregates, crystallite size is not generally the same thing as particle size. When crystallites are less than approximately 100 nm in size, appreciable broadening in x-ray diffraction lines will occur. These regions may, in fact, correspond to the actual size of the particles. At other times, however, these regions form "domains" in a larger particle and may be a distinguishing and important feature. In either case, the observed line broadening can be used to estimate the average size. In the simplest case where the particles are stress-free, the size is estimated from a single diffraction peak. However, in cases where stress may be present, a more robust method involving several diffraction peaks is required.

Sample Used and their Preparation

All the diffractograms are for powder form. We have made powder form of our sample by centrifuging the sample at 10000 rpm for 15 minutes. The precipitate was dissolved in water and then dried in oven at 110°C so that the water gets evaporated and the powder form of the sample is obtained.

2.3 Scanning electron Microscopy

Sem is done to study the surface morphology of the synthesized nanoparticles. Scanning electron microscope was first discovered in the year 1942, but it was used commercially in the year 1965. It is a type of electron microscope which is used to get highly magnified image of an object. Electron microscopes are similar to light microscope except that it uses beam of electron produced from electron gun, made up of Tungsten (W), instead of light.

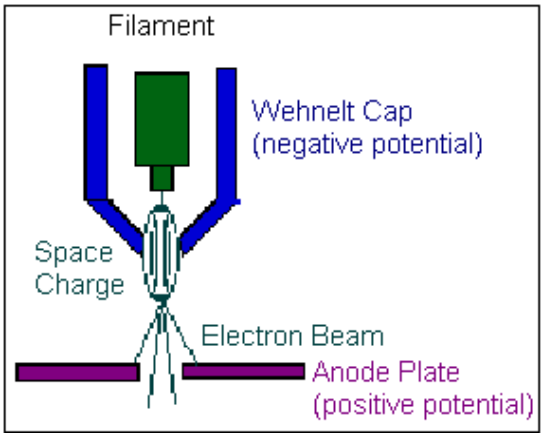


Fig 2.2 Electron Gun

Scanning electron microscope is used to study the surface morphology of an object. It does not give information about the internal structure of the object. A beam of electron is focused on the specimen at a voltage between 2 to 50kV. The diameter of the electron beam is 5nm-2μm. When the electron strikes the specimen secondary electrons, backscattered electrons and X-rays are produced which helps in forming the final image.

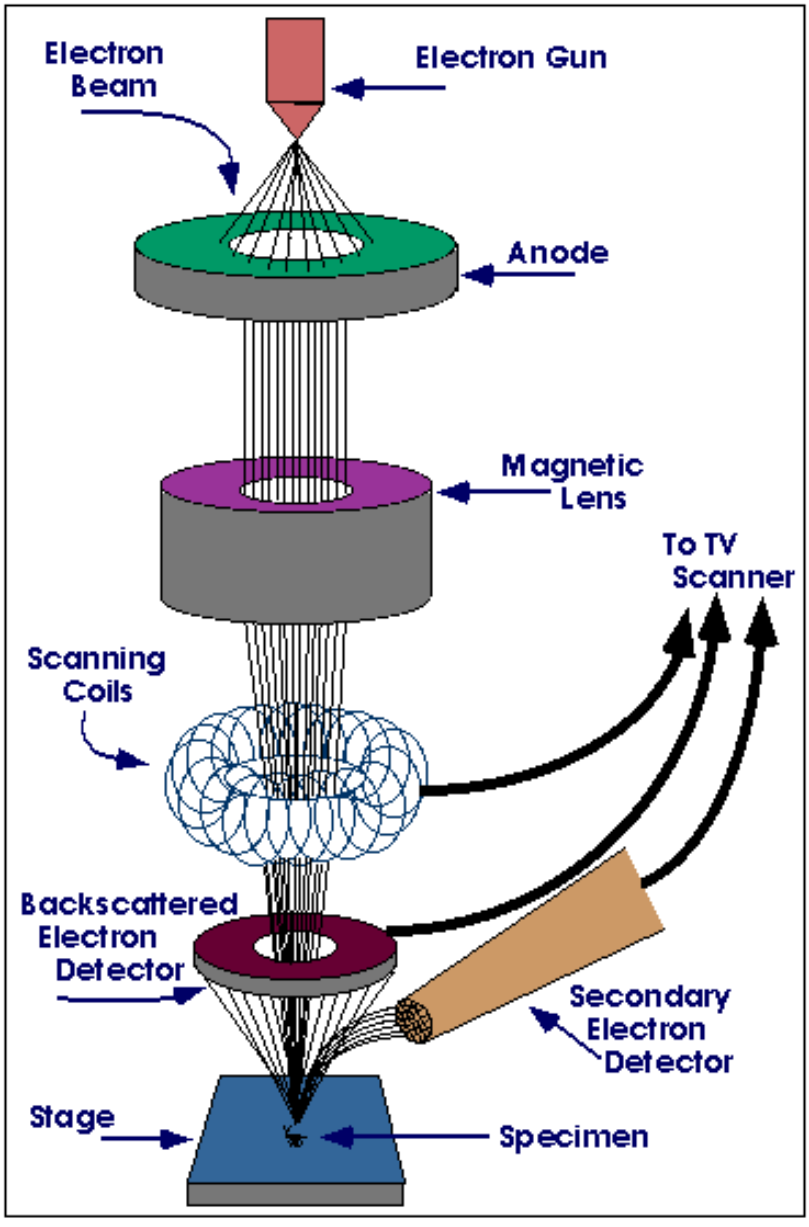


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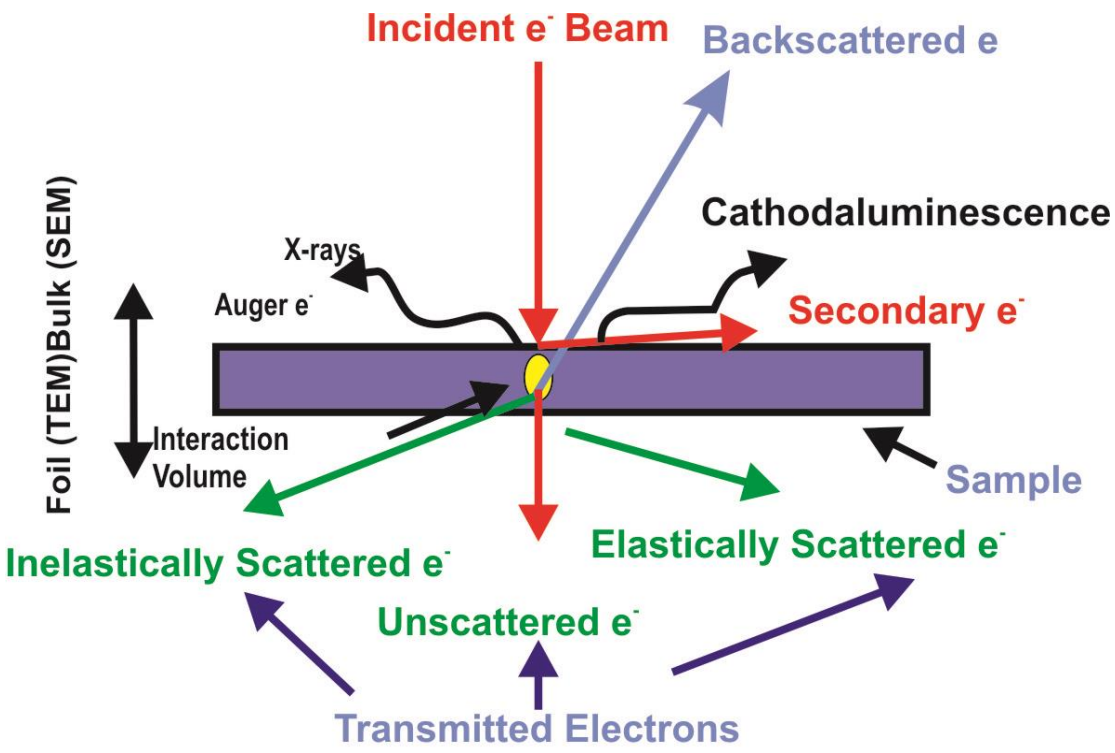
Fig 2.3: Scanning Electron Microscopy



Interaction of high energy electrons with solid

Fig 2.4 : Interaction of electrons with solid in SEM

Electron beams interacts with the conductive specimen and the electrons scattered are detected by a detector where the detected signal is transform into image obtained on the screen.

The magnification system is given by:

M=L/l

Where, L = raster's length of CRT monitor

l = raster's length on the surface of the sample.

SEM is used to study the chemical composition and surface topology of the specimen. However, the sample has to be conductive or should be made conductive before analysis.

2.4 Optical Absorption Spectrometer (Ultraviolet-visible-Near Infra Red)

Absorption Spectroscopy is a powerful technique which is used to study about the metals, semiconductors and insulators in bulk, colloidal and nanostructure form. Semiconductors and insulators have an energy gap between their valence and conduction band. When the energy of photons is sufficient enough to excite the electrons from valence to conduction band, a sudden rise in absorption is observed. The light is in the visible region and near infrared and ultraviolet region. When the particle size decreases, a shift in the absorption to shorter wavelength i.e. blue shift is observed. The absorbed intensity with respected to the wavelength is useful to understand the transition between valence and conduction band and helps to understand the electronic structure.

In case of metals, there is strong surface Plasmon resonance (SPR) due to resonant absorption of photons. The peak in this case is broad and the position of peak is size dependent. Moreover, the peak width depends on the size and the distribution of the particles.

The concentration of analyte in the solution can be determined by applying Beer-Lambert law according to which, the absorbance of any solution is directly proportional to the path length and concentration of the absorbing species. Therefore, when the path length is fixed or constant, the concentration of the absorber in a solution can be easily determined this method. Beer-Lambert law can be given by:

$$A = \log_{10}(I_0/I) = \epsilon c L,$$

Where,

A= Absorbance

I_0=Incident light intensity

I =Transmitted intensity

C=concentration of the sample

L=pathlength

In UV-Vis-NIR spectrometer, the high intensity incident light is selected by a monochromator at different wavelengths. Depending on the properties of the sample, it reflects or absorbs certain wavelength of light. The transmitted intensity is then measured using a photodetector and result is obtained. X-axis is the wavelength of light falling on the sample and Y-axis corresponds to the absorbance or optical density. To eliminate the path travelled by photons in sample holder, a reference containing the liquid in which the

nanoparticles are dissolved without having the nanoparticles is used. Photon source is generally a Tungsten lamp.

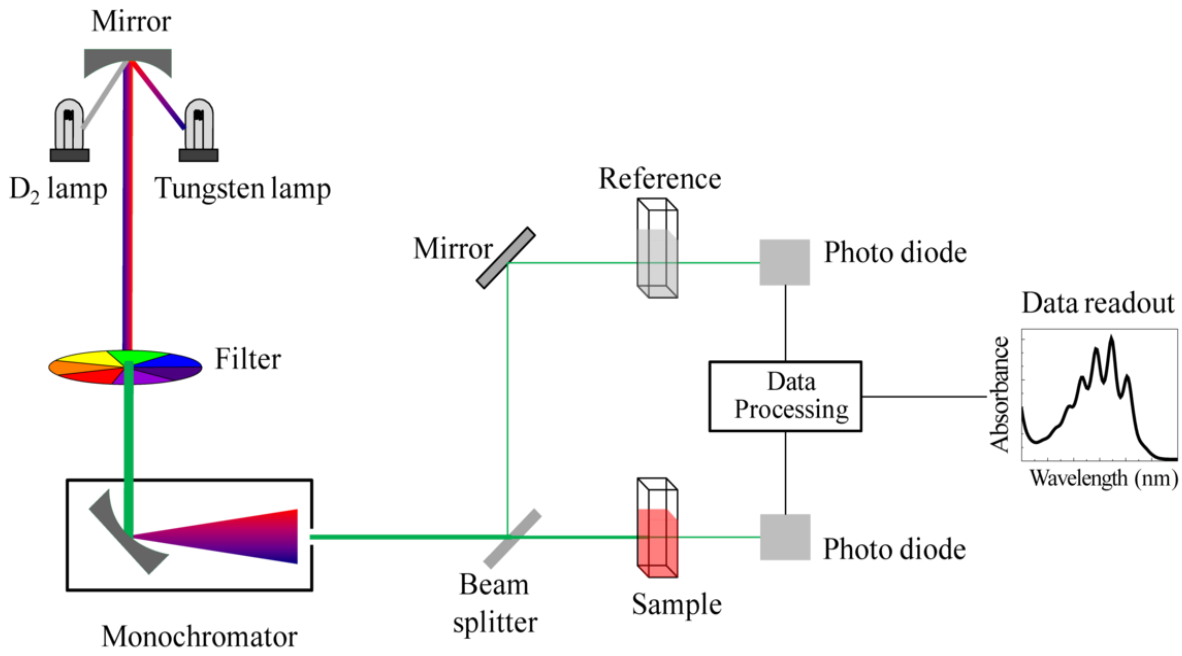


Fig 2.5 UV-VIS spectroscopy

2.5 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (**FTIR**) is a technique for getting IR spectrum of absorption or emission of a specimen. It is a Michelson interferometer. In this technique, light from a polychromatic IR source is focused on a beam splitter, usually made up of thin plastic film, from where the light is split and refracted and transmitted equally to the fixed and movable mirror respectively. The light is then reflected back from the two mirrors and focused on the sample. The light passing through the sample is then collected by the detector and an image is obtained. The peak in the image corresponds to the groups present in the sample. This method is useful in recognizing the chemical groups taking part in a reaction, the capping agent involved in the reaction.

Theory:

FTIR spectroscopy is based on the principle that almost all the molecules absorb infrared (IR) light (λ> 800nm) . However only the monoatomic (He, Ne, Ar, etc) and homopolar diatomic (Hydrogen, Nitrogen, Oxygen) molecules do not absorb IR light. Molecules only absorb IR

light at those frequencies where the IR lights affect the dipolar moment of the molecule. The differences in charges on the electronic field in the atoms of a molecule, produces the dipolar moment of the molecule. Molecules with the dipolar moment allow the infrared photons to interact with the molecules which lead to excitation of these molecules to higher vibrational states. When the frequency is matching, an oscillating dipole can absorb the IR. The intensity of the absorption is proportional to the square of the change in dipole moment μ.

If the stretch does not change the dipole moment, there would not be any infrared band if the stretch do not change the dipole moment. The FTIR spectrum is usually presented in the form of a graph of transmittance (T) and absorbance (A) verses frequency (λ). Transmittance is defined as the ratio of intensity of light at depth l in the medium (I_o) to the intensity of the incident ray (I).

$T = I/I_o$

$$A = \log_{10}\frac{\mathbf{1}}{T} = -\log 10\ T = -\log_{10}\frac{I}{I\mathbf{0}} \qquad \ldots. \qquad (2.2)$$

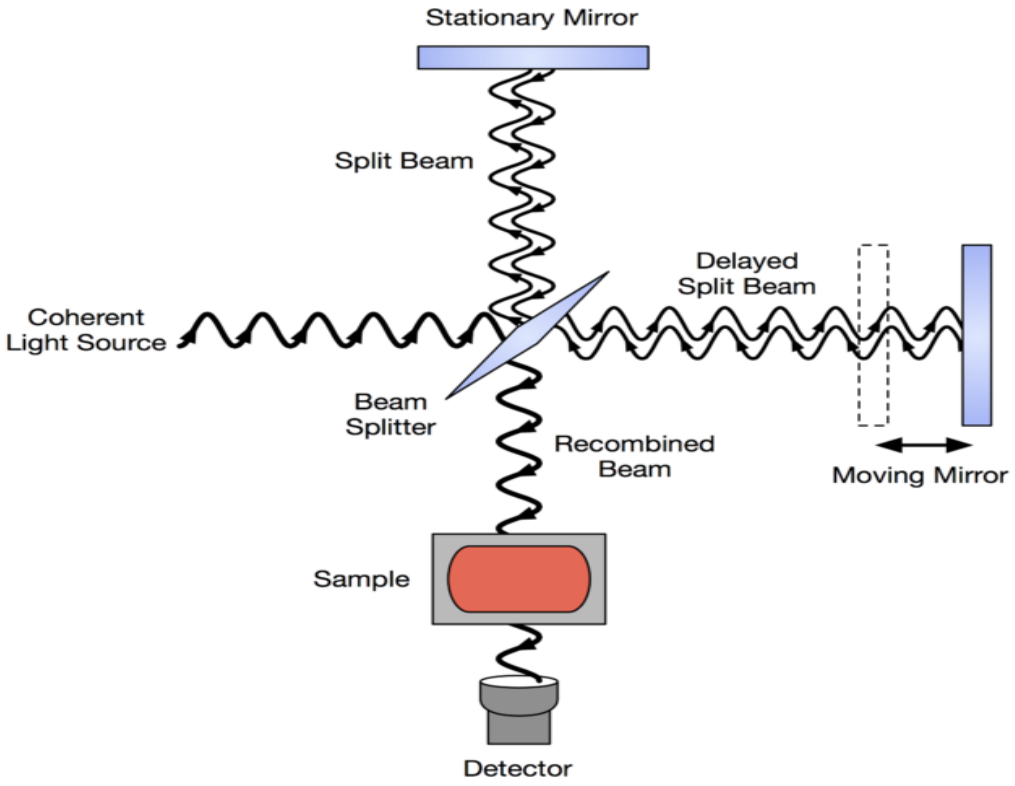


Fig 2.6 FTIR

FTIR spectrometer is an instrument which has broadband near –IR (<650 cm^{-1}) to the far-IR (>4000 cm^{-1}) spectra. A FTIR spectrometer collects all wavelengths simultaneously unlike a dispersive instrument like grating monochromator.

When the metal nanoparticles form in solution, they must be stabilized against the Van der Waals force that may cause coagulation. The stabilization may occur in a number of ways: physisorbed surfactant and polymers may create steric or electrosteric barriers or purely electrostatic barriers around the particle surface. In many cases, the distinction between chemical adsorption (involving direct covalent bonding with the surface metal atoms) and more subtle electrostatic mechanisms (e.g., charge-induced dipole mechanisms and dispersion force mechanisms) is largely a matter of degree. The problem distinguishing chemisorption and physisorption is further exacerbated in the case of metal clusters containing only a few tens of metal atoms, where it is not clear whether the stabilizing molecules should be denoted as ‘‘adsorbates’’ or ‘‘ligands’’.

2.6 Photoluminescence

Photoluminescence (PL) spectroscopy is one of the most powerful tools to study to study the optical properties of semiconductors and metals. A typical PL experiment in semiconductor can be divided into three stages: First, the sample is excited from its ground state, which is completely filled valence band (VB) to the empty conduction band (CB). Energy pumped for excitation is hν. The laser creates electron-hole pairs due to a transfer of electrons from VB to CB, Second, the non-equilibrium electron and hole distributions tend to relax into ground sate. The initial intraband relaxation is caused by energy transfer to the crystal lattice, i.e. a step by step excitation of lattice vibration. Third, the electron hole pair recombines accompanied by the emission of light which is a photoluminescence process.

Due to attractive Coulomb interaction between the charge carriers, the emission does not only contain contributions from states at or above the fundamental energy gap (E_{gap}) but also sharp discrete lines (transitions) just below E_{gap} , which originates from bound excitonic states.

Utility of PL spectroscopy for nanomaterials

The technique of photoluminescence (PL) has become a standard tool for obtaining information about the nature of nanomaterial such as quantum dots. In bulk materials, the luminescence often resembles a standard direct absorption spectrum, so there is little

advantage in studying the details of the both. High photon excitation energies above the band gap can be most effective for luminescence studies of bulk material, but it has been found that for the case of nanoparticles the efficiency of luminescence decreased at high incoming photon energies. Non radiative relaxation pathways can short circuit the luminescence at these higher energies, and it is of interest to investigate the nature of these pathways.

Instrumental details

PL is a static process in which input energy for excitation is fixed and output is varied i.e. response (PL emission intensity) is recorded for all available range λ.

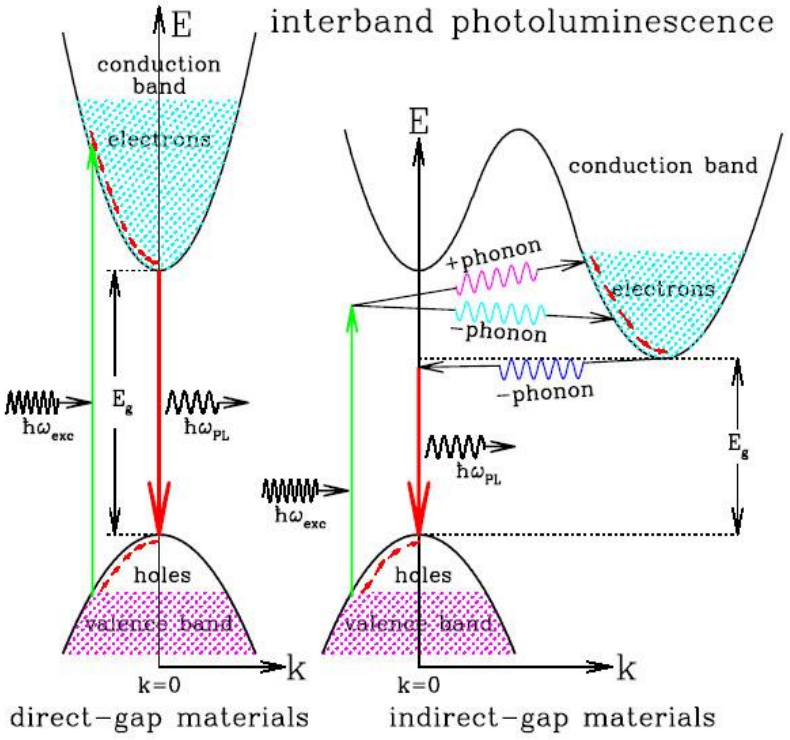


Fig 2.7 Photoluminescence

2.7 EXPERIMENTAL DESIGN

2.7.1 Synthesis of Silver Nanoparticle using extract of Neem Leaf

Sample Collection: Fresh leaves of Neem were collected from nearby area of Delhi Technological University (DTU), Rithala. Leaves were washed thoroughly and allowed to air dry at room temperature.

Method

2.7.1. (A) Preparation of Leaf Extract

Materials

Beaker, distilled autoclaved water, Leaves

Method

1) 25g of neem leaves were weighed and washed thoroughly with distilled water.
2) Neem leaves were then cut into fine pieces and were boiled with 100 ml of distilled water for 20 minutes.
3) After cooling the sample was filtered through Whatman filter paper and filtrate was obtained.

2.7.1. (B) Synthesis of silver nanoparticle

Materials

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth,

Method

1) 5ml of Neem leaf broth was added to 45ml of 10^{-3}M aqueous $AgNO_3$ solution.
2) Colour change was observed after 15minutes.
3) A reduction of silver ions was monitored by measuring the absorption spectra of the solution at regular intervals after diluting a small aliquot (0.2 mL) of the sample 20 times
4) Emission spectra was measured of the $AgNO_3$- Neem broth.
5) After 24 hours, the sample was centrifuged at 10,000 rpm for 15 minutes and the pellet was dissolved and heat dried to obtain silver nanoparticles.
6) $AgNO_3$ solution was taken as negative control.

2.7.1. (C) Effect of time

Material

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth,

Method

1) 5ml of Neem leaf broth was added to 45ml of 10^{-3}M aqueous $AgNO_3$ solution.
2) Colour change was observed after 5 minutes.
3) A reduction of silver ions was monitored by measuring the UV–vis spectra of the solution at different time (5mins, 15 mins, 25 mins, 35 mins, 45 mins) after diluting a small aliquot (0.2 mL) of the sample 20 times
4) Emission spectra of the solution was recorded at different time (5mins, 15 mins, 25 mins, 35 mins, 45 mins) after diluting the sample 20 times.
5) After 24 hours, the sample was centrifuged at 10,000 rpm for 15 minutes and the pellet was dissolved and heat dried to obtain silver nanoparticles.

2.7.1. (D) Effect of pH

Material

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth, KOH

Method

1) Neem leaf broth and $AgNO_3$ were maintained at 5 different pH using pH meter. KOH was added to change the pH of the solution.
2) 5ml of Neem leaf broth was added to 45ml of 10^{-3}M aqueous $AgNO_3$ solution.
3) Step 2-6 of 4.was repeated.

2.7.1. (E) Effect of temp

Material

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth, Refrigerator, thermometer, Hot Plate

Method

1) 5 samples were prepared by mixing 5ml of Neem leaf broth to 45ml of 10^{-3}M aqueous $AgNO_3$ solution.
2) The samples were maintained at 5 different temperatures (10°C, 20°C, 30°C, 40°C, 50°C).

3) Colour change was observed.
4) Absorption and Emission spectra was recorded by diluting the sample 20 times.

2.7.1. (F) Effect of Concentration

Material

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth

Method

1) Neem leaf broth and $AgNO_3$ were mixed at different concentration (1:2, 1:4, 1:8,1:12, 1:16) respectively and 5 samples of different concentration was prepared.
2) Colour change was observed.
3) Absorption and Emission spectra was recorded for all 5 samples of different concentration after diluting the sample 20 times.
4) The sample was centrifuged at 10,000 rpm for 15 minutes and silver nanoparticles were obtained.

2.7.1. (G) Effect of change in $AgNO_3$ concentration

Material

Beaker, Silver Salt ($AgNO_3$), Neem leaf broth

Method

1) Different concentrations of $AgNO_3$ were prepared (0.5Mm, 1Mm, 10 mM).
2) 5 ml of Neem leaf broth was added to 45 ml of $AgNO_3$ solution of 3 different concentration..
3) Colour change was observed.
4) Absorption and Emission spectra was recorded for all 5 samples of different concentration after diluting the sample 20 times.
5) The sample was centrifuged at 10,000 rpm for 15 minutes and silver nanoparticles were obtained.

2.8 Characterization of silver nanoparticles

2.8(A) UV-Vis Spectroscopy: A reduction of silver ions was monitored by measuring the UV–vis absorption spectra. UV-Vis spectroscopic analysis was carried out on Perkin Elmer spectrophotometer. The measurements were carried out at different time, pH, temperature and concentration..The bioreduction of silver ions in aqueous solution was monitored by UV-VIS spectra of the solution between 250 nm – 700 nm.

Material

Quartz cuvette, UV-vis spectrometer, beaker,distilled water

Method

1) UV-vis spectra of the solution at regular intervals was obtained after diluting a small aliquot of the sample 20 times.

2) Distilled water was used to adjust the baseline.

2.8 (B) Photoluminescence: Emission and excitationspectrawere measured with Horiba jobin yvon Florolog-3 spectrofluorometer. The measurements were carried out for different time, pH, temperature and concentration.The excitation wavelength was decided by observing the absorption peak obtained of respective samples. The emission spectra of silver ions in aqueous solution were monitored in the range of 390 nm – 700 nm.

Material

Quartz cuvette, beaker,distilled water

Method

1. Excitation wavelength was decided by observing the absorption peak of respective sample.
2. Emission spectra of the solution at different pH, concentration, time and temperature was obtained after diluting a small aliquot of the sample 20 times.

2.8.(C) X-Ray Diffraction: XRD measurements were recorded on X-ray difractometer (Bruker D8 advanced). The AgNPs dried powder was analyzed on XRD for their phase structure and exact material identification.

Material

Dried sample, X-Ray diffractometer

Method

1. Sample containing $AgNO_3$ – Neem leaf extract was centrifuged at 10,000 rpm for 15 minutes and silver nanoparticles were obtained.
2. The precipitate obtained after centrifugation was thoroughly washed with water to remove the impurities.
3. The silver nanoparticles were dried at 60° in oven and then XRD analysis of the dried sample was done.
4. The Cu αradiation (k= 1.5418Å) was selected and the diffractogram was obtained in the 2θrange of 10–70 degree.

2.8(D) Scanning Electron Microscopy: The freeze-dried silver nanoparticles were mounted on specimen stubs with double-sided tapes and examined under a Hitachi S700N operated at a voltage of 15KV.

Material

Dried sample, SEM

Method

1) The dried sample was used for SEM analysis and the results were obtained.

2.8(E) FTIR: FTIR analysis was done to study about the functional groups attached to the synthesized nanoparticles . It is used to study about the biomolecules present in Neem leaf extract which are responsible for the reduction of silver salt.

Material

Neem leaf broth, Silver nanoparticles, FTIR (Thermoscientific Nicolet 380)

Method

1. FTIR analysis of Neem leaf broth and synthesized silver nanoparticles were done and the results were obtained.
2. The results were then analyzed and the peaks indicated the functional groups present and hence capping and stabilizing agent in the plant was known.

2.9 Isolation of Bacteria

2.9. (A) Serial Dilution

A serial dilution is the stepwise dilution of a substance in solution. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion

Materials

Test tubes, test tube stand, pipette, soil sample, distilled autoclaved water

Method

1. Collected the soil sample and performed serial dilution.
2. Dissolved 1gm of soil in 9ml water in first test tube.
3. Took 1gm of sample from test tube 1 and added to second test tube then added 9ml of water to the same to obtain dilution 10^{-1}.
4. Repeated the above steps to obtain dilutions till 10^{-6}.

2.9. (B) Pour Plating

Material

Petriplates, nutrient agar, distilled water(autoclaved)

Method

1) Preparation of agar plates

1.8% agar was prepared and agar plates were prepared for pour plating.

2) Pour Plating

1) The plates prepared were marked for each dilution.

2) The dilutions were poured into respective plates as marked accordingly.

3) The plates were kept for overnight incubation at 37°C

2.10 Antimicrobial Activity of silver nanoparticles

Material

Bacterial colony, Silver Nitrate, Silver nanoparticles

Method

1) Petri plates containing bacterial colony was taken.

2) Negative control (intact bacterial colony),

3) Different quantity of Silver nanoparticles were added (2,4,8,12 μg/ml) to the bacterial colony

4) Zone of clearance was observed and the diameter of clear zone was recorded.

CHAPTER 3

Green Synthesis of Silver Nanoparticles using Neem Leaves and their Antimicrobial Activity

Abstract: In the present work, Silver nanoparticles were synthesized using aqueous extract of Neem (*Azadirachta indica*) leaves. Silver salt was added to aqueous leaf extract, which was used as reducing as well as capping agent. Characterizations of the synthesized nanoparticles have been done using XRD, SEM, optical absorption and emission spectra. The absorbance maximum was observed at 410nm for Neem. The presence of biomolecules responsible for reduction of metal ions in Neem leaves was studied using FTIR. The effect of various parameters like extract concentration, reaction pH, mixing ratio of the reactants, temperature and interaction time on the morphology and size of synthesized silver nanoparticles were studied. Green synthesis of silver nanoparticle were found to have enhanced antimicrobial property and showed zone of inhibition against isolated bacteria from garden soil sample. Based on the results obtained it can be said that the resources obtained from plants can be efficiently used in the production of silver nanoparticle and it could be utilized in various fields such as biomedical, nanotechnology and so on.

Introduction

Richard Feynman's famous talk “There is plenty of room at the bottom” laid the foundation to revolutionize the field of nanotechnology [1]. Nanotechnology is an interdisciplinary field which includes physics, chemistry, biology, material science and medicine. The field of nanotechnology is one of the most active research field and is based on synthesis of small sized nanoparticles which has at least one dimension in the range of 1-100nm(10^{-9}) .Nanotechnology is offering wide range of applications in the field of biosensors, bionanotechnology, biomedicine etc. Nanoparticles are being used for the treatment of various diseases like cancer and also in gene therapy and targeted drug delivery

Silver nanoparticles are one of the promising researches in the nanotechnology industry. Researchers are trying to use economical and environment friendly method for the synthesis of silver nanoparticles, where green synthesis has proved to be a promising process. Green synthesis of metal nanoparticles is gaining importance because of their biocompatibility, low toxicity and eco-friendly nature [2]. The green synthesis methods include synthesis of nanoparticles from bacteria, fungus, yeasts [12], plants [13] and DNA [14]. The biosynthesis method employing plant extracts like *Pelargonium graveolens* , *Medicagosativa, Azadirachta*

indica, *Lemongrass*, *Aloevera*, *Cinnamomum Camphora* [15][18], have drawn great attention as an alternative to conventional methods because plants are found in abundance in nature .

Azadirachta indica is common plant also known as Neem, which is found abundantly in India and nearby Indian subcontinents like Nepal, Bangladesh, Sri Lanka etc. It belongs to *Meliaceae* family and is known for its various applications especially its medicinal property [17]. *Azadirachta indica* leaf extract is used in the synthesis of various nanoparticles like gold, zinc oxide, silver etc. The photochemicals present in Neem is terpenoids and flavones which act as reducing as well as capping agent and help in stabilizing the synthesized nanoparticles. When silver salt is treated with Neem leaf extract, the silver salt is reduced to silver nanoparticles. The synthesized nanoparticles are capped with neem extract and also exhibit enhanced antibacterial activity.

Silver nanoparticles are found to be non-toxic to humans but at low concentration they are effective against microorganism like bacteria, virus and fungi. Antimicrobial capability of SNPs allows them to be suitably employed in numerous household products such as textiles, food storage containers, home appliances and in medical devices [19]. The most important application of silver and SNPs is as tropical ointments to prevent infection against burns and open wounds.

Materials and Methods

20 grams of finely cut Neem leaves were boiled in 100ml water for 10 minutes and filtered to obtain Neem leaves extract. The extract of Neem leaves (5.0 ml) were mixed with 45 ml of 1mM silver nitrate ($AgNO_3$) and colour change was observed indicating the formation of silver nanoparticles. The effect of various physic-chemical parameters was studies by varying the reactant concentration, pH, temperature and reaction time. Reduction of Ag^+ ions was monitored after diluting a small amount of sample 20 times using spectrophotometer (Perkin Elmer Lambda 750) and spectrofluorometer (Horiba Jobin yvon). Neem broth containing silver naoparticles were centrifuged at 10,000 rpm for 15 mins and the precipitate was thoroughly washed with sterile distilled water to get rid of any unwanted impurities. The purified pellet was then dried and the sample was characterized using SEM (Hitachi S7000N) and XRD (Brooker D8 advanced). Biomolecules responsible for the reduction of silver salt was studied using FTIR (Thermoscientific Nicolet 380).The synthesized silver nanoparticles were then tested for their antibacterial property against bacteria obtained from garden soil samples. The bacteria were grown on 1.8% agar plates and small amount of silver nanoparticles were added and the antibacterial property was studied.

Results and Discussion

3.1 Colour Change

When Silver salt is added to aqueous Neem leaf extract, a colour change is observed from pale yellow to yellowish brown and finally to dark brown colour. The change in colour of the solution indicated the formation of nanoparticles, since Silver nanoparticles exhibit yellowish brown colour in aqueous solution due to the excitation of surface plasmon vibrations in silver nanoparticles. Fig 3.1 shows the change in colour of $AgNo_3$-Neem leaf extract.

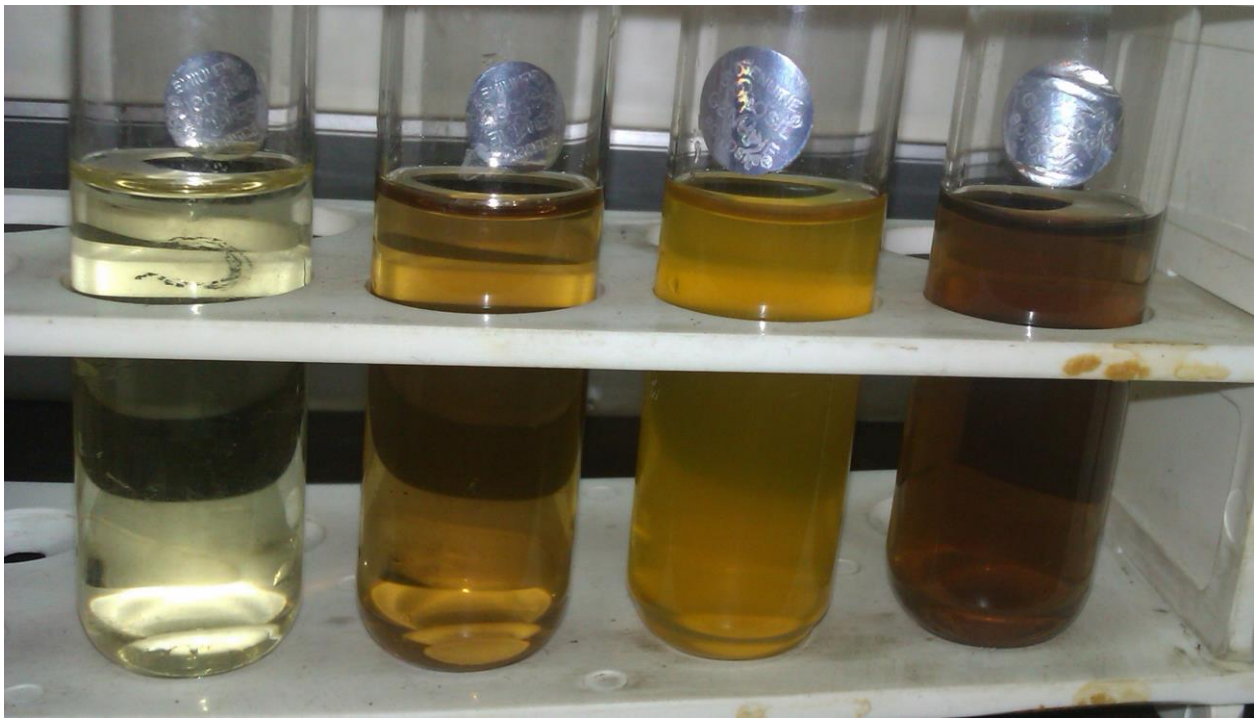


Fig 3.1: Change in colour of the solution with time.

3.2 UV-Visible Spectroscopy (Absorption and Emission)

The reduction of silver salt ($AgNo_3$) when treated with boiled *Azadirachta indica* leaf broth, result in the formation of silver nanoapticles. The formation of the metal nanoparticles is initially confirmed using UV-Vis-NIR spectroscopy. Fig 3.2 shows the absorption spectra obtained from the reaction of Neem leaf extract and $AgNo_3$, and shows absorption maximum at 400nm. It is observed that there is an increase in the optical density with respect to time (as

shown in Fig. 3.2 for 5 different time) without any shift in the peak wavelength. This indicate enhancement in the formation of silver nanoparticles without any change in particle size. Change in colour is observed initially after 5 minutes of adding the salt solution to the Neem leaf broth. After 30 minutes, the colour of the solution becomes nearly constant.

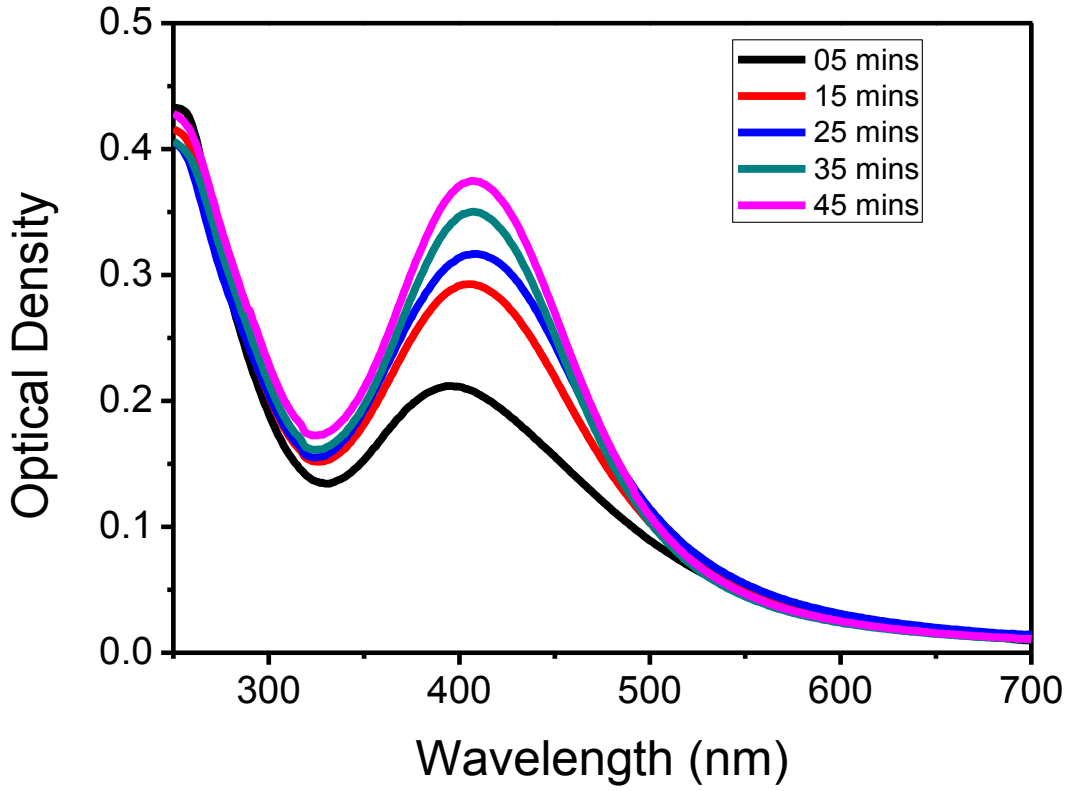


Fig 3.2: Absorption spectra of silver nanoparticles at 5 different time (maximum absorption at 400nm)

Fig.3.3 shows photoluminescence (PL) spectra of silver nanoparticles as a function of time. The excitation wavelength is selected at 380 nm. The photoluminescence spectrum shows a peak at around 450 nm. With increase in time, there is an increase in the intensity of the PL peak, without any shift in peak wavelength, thus indicating no change in the size of the particle. The emission at 450 nm corresponds to blue emission.

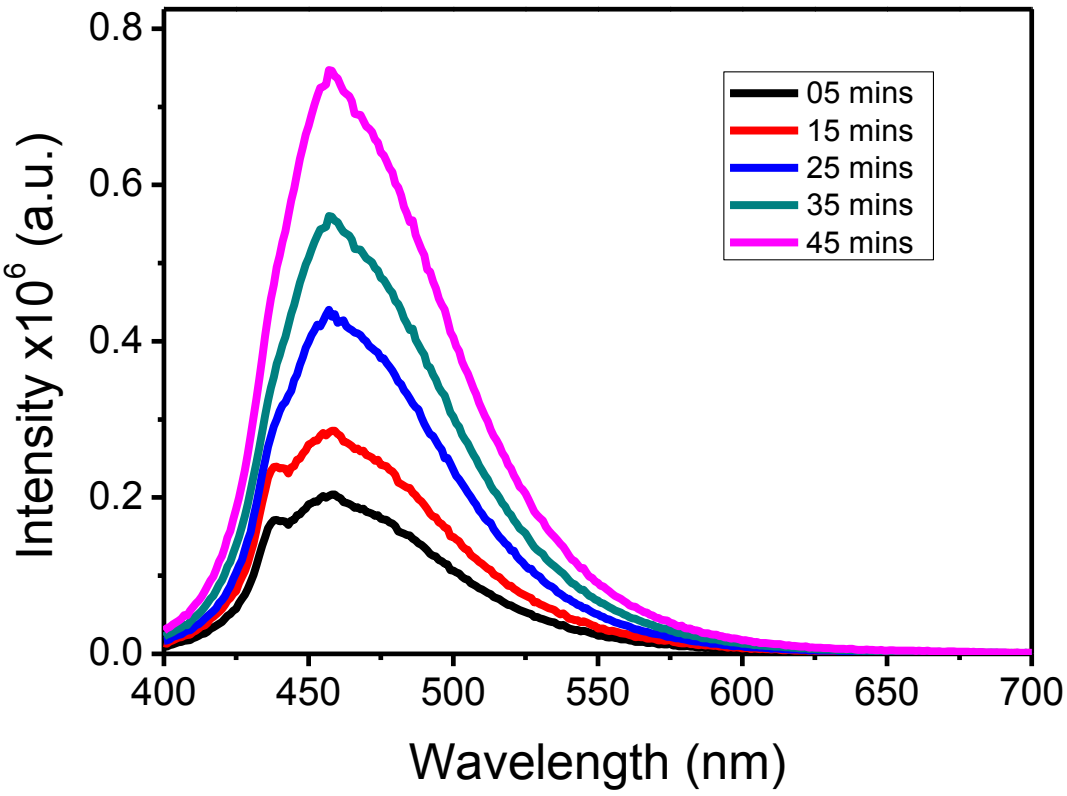


Fig 3.3: Photoluminescence spectra recorded at 5 different time of 10^{-3}M aqueous solutions of silver nitrate with Neem leaf broth (λ_{ex}= 380nm).

Fig 3.2(a) shows a plot between changes in peak absorption intensity as a function of time. It is observed that the intensity of absorption maxima at 400 nm increases with increasing reaction time. It appears that the absorption intensity is nearly linear with the passes of reaction time

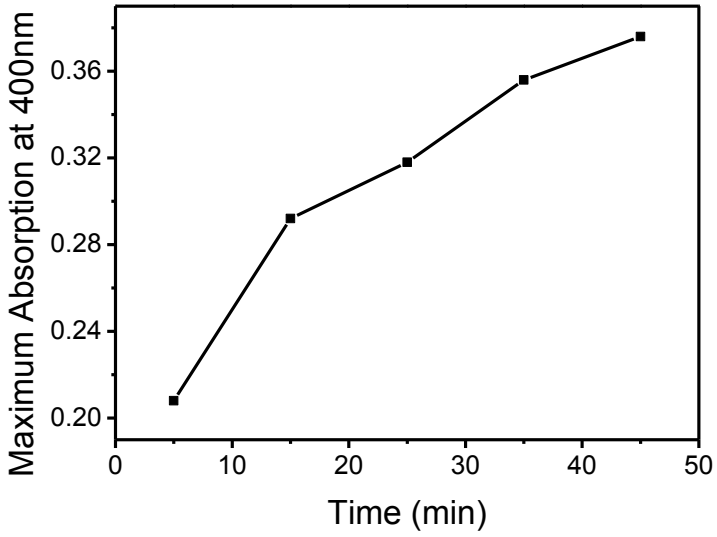


Fig3. 2(a): Effect of time on the optical density when silver salt was treated with Neem leaves extract.

Fig 3.3 (a) shows photoluminescence intensity at 450 nm as a function of time. It is noted that the photoluminescence intensity increases with increasing reaction time. It is important to note that the peak PL intensity is nearly linear with reaction time.

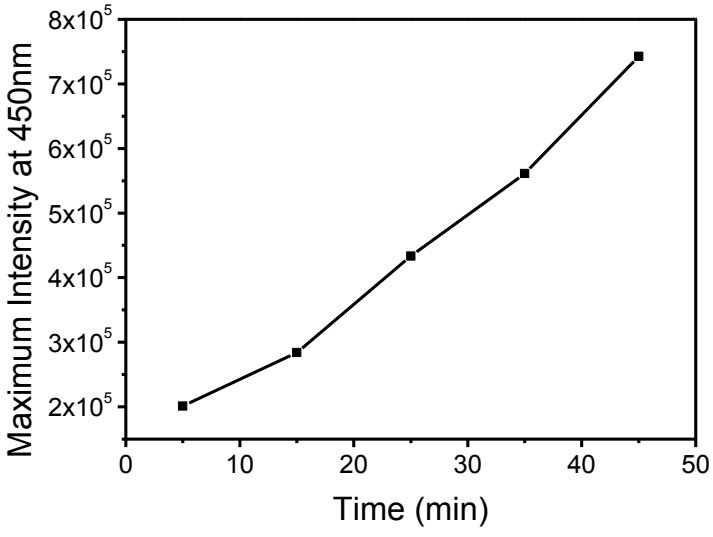


Fig 3.3(a): Effect of time on the PL intensity when silver salt was treated with Neem leaves extract.

3.3 EFFECT OF CONCENTRATION

Fig 3.4 shows the absorption spectra of silver nanoparticles obtained on changing the concentration of Neem Broth and silver salt. When the concentration of Neem leaf broth and silver salt is in the ratio 1:16, a weak absorption band with maximum at 400 nm is observed. With increase in concentration of reaction mixture to the ratio 1:02, the peak intensity increases, indicating enhancement in the production of Silver nanoparticles. The absorption intensity increases monotonically with increasing the concentration of Neem broth (as given in fig. 3.4).

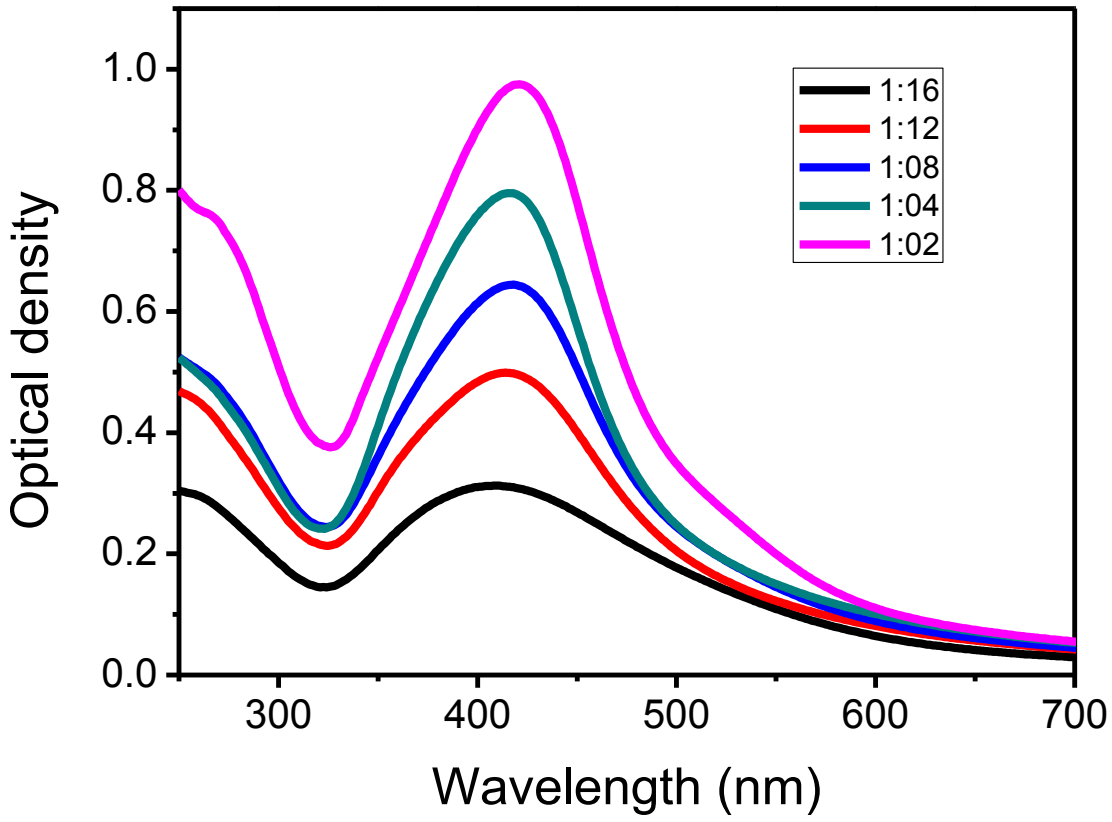


Fig 3.4: Effect of different concentrations of the Neem broth: Absorption spectra of production of silver nanoparticles against various concentrations of Neem Broth (1:2, 1:4, 1:8, 1:12, 1:16)

Fig 3.5 shows the photoluminescence spectra of silver nanoparticles obtained on changing the concentration of Neem Broth and silver salt. The excitation wavelength was 350 nm. The photoluminescence appeared in the wavelength range of 400 - 700nm. The PL peak is observed at 454nm. When the concentration of Neem broth and silver salt was in the ratio 1:20, a weak PL was observed. Whereas with increase in concentration of Neem broth and silver salt to the ratio 1:02, the peak intensity increased indicating enhancement in the production of Silver nanoparticles having the same size. The change in intensity with increasing concentration of Neem broth can be explained by the presence of large number of functional groups that react with silver salt.

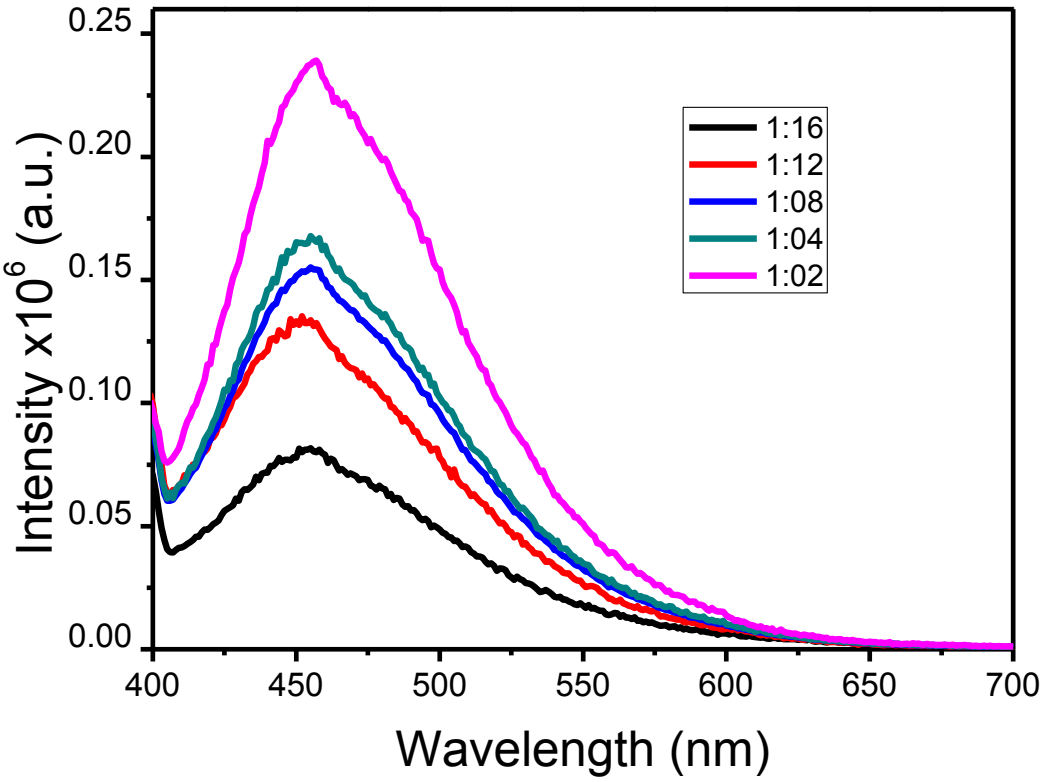


Fig 3.5: Effect of different concentrations of the Neem broth: PL spectra of production of silver nanoparticles against various concentrations of Neem Broth (1:2, 1:4, 1:8, 1:12, 1:16). Excitation wavelength λ_{ex} = 350nm.

Fig 3.4(a) shows a plot obtained between the maximum absorbance monitored at 400 nm and reaction concentration. It is observed that optical density increases with increasing reactant concentration.

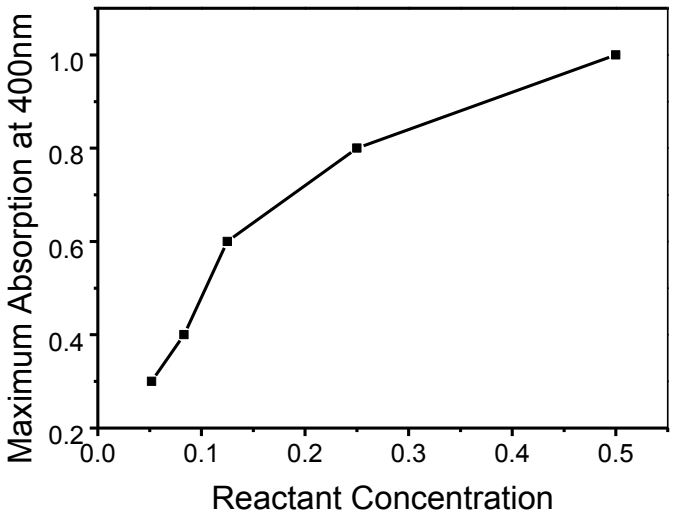


Fig 3.4 (a): Effect of concentration on absorption intensity at 5 different reactant concentrations

Fig 3.5 (a) shows a plot obtained between maximum PL intensity monitored at 450 and reaction concentration. It is observed that with increase in concentration, emission intensity increased in the same manner as the absorption intensity.

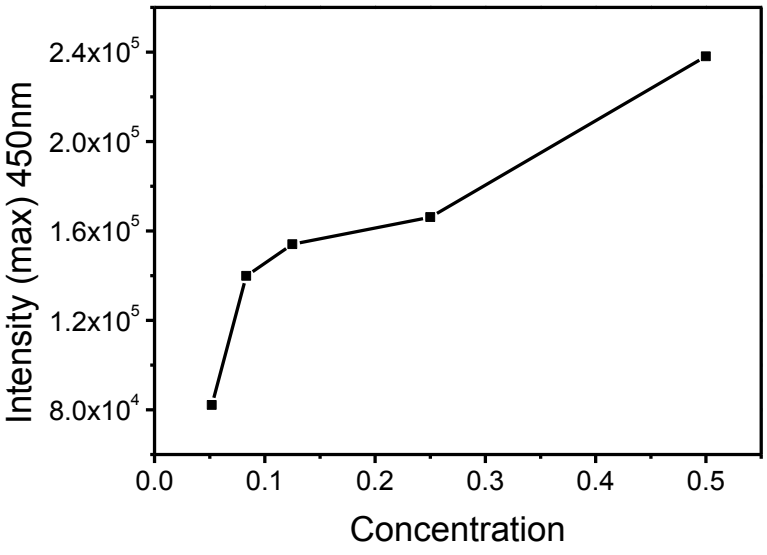


Fig 3.5 (a): Effect of concentration on PL intensity at 5 different reactant concentrations.

3.4 EFFECT OF pH

Another important parameter which affects the formation of nanoparticles is the pH of the solution. Change in pH affects the shape and size of the particles. Fig 5.6 shows change in peak absorption wavelength and intensity with change in pH. As the pH increased from 8 to 12, blue shift was observed, the peak wavelength shifted from 382nm at pH 8 to 417nm at pH 12. In addition to the spectral shift, the absorbance intensity also increased with increase in pH. pH 13, i.e. maximum pH was found favorable for the synthesis of nanoparticles. Moreover it was observed that pH enhances the rate of reduction reaction, as the colour change was observed very fast when $AgNO_3$ was mixed with aqueous Neem leaf extract, i.e. within few seconds the colour changed to dark brown. Shift in peak wavelength indicate that the size of the particle increased with decrease in pH. It can be concluded that at acidic pH, formation of nanoparticles was suppressed. At high pH, large numbers of nanoparticles were present due to bioavailability of functional groups in Neem leaf extract. However, at very high pH, the particles became unstable.

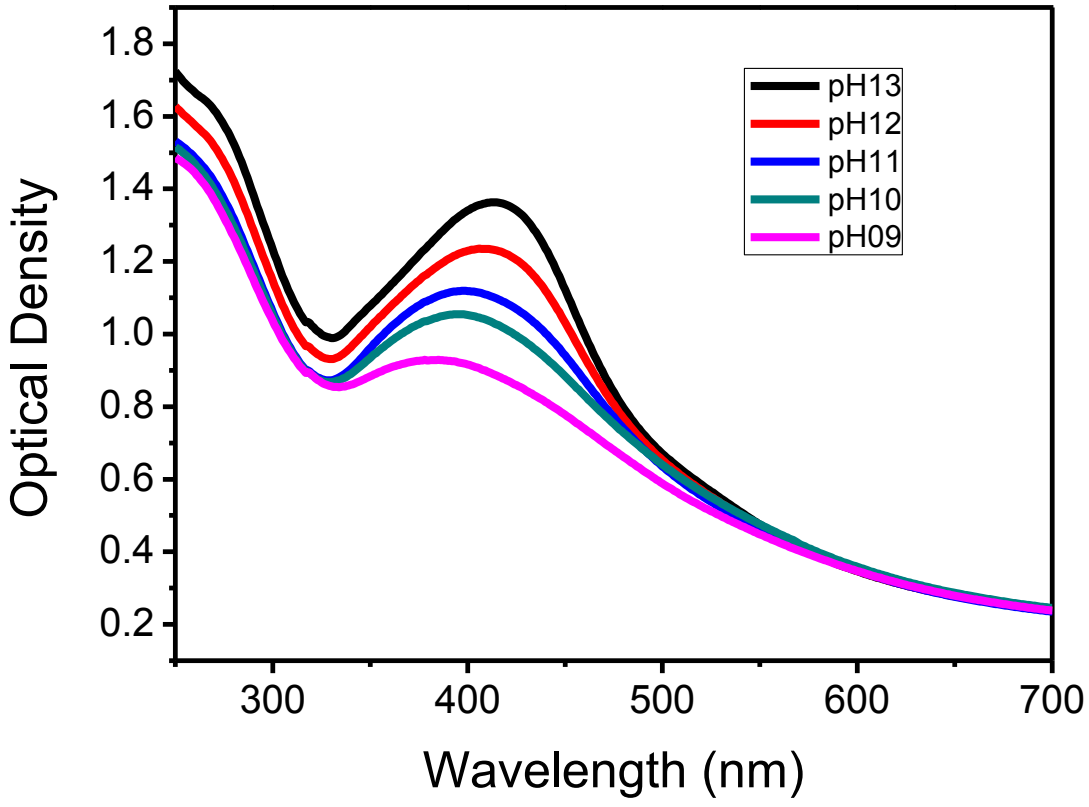


Fig 3.6: Absorption spectra of silver nanoparticles against different pH: Effect of pH on nanoparticle production from Neem broth.

Fig 3.7 shows the photoluminescence spectra obtained at different pH with 350 nm excitation. PL spectrum shows a peak at around 450 nm. With change in pH from acidic to alkaline, the intensity of PL band increased, indicating increased rate of reduction at high pH. Together with the increase in PL intensity, a slight shift in peak wavelength is also observed indicating change in size of the nanoparticle.

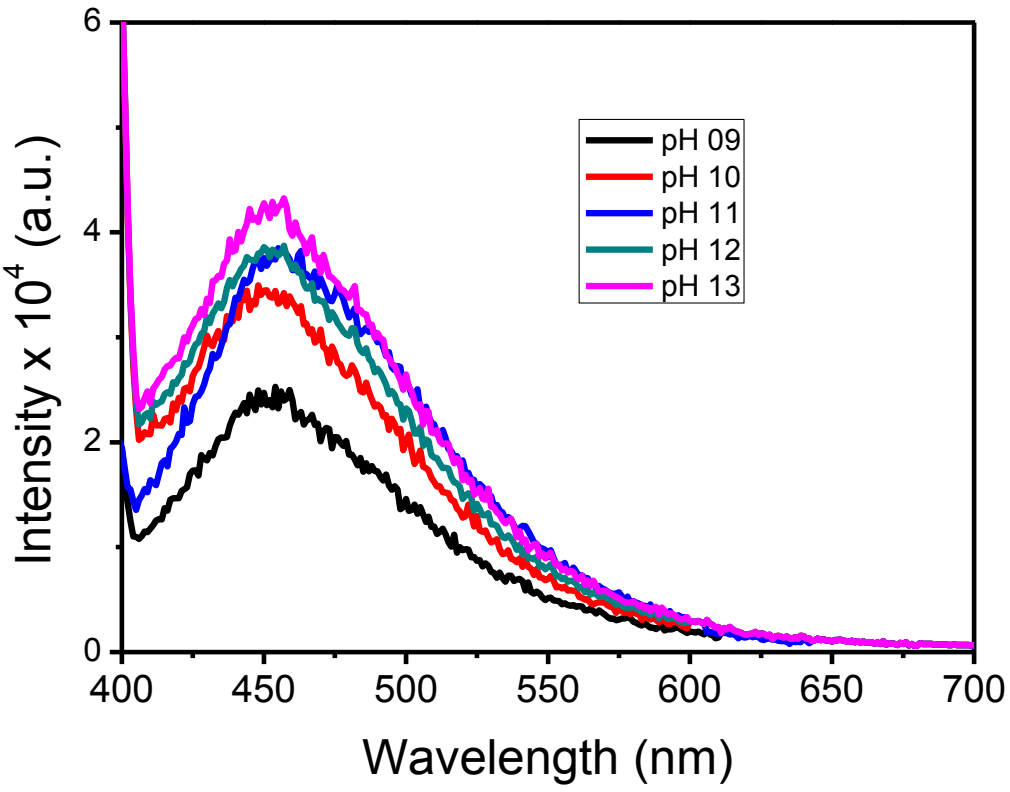


Fig 3.7: Photoluminescence spectra of silver nanoparticles at different pH values of the reaction mixture: Effect of different pH on nanoparticle production. Excitation wavelength is λ_{ex} = 350nm.

Fig 3.6(a) shows a plot obtained at peak absorption wavelength as a function of pH**.** It is inferred that with increase in pH there is a red shift in the absorption maximum, i.e. an increase in λ_{max} from 390 to 420. A linear relation is observed between maximum absorption and pH.

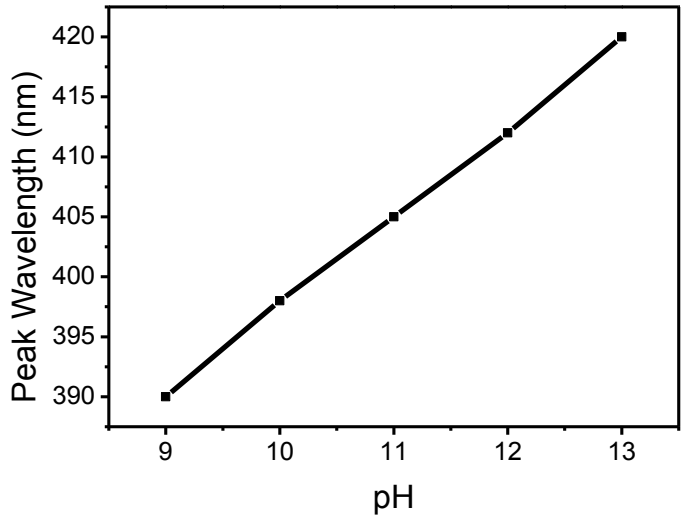


Fig 3.6(a): Plot between peak PL (λ_{max}) and pH of the reaction mixture: Effect of different pH on nanoparticle synthesis.

Fig 3.6(b) shows a plot between maximum photoluminescence intensity and pH. This indicates that synthesis of nanoparticles increases with increasing pH, hence maximum at alkaline pH.

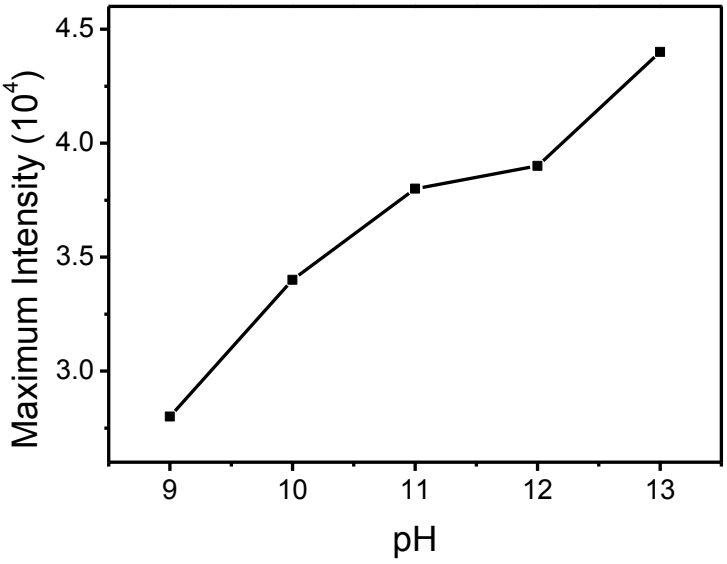


Fig 3.6(b): Plot between maximum PL intensity and pH of the reaction mixtutre

3.5 EFFECT OF TEMPERATURE

Temperature is very important factor that affects the synthesis of nanoparticles. Fig 3.8 shows the absorption spectra observed at 5 different temperatures. It is observed that with increase in temperature, the peak wavelength shift to left from 433nm at 10º C to 397nm at 50º C. The shift in band maximum is due to localization of surface plasmon resonance of the silver nanoparticles. The reduction of silver salt was enhanced at higher temperature, indicated by rapid change in colour.

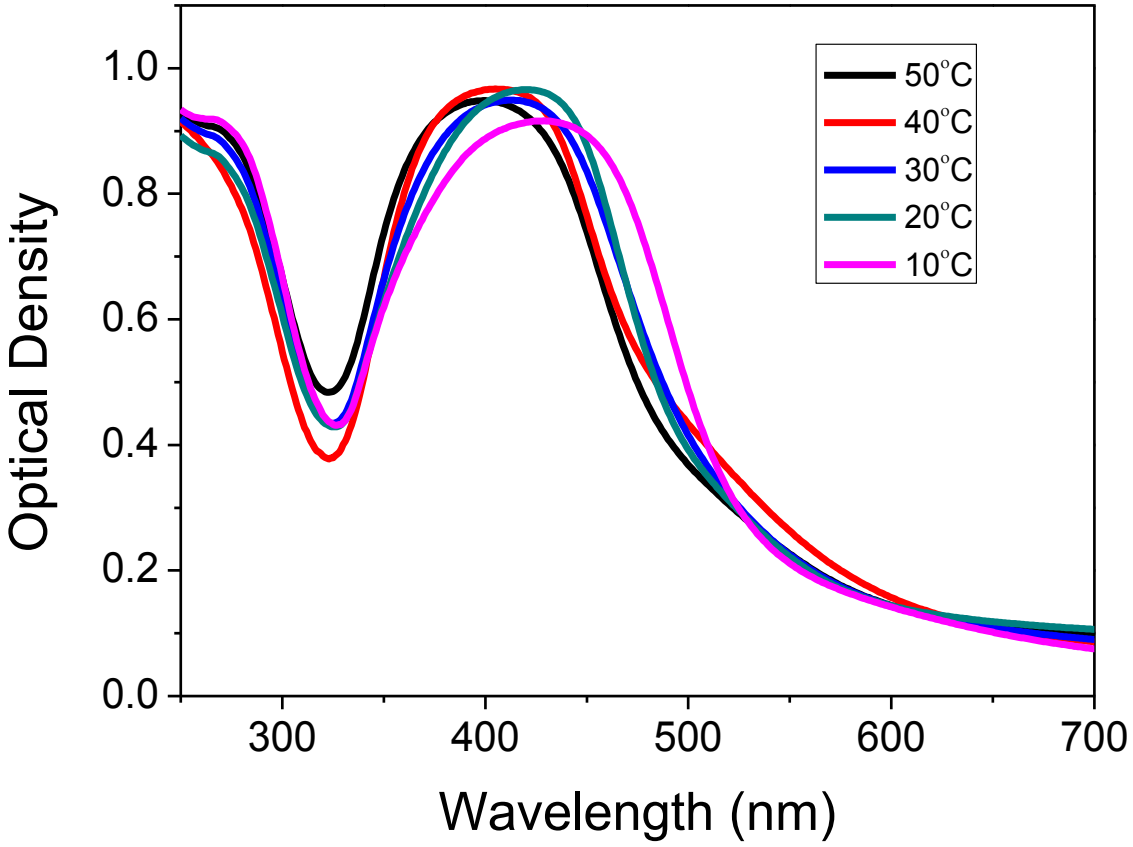


Fig 3.8: Absorption spectra of silver nanoparticles obtained at different reaction temperature 10º,20º,30º, 40ºand 50ºC.Effect of different reaction temperature on nanoparticle production from Neem leaves.

Fig 3.9 shows the photoluminescence spectra of silver nanoparticles at different temperatures. Two simultaneous effects were observed. With increasing temperature, the intensity of PL band increases rapidly and at the same time shifted towards blue. As temperature increases, the size of the nanoparticle decreases however the amount of nanoparticles increases having the same size.

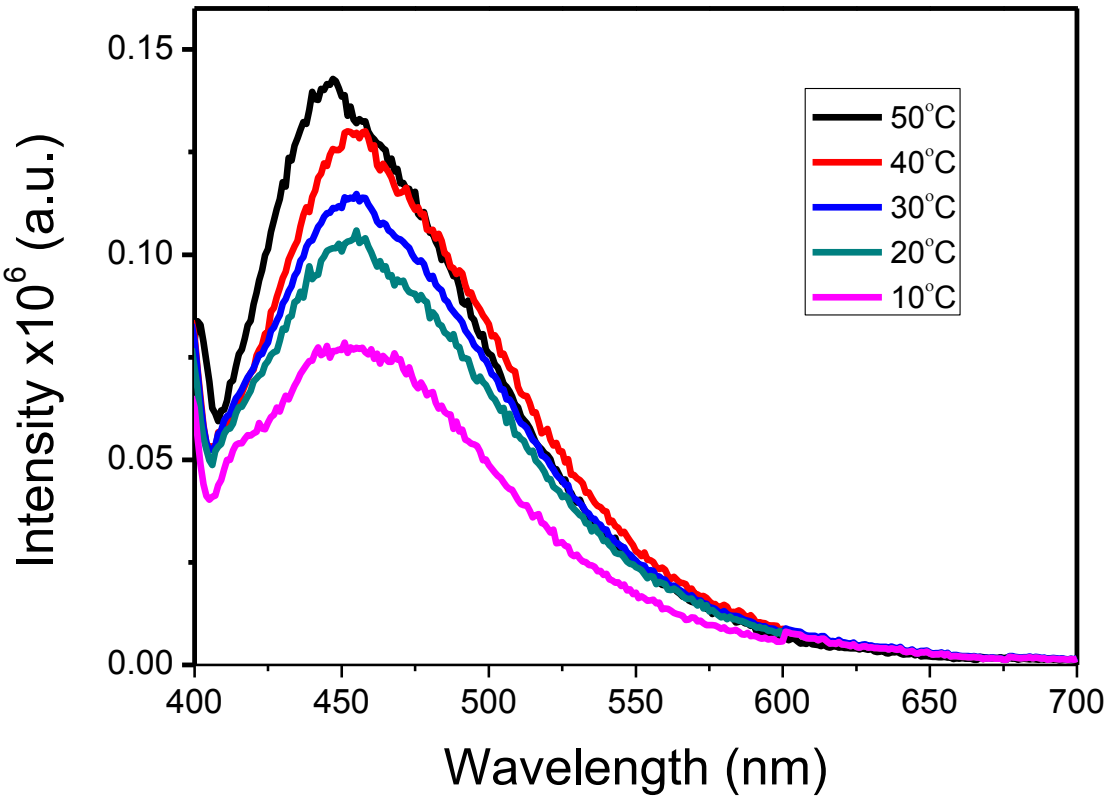


Fig 3.9: Emission spectra of silver nanoparticles against different reaction temperature 10°,20°,30°, 40°and 50°C. Effect of different reaction temperatures on nanoparticle production from Neem leaves. Excitation wavelength λ_{ex} = 350nm.

Figs. 3.8(a) and 3.9(a) show the plot of absorption maximum and PL maximum as a function of temperature. With increasing temperature, a blue shift is observed both in absorption maximum and PL maximum. Absorption peak shifted from 430nm to 395 nm whereas PL peak shifted from 440nm to 460 nm as temperature increases from 10-50 ^{0}C.

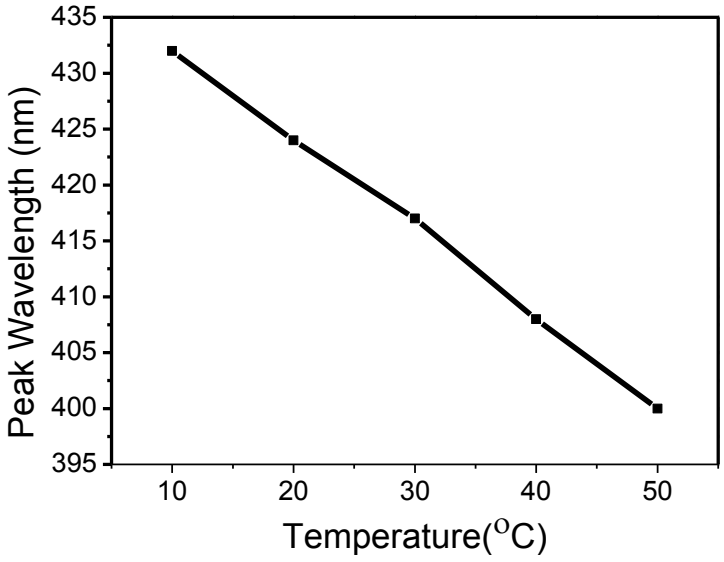


Fig 3.8(a): Plot between peak absorption intensity (λ_{max}) and temperature of the reaction mixture. Effect of different temperature on nanoparticle synthesis.

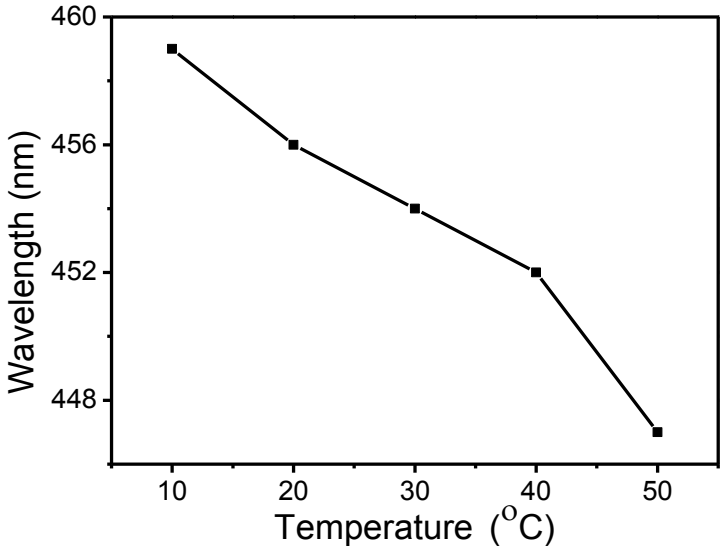


Fig 3.9(a): Plot between peak PL intensity (λ_{max}) *vs* temperature of the reaction mixture. Effect of different temperature on nanoparticle synthesis.

3.6 Surface morphologyof silver nanopartilces

X-ray diffraction (XRD) of the Ag nanoparticles obtained from Neem leaf broth are carried on Bruker D8 Advanced instrument operating at a voltage of 40kV and a current of 30 mA with Cu$K\alpha$ radiation.

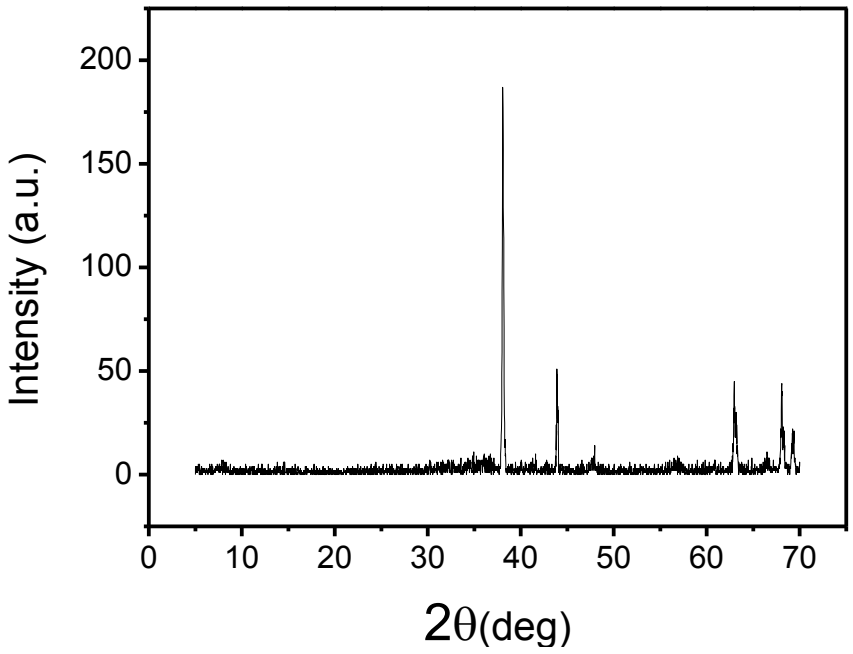


Fig 3.10: X-Ray Diffraction pattern of Silver Nanoparticles synthesized by reduction of silver salt by neem leaf broth.

The XRD result shows high intensity peaks at around 38°,44° and 64° corresponding to three diffraction faces of silver.

The crystallite size of the dried powder is calculated using Scherrer's equation:

$$\tau = \frac{K\lambda}{\beta \cos\theta}$$

The crystallite size is calculated to be approximately 85nm.

SEM is used to study the surface morphology of sythesized Silver nanoparticles obtained from *Azadirachta indica* leaf extract. Figs. 3.11 and 3.12 show the SEM images of the synthesized nanoparticles.The obtaiend nanoparticles are spherical in shape. From the SEM image the average size of the particle was obtained to be around 190 nm. The size and shape of the particles changed with change in the temperature, as represented by Fig. 3.12, which shows increase in the size of the particle at high pH.

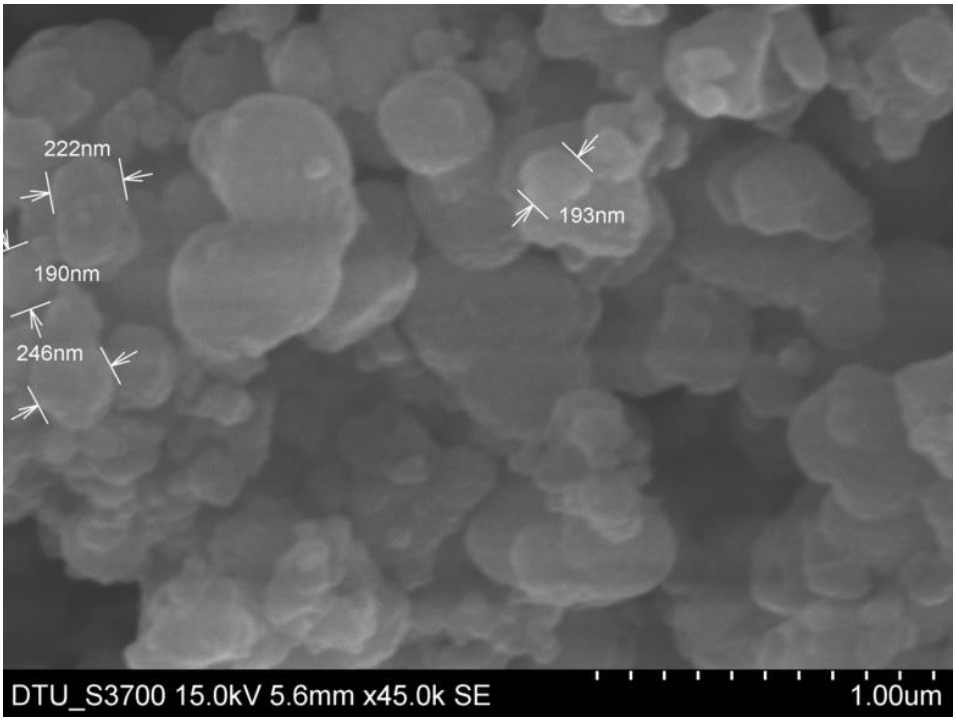


Fig. 3.11: SEM image of the synthesized nanoparticles showing spherical shape of the particles.

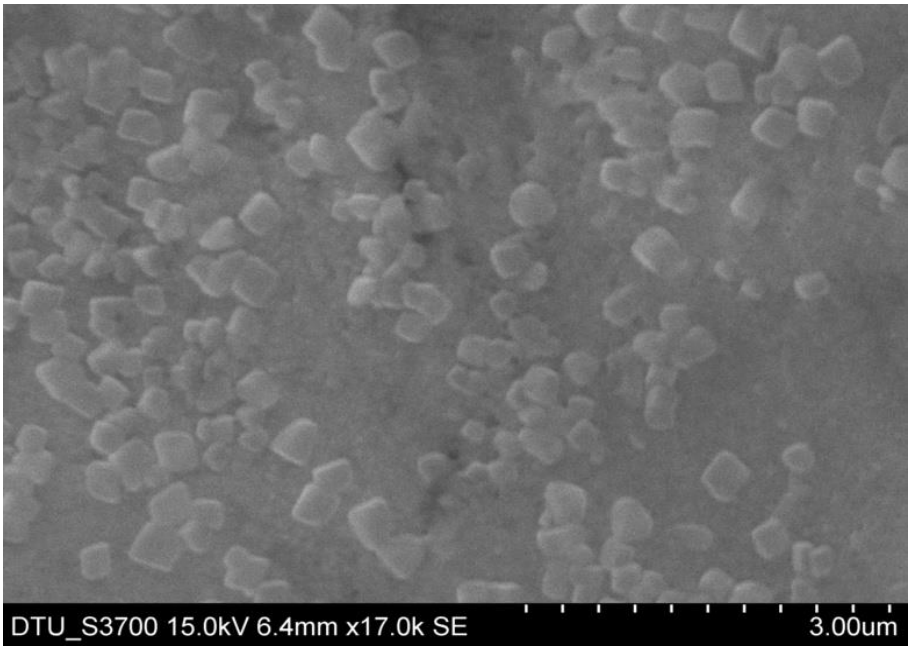


Fig. 3.12: SEM image of the synthesized nanoparticles showing larger particles and different shape at alkaline pH

Furthermore, to identify the possible biomolecules present in Neem leaf broth that are responsible for capping and stabilizing the metal nanoparticles FTIR measurements were carried out. FTIR analysis of pure Neem leaf broth and purified silver nanoparticles obtained after centrifugation of sample at 10, 000rpm is done. Figs.5 13 and 5.14 show the FTIR spectra of Neem leaf broth, which show peak at 3389, 1635, 2117, 1390 cm^{-1} .These peaks represent the bonds due to O–H stretching (around 3,389 cm^{-1}), aldehydic C–H stretching (around 2,117 cm^{-1}), C=C group (around 1,635 cm^{-1}) and geminal methyl group (around 1,380 cm^{-1}). No peaks are observed corresponding to amide I and amide II groups. These bands suggest the presence of terpenoids present in Neem leaf. Terpenoids act as stabilizing as well as capping agents. Besides Terpenoids, presence of Flavanones is also possible. Terpenoids and Flavanones are adsorbed on the surface of the metal ions by interaction through carbonyl groups or π-electrons in sufficient concentration. Terpenoids help in reduction of metal ions by oxidation of aldehydic groups in the molecules to carboxylic acid.

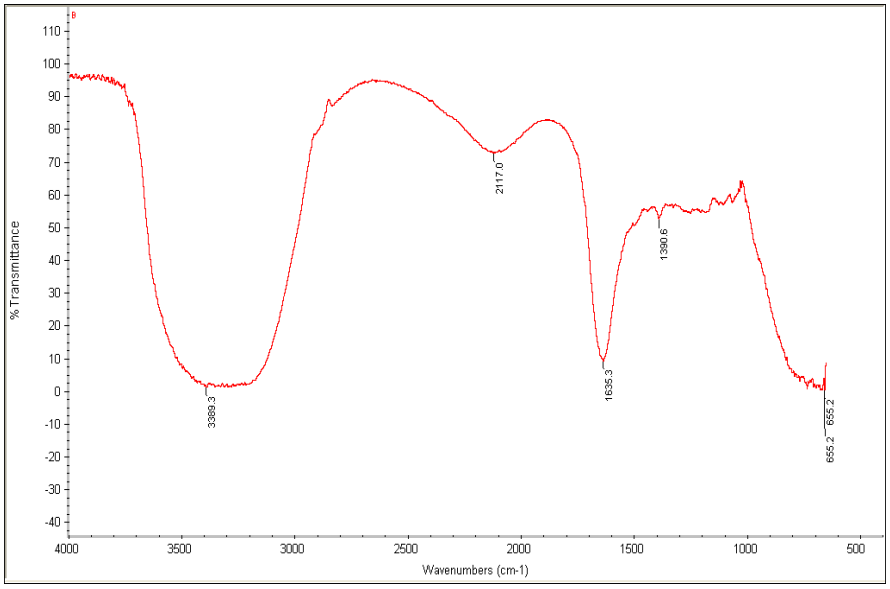


Fig 3.13: FTIR recorded with fresh Neem leaves extract

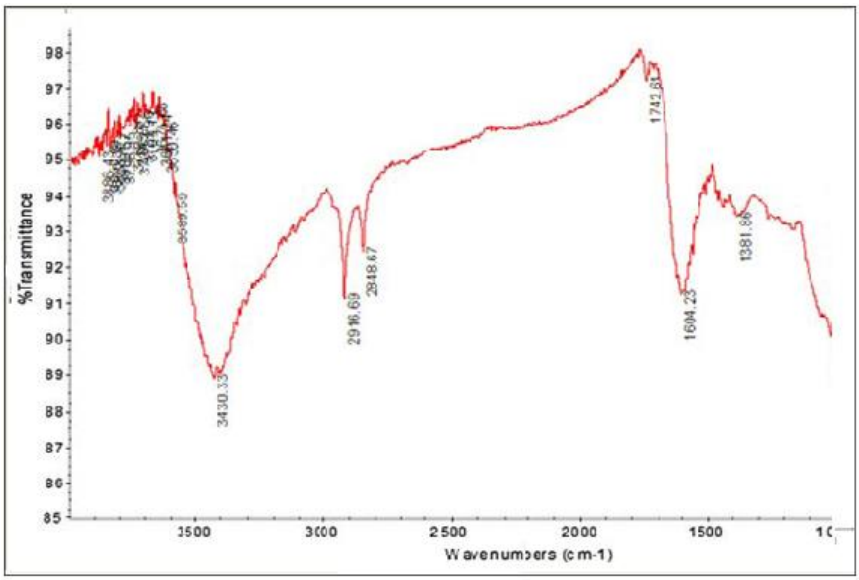
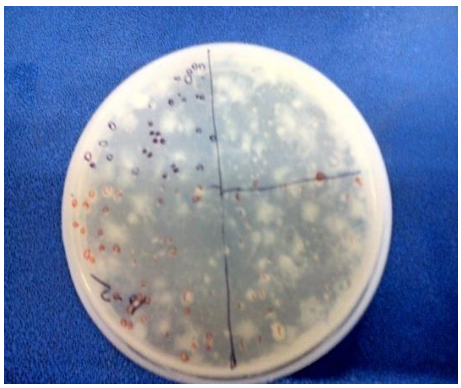


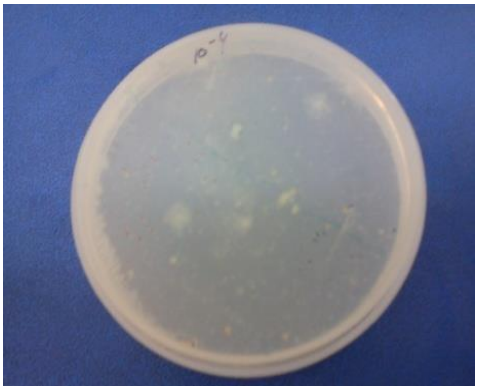
Fig 3.14: FTIR recorded with synthesized silver nanoparticles

3.7 STUDY OF ANTIBACTERIAL ACTIVITY

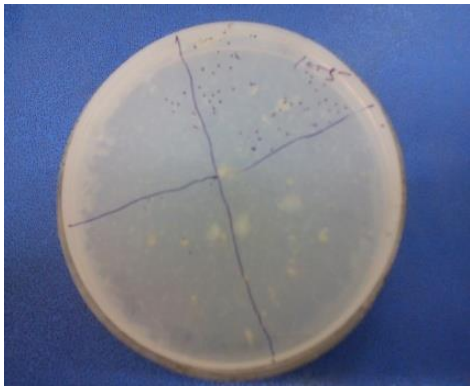
The soil sample is taken and serially diluted on 1.8% agar plates (1g beef extract, 1g peptone, 0.5 g NaCl dissolved in 100 ml of double distilled water) and pour plating technique is used to culture the microorganisms present in different dilutions. The plates are marked for each dilution Bacterial colony is observed after incubating the agar plates overnight at 37^{o}C.



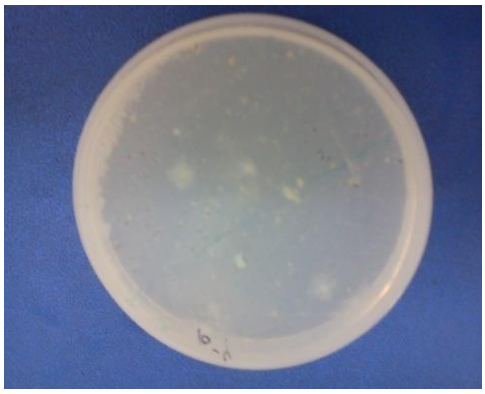
(A)



(B)



(C)



(D)

Fig 3.15: Colony morphology of the strains on the basis of serial dilution (A) 10^{-2} (B) 10^{-4} (C) 10^{-5} (D) 10^{-6}

Bacterial colonies were obtained from the plate which was serially diluted at 10^{-6} ml. Agar plates were streaked four quadrants to isolate different colonies and to obtain pure culture. The two pure colonies obtained from streaking are shown in Fig. 3.16.

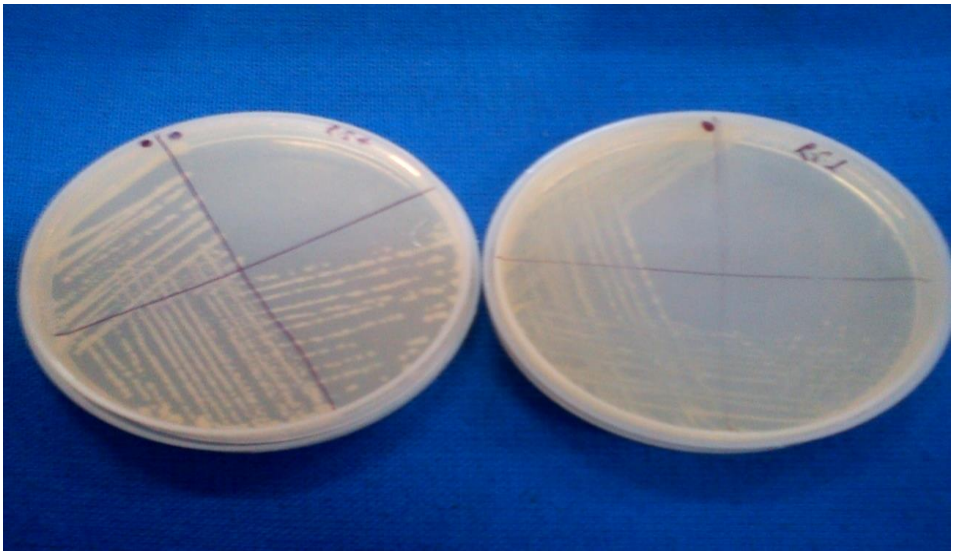


Fig 3.16: Pure colonies obtained from garden soil sample using streaking method from the plates at dilution 10^{-6} ml.

The antibacterial activity of the synthesized silver nanoparticles was tested against bacterial colony obtained from soil sample. Different quantity of nanoparticles (2, 4, 8, 12 μg/ml) were added to the agar plates containing bacterial colony. Zone of clearance was observed maximum at 12μg/ml of AgNPs. The antibacterial activity of Silver nanoparticles can be explained due to the change in the cell membrane permeability or degradation of enzymes in bacteria. The Zone of clearance observed at 12 μg/ml of AgNPs is 6 mm.

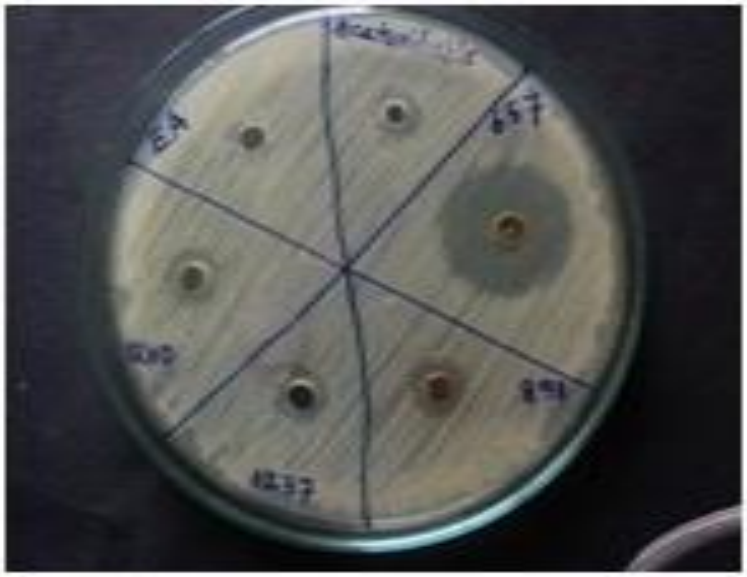


Fig 3.17: Zone of clearance is observed when silver nanoparticles are added to the bacterial colony isolated from garden soil. Nanoparticles are added at different concentration and zone of clearance is maximum at 12 µg/ml of AgNps, with diameter of 6mm.

CONCLUSION

Silver nanoparticles of few hundred nanometers are synthesized using Neem leaf extract, well-known medicinal plants. The synthesis is found to be efficient in terms of reaction time as well as stability of the synthesized AgNPs. Effect of various physical and chemical parameters is studied like pH, reactant concentration, temperature, reaction time. Synthesis of silver nanoparticles is enhanced with time at low temperature and alkaline pH. Nanoparticle synthesizes faster in case of Neem as compared to use of microbes, highlighting the probability that biological method of synthesis will achieve rate of synthesis comparable to those of chemical method. Investigation on the antibacterial activity of green synthesized Silver nanoparticles against bacterial colony isolated from soil sample reveals high potential of Neem extract stabilized Silver nanoparticles to be used as antimicrobial agent in medical field as well as food and cosmetic industries.

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