Chapter III

MATERIALS AND METHODS

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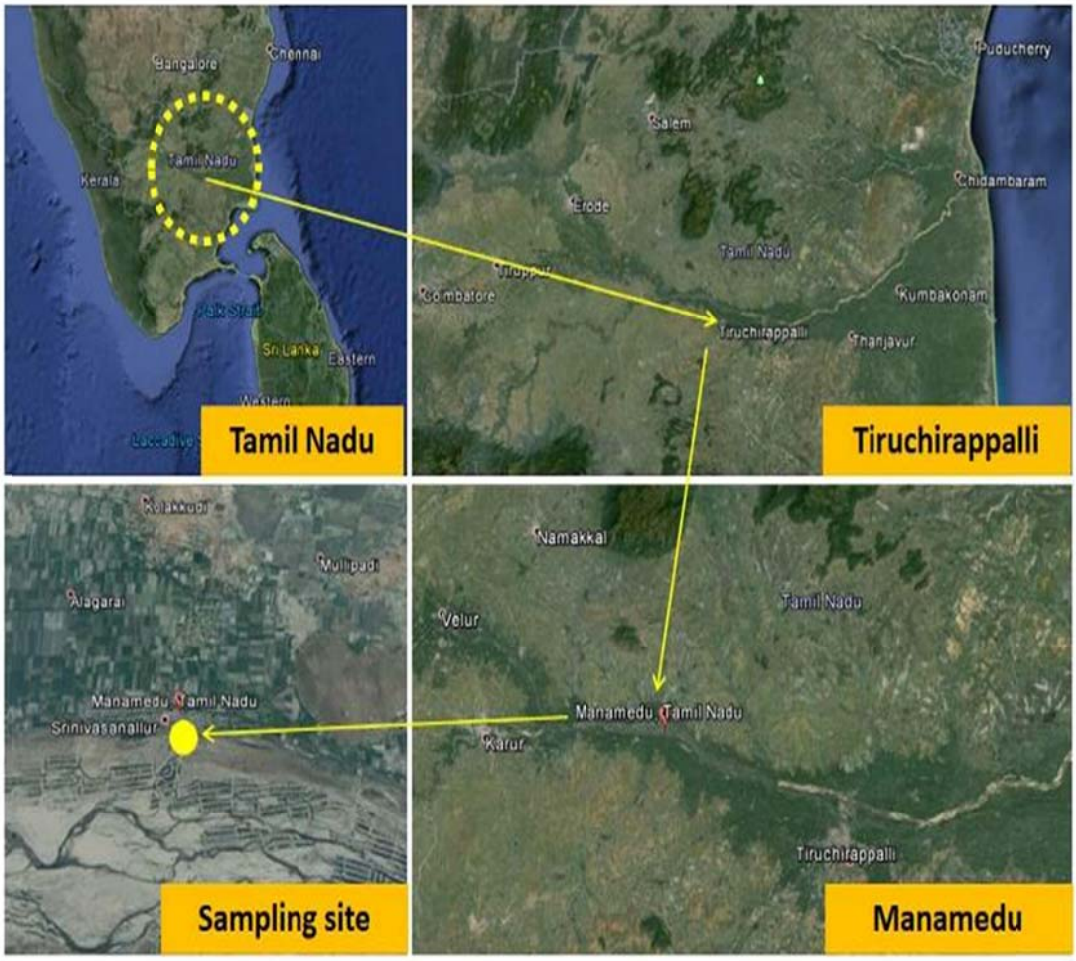
3.1. Study Area

The plant materials were collected from Manamedu village (10°58’22.77” N 78°23’09.78” E) of Tiruchirappalli District, Tamil Nadu (Plate 1). The Manamedu is the belongsto Musiri Taluk in Tiruchirappalli District of Tamil Nadu State, India. It is located 5 KM towards west from District headquarters. Musiri is surrounded by Kulittalai Taluk towards west, Thottiam Taluk towards west, Krishnarayapuram Taluk towards west, Andanallur Taluk towards East. This district lies at the Cauvery delta region, the most fertile region in the state. The district is the main rice producing region in the state and hence known as the rice bowl of Tamil Nadu. Cauvery River and its tributaries irrigate the district. Apart from paddy, farmers here grow coconut and sugarcane and it is the largest producer of coconut in Tamil Nadu. Nearly one lakh people are totally dependent on agricultural resources for their livelihood. The district of Tiruchirappalli is one of the richest herbal valleys of the Tamil Nadu. The important medicinal plant of *C. gynandra* was collected from Manamedu village of Tiruchirappalli district.

Plate 1. Sampling site of the study area



3.2. Taxonomic position of plant

Kingdom	:	Plantae	–	Plants
Subkingdom	:	Tracheobionta	–	Vascular plants
Super division	:	Spermatophyta	–	Seed plants
Division	:	Magnoliophyta	–	Flowering plants
Class	:	Magnoliopsida	–	Dicotyledons
Subclass	:	Dilleniidae		
Order	:	Capparales		
Family	:	Capparaceae	–	Caper family
Genus	:	*Cleome*	–	Spider flower
Species	:	*gynandra Linn.*		

3.3. Sample Collection and Processing

The plant materials were collected from the Manamedu village of Tiruchirappalli district, Tamil Nadu during the summer 2015. The shade dried plant powders (100 g) were successively extracted with ethanol and aqueous by soxhelt apparatus and is used as test sample for further studies such as phytochemical, heavy metal, secondary metabolites screening, silver nanoparticles, antimicrobial and anticancer studies.

Plants of *Cleome gynandra L.*

3.4. Phytochemical Screening

The solvent extracts (solid-liquid extraction) of raw plant sample was subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents (steroids, triterpenoids, reducing sugars, sugars, alkaloids, phenols, flavonoids, saponins anthroquinones, amino acids and catechins) present in the sample.

3.4.1. Steroid

Three ml of test solution and minimum quantity of chloroform was added with 3-4 drops of acetic anhydride and one drop of concentrated H_2SO_4. Purple colour thus formed changes into blue or green colour indicating the presence of steroids.

3.4.2. Triterpenoid

A 3 ml of test solution was added with a piece of tin and 2 drops of thionyl chloride. Formation of violet or purple colour indicates the presence of triterpenoids.

3.4.3. Sugars

A 3 ml of the test solution was added with very small quantity of anthrone reagent and a few drops of concentrated H_2SO_4 and heated. Formation of green or purple colour indicates the presence of sugars.

3.4.4. Reducing Sugars

A 3 ml of test solution was added with a 2 ml of Fehling's reagent and 2 ml of water. Formation of reddish orange colour indicates the presence of reducing sugar.

3.4.5. Alkaloid

A 3 ml of test solution was taken with 2N HCl. Aqueous layer formed was decanted and then added with one or a few drops of Mayer's reagent. Formation of white precipitate or turbidity indicates the presence of alkaloids.

3.4.6. Phenol

A 3 ml of test solution in alcohol was added with one drop of neutral ferric chloride (5%) solution. Formation of intense blue colour indicates the presence of phenols.

3.4.7. Flavonoid

A 3 ml of test solution in alcohol was added with a bit of magnesium and one (or) two drops of concentrated HCl and heated. Formation of red or orange colour indicates the presence of flavonoids.

3.4.8. Saponins

A 3 ml of test solution was added with water and shacked. Formation of foamy lather indicates the presence of Saponins.

3.4.9. Tannins

A 3 ml of test solution was added with water and lead acetate. Formation of white precipitate indicates the presence of tannins.

3.4.10. Anthroquinone

A 3 ml of test solution was added with magnesium acetate. Formation of pink colour indicates the presence of anthroquinones.

3.4.11. Amino Acids

A 3 ml of test solution was added with 1% ninhydrin in alcohol. Formation of blue or violet colour indicates the presence of amino acids.

3.4.12. Catechins

A 3 ml of test solution in alcohol was added with Ehrlich reagent and a few drops of concentrated HCl. Formation of pink colour indicate the presence of catechins.

3.5. Quantitative Analysis of Phytoconstituents

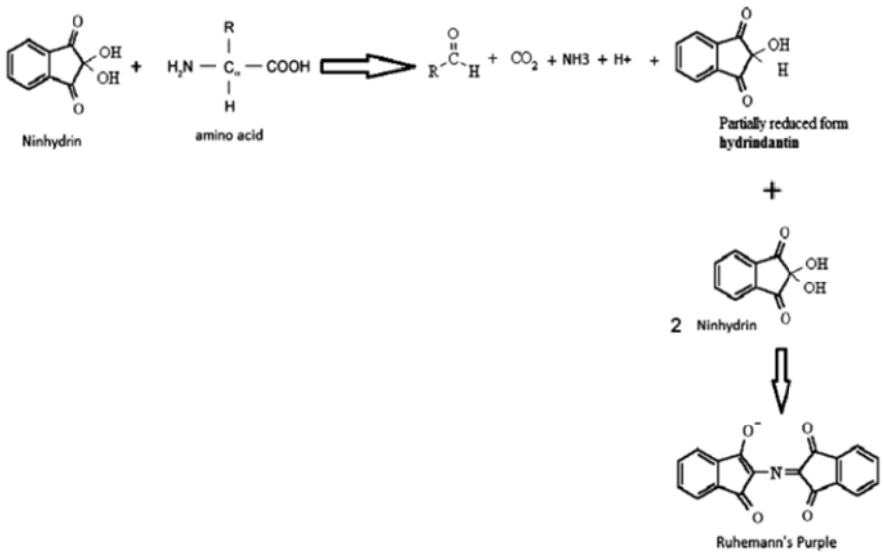
3.5.1. Estimation of Chlorophyll Pigments by (Arnon, 1949) Method

The chlorophyll pigments in the leaves were estimated following the method of Arnon (1949). After pre-cleaning, weighted fresh leaf material was homogenized and extracted thrice in chilled 80% acetone (v/v). The volume of the acetone extract was made up to a known one and the optical density was read at 645nm and 663nm wavelengths on a spectrophotometer. The concentration of the chlorophyll pigments was calculated and is expressed in mg/g fresh weight.

3.5.2. Estimation of Amino Acid by Ninhydrin Method, (Hwang, 1975).

Amino acids were estimated by Ninhydrin method which is calorimetrically measured at 570nm (Hwang, 1975). The amino acids are colourless ionic compounds that form the basic building blocks of protein. Apart from being bound as proteins, amino acids also many tissues and are known as free amino acids. They are mostly water soluble in nature. Very often in plants during disease conditions, the free amino acid composition exhibits a change hence, the measurement of the total free is the plants. Ninhydrin, a powerful

oxidizing agent, decarboxylates the alpha yields an intensely coloured bluish purple product which is colourimetrically measured.



Procedure

Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of standard amino acid solution to the respective labelled test tubes. Added distilled water in all the test tubes to make up the volume to 1ml. Added 1ml of distilled water to the test tube labelled Blank. Then added 1ml of ninhydrin reagent to all the test tubes including the test tubes labelled 'blank' and 'unknown'. Mix the contents of the tubes by vortexing /shaking the tubes. Then cover all the test tubes with paper/marble. Place all the test tubes in boiling water bath for 15 minutes. Cool the test tubes in cold water and add 5ml of diluents solvent to each test tube and mix well. Now record the absorbance at 570 nm of each solution using a colourimeter. Then plot the standard curve by taking concentration along X-axis and absorbance at 570 nm along Y-axis.

3.5.3. Estimation of Protein by Bradford Method

The assay is based on the ability of protein to bind coomassie brilliant blue G250 and form a complex whose extinction coefficient is much greater than that of the free dye.

3.5.3.1. Dye Concentrate

Dissolve 100 mg of coomassie brilliant blue G250 in 50 ml of 95 % ethanol. Add 100 ml of concentrated orthophosphoric acid. Add distilled water to a final volume of 200 ml. store refrigerated in amber bottles; the solution is stable at least 6 months. Mix 1 volume of concentrated dye solution with 4 volumes of distilled water for use. Filter with Whatman No. 1 paper.

3.5.3.2. Protein Standard

100 μg/ml in Phosphate Buffer Solution (PBS).

3.5.3.3. Procedure

Pipette out 0.0 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labelled test tubes. Also, Pipette out 1 ml of the given sample in another test tube. Make up the volume to 1 ml in all the test tubes with PBS. A tube with 1 ml of distilled water serves as the blank. Now add 5 ml of diluted dye solution to all the test tubes including the test tubes labelled 'blank' and 'unknown'. Mix the contents of the tubes by vortexing / shaking the tubes and allow the colour to develop for at least 5 min but not more than 30 min. The red dye turns blue when it binds protein. Now record the absorbance at 595 nm against blank. Then plot the standard curve by taking concentration of protein along X-axis and

absorbance at 595 nm along Y-axis. Then from this standard curve calculate the concentration of protein in the given sample.

3.5.4. Estimation of Carbohydrate by Anthrone Method (Hedge and Hofreiter, 1962)

The anthrone reaction is the basis of a rapid and convenient method for the determination of carbohydrates, either free or present in polysaccharides. Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm. Weigh 100 mg of the sample into a boiling tube.

3.5.4.1. Procedure

Hydrolyse by keeping it in a boiling water bath for three hours with 5 mL of 2.5 N HCl and cool to room temperature. Neutralise it with solid sodium carbonate until the effervescence ceases. Make up the volume to 100 mL and centrifuge. Collect the supernatant and take 0.5 and 1 mL aliquots for analysis. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard.'0' serves as blank. Make up the volume to 1 mL in all the tubes including the sample tubes by adding distilled water. Then add 4 mL of anthrone reagent. Heat for eight minutes in a boiling water bath. Cool rapidly and read the green to dark green colour at 630 nm.

3.5.5. Determination of Total Phenolic Contents in the Plant Extracts

The concentration of phenolic in plant extracts was determined using spectrophotometric method (Singleton et al., 1999). Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45^0 C for 45 min. The absorbance was determined using spectrophotometer at λ max = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolic was read (mg/ml) from the calibration line; then the content of phenolic in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

3.5.6. Secondary Metabolites Screening by GCMS

3.5.6.1. Ethanol Extraction of Plant Samples

Sterilized fine powder, 20 g each was taken each plant, mixed with 200 ml of Milli Q water and kept in boiling water bath at 60°C for 10 min. The extracts were filtered with whatman No. 1 filter paper and the filtered extracts were stored in a refrigerator at 4°C and it's used as test samples for basic preliminary study. The 250 g of sterile fine powder was processed in soxhelt apparatus for attaining

of ethanol extraction of the plant sample. The ethanol extraction of plant sample was used for analysis of bioactive compounds through several techniques.

3.5.6.2. Screening

This ethanol extracts of plant sample were sonicated for 20 min in sonicator 20 µl from sonicated extracts was passed through 0.45 µm filters. Filtrate was used for GCMS analysis. Gas Chromatography Mass Spectrometry (GC-MS) is a technique for the analysis and quantitative of organic volatile and semi-volatile compounds. Gas chromatography (GC) is used to separates mixtures into individual components using a temperature-controlled capillary column. Smaller molecules with lower boiling points travel down the column more quickly than larger molecules with higher boiling points. The maximum allowable temperature for this method is 300ºC. The GCMS system (Agilent 7890A GC-MS QToF 7200 series) was used. Chromatographic analysis was carried out using an INNOWAX 30 m x 0.250 mm x 0.25 um column at temperature: ambient. Running conditions included: injection volume HS 2.5 mL syringe, HS SPME injection technique; mobile phase: Helium. Samples were filtered through an ultra-membrane filter (pore size 0.45 µm) prior to injection in the sample loop. Retention time and concentration of Metabolites were analysed by using in-built GCMS software.

3.6. Screening of Trace Metals

3.6.1. Sampling and Processing

The ground water, surface soil and plant (*Cleome gynandra*) samples were collected during summer and monsoon 2014. The 2000 ml of water samples were collected with a 2500 ml sterile container and 250 g of surface soil

samples were collected with a sterile spatula. All samples were kept in iceboxes and processed within 12 h of collection. For heavy metal analysis, the one liter of sea water was acidified immediately with concentrated nitric acid (HNO_3).

3.6.2. Trace Metal Analysis

For trace metal study, acidified the water samples were filtered by Whatman No.1 filter paper and processed (APDC + MIBK) for metal analysis. The soil samples were air-dried and smaller than 63 μm in size were retained in pre-cleaned properly. Simultaneously, the three different parts (root, stem and leaves) of *Cleome gynandra* plant were collectedThe plant leaves were carefully removed and washed with sterile distilled water, separately. Thereafter, the dried soil and plant samples were crushed by agate mortar and pestle. The samples were treated with aqua-regia mixture (i.e. HCl: HNO_3= 3:1) in Teflon bomb and were incubated at 140 °C for 2-3 days after dried and sieved samples. After incubation, the reaction mixture was filtered with nitrocellulose (0.45 μm) filter paper by Millipore vacuum filtration unit. Then the extraction was test for trace metals (Fe, Cu, Zn, Pd, Cd, Cr and Ni) analysis. The trace metals in the water and soil samples were determined by the atomic absorption spectrophotometry (GBC SensAA - AAS, Australia) in flame mode.

3.7. Biosynthesis and Characterization of Silver Nanoparticles (AgNPs)

Biosynthesis of silver nanoparticles (AgNPs), Silver nitrate prepared at the concentration of 10^{-3} M with pre-sterilized Milli Q water. A quantity of 10 mL *C. gynandra* extract was mixed with 90 mL of 10^{-3} M silver nitrate for the synthesis of silver nanoparticles. Silver nitrate has taken in similar quantities without adding plant extracts to main respective controls. The saline bottles were tightly

covered with aluminium foil in order to avoid photo reduction of ions, incubated at room temperature under dark condition and observations were recorded. After AgNPs formation, it was characterized by UV-vis spectroscopy (UV spec), Fourier transform-infra red spectroscopy (FTIR), Scanning electron microscope (SEM), energy dispersive spectroscopy (EDS), X-ray diffraction (XRD) and dynamic light scattering (DLS - particles analyzer/ 'Z' potential) methods.

3.8. Characterization of Nanoparticles

3.8.1. UV-Vis Spectroscopy

Absorbance spectroscopy is used to determine the optical properties of a solution. A Light is send through the sample solution and the amount of absorbed light is measured. When the wavelength is varied and the absorbance is measured at each wavelength. The absorbance can be used to measure the concentration of a solution by using Beer-Lamberts Law. The examination of nanoparticles, the optical properties are much more complicated. For instance, the measured absorbance spectrum does not necessarily show the actual absorbance but the extinction of the light is both the absorbed and the scattered light from the particles. These wave lengths arise due to the surface Plasmon resonance of the particle.

3.8.2. Fourier Transform-Infra Red (FT-IR) Spectroscopy

FTIR is a chemical analytical method which measures infrared intensity v/s wavelength or wave number of light. It used to analysis of possible bio molecule and also bonding interaction between themselves. IR spectroscopy detects the vibration characteristics of chemical functional groups of the sample. When an infrared light interacts with matter, chemical bonds will show stretch,

contract and bend form. This chemical functional group tends to adsorb infrared radiation in a specific wave number range of the structure of the rest of the molecule. The silver nanoparticle synthesis, FTIR data measures interaction between Ag salts and proteins molecules, which accurate for the reduction of silver ions and stabilization of Ag NPS formed. The analysis of bio-reducing agent present in each of the extracts was measured by FT-IR. After the reaction, a small aliquot of the concentrated reaction mixture was measured in the transmittance mode at 400 to 4000 cm-1. The spectra of the extracts taken after the biosynthesis of nanoparticles were analyzed.

3.8.3. Scanning Electron Microscope (SEM) and Energy Dispersive Spectroscopy (EDS)

Scanning electron microscope (SEM) analysis the employed to characterization of size, shape & morphologies of formed nanoparticle SEM gives high-resolution images of the surface of a sample is desired. The scanning electron microscope works as same principle as an optical microscope, but it measures the electrons scattered from the sample rather than photon. Because electrons can be accelerated by an electric potential, the wavelength can be made shorter than the one of photons. This makes the SEM capable of magnifying images up to 200.000 times. At the same time, it is possible to achieve high resolution pictures of the surface, making the instrument very useful in determining the size distribution of nanoparticles. In this research work, Joel JSM-6480 LV SEM machine was used to characterize the mean particle size and morphology of nanoparticles. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM.

The EDS analysis of Ag sample was done by the SEM (JEOLJSM 5800) machine. The EDS normally reveals the presence of phases.

3.8.4. Dynamic Light Scattering

The DLS technique uses light to determine the size of particles in a solution. Light at a given frequency is sent through the solution from a laser. When the light interacts with the moving particles in the solution and is scattered, the frequency of the light is also changed. This change of light frequency is directly related to the size of the particles in the solution; the smaller the particles, the greater the shift in the light frequency. This difference in the light shift is used to determine the size of the particles in the solution. DLS is capable of measuring particles in the size range from a few nanometers to a few micrometers. It is therefore applicable for determining the size of silver nanoparticles.

3.8.4.1. Dynamic Light Scattering *Particle size analyzer*

In order to find out the particle size distribution the Ag powder was dispersed in water by horn type ultrasonic processor (Vibronics, model: VPLP1). Then an experiment was carried out in computer controlled particle size analyzer [ZETA Sizers Nanoseries (Malvern Instruments Nano ZS)] to find out the particle size distribution.

3.8.4.2. Dynamic Light Scattering *Zeta Potential Measurement*

Zeta potential describes the electrical potential in the double layer of ions surrounding a particle at the boundary of the particle surface and the adsorbed

ions in the diffuse layer (Ives, 1956; Henderson, 2008). Zeta potentials were determined with a Zetaphorementer IV (CAD, France).

3.8.5. X-ray Diffraction Method

XRD is a technique to used go study phase composition of a sample, crystal structure, texture or orientation. The principle of XRD is that the X-rays are passed through a material and the pattern produced give information of size and shape of the unit cell. The atoms are crystal in structure arranged in a periodic array and thus can diffracted light at different angle. When X-ray passing through a crystal it produces a diffraction pattern, that diffraction gives the information about the atomic arrangement within the crystals. In silver nanoparticle XRD gives phase structure and purity of the particle. The phase evolution of calcined powder as well as that of sintered samples was studied by X-ray Diffraction Technique (Philips PAN analytical, The Netherlands) using Cu radiation. The generator voltage and current was set at 40 KV and 30 mA respectively. The Au sample was scanned in the range 10.0000 - 90.0000^{o} in continuous scan mode. The scan rate was 0.60/sec.

3.8.6. Applications of *C. gynandra* Mediated AgNPs

3.8.6.1. Determination of Antimicrobial Activity

The two different test samples (raw and AgNPs) were challenged against certain microbial strains (procured from MTCC and NCIM, India) for antimicrobial sensitivity using the disc diffusion method (Bauer and Kirby, 1966 and Vignesh *et al*., 2013). The test strains were: *Aeromonas liquefactions* MTCC 2645 (B1), *Micrococcus luteus* NCIM 2871 (B2), *Salmonella typhimurium* NCIM 2501 (B3), *Candida albicans* MTCC 1637 (F1), *Cryptococcus* sp. MTCC

7076 (F2), *Trichophyton rubrum* MTCC 3272 (F3). A sterile cotton swab was used to inoculate the bacterial and fungal suspension on surface of MHA and PDA agar plates. The 15 and 30 µL of sample coated disc were placed in agar plates, separately. For negative control study, the sterile triple distilled water was used. The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungus). After incubation, the zone of inhibition was measured with ruler. All the trial was performed thrice and means values were presented

3.8.6.2. Determination of Anticancer Activity

For anticancer, AgNPs samples were dissolved in DMSO, diluted in culture medium and used to treat the chosen cell line (MG63) (obtained from NCCS) over a sample concentration (10 different concentrations – 0.1, 1.0, 10 25 and 50 µg/mL) range of 0.1 - 50 µg/mL for a period of 24 h and 48 h. The DMSO solution was used as the solvent control. A miniaturized viability assay using 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2H-tetra-zolium bromide (MTT) was carried out according to the method described by standard procedure (Mosmann, 1983) To each well, 20 µl of 5 mg/mL MTT in phosphate-buffer (PBS) was added and wrapped with aluminium foil, and incubated for 4 h at 37 ^{0}C. The purple formazan product was dissolved by addition of 100 µl of 100 % DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for four replicates. Each and used to calculate the respective means.

The percentage of inhibition was calculated, from this data, using the formula:

$$\frac{\text{Mean absorbance of untreated cells (control)} - \text{mean absorbance of treated cells (test)}}{\text{Mean absorbance of untreated cells (control)}} \times 100$$

The IC_{50} value was determined as the complex concentration that is required to reduce the absorbance to half that of the control.