Chapter 3

Materials and Methods

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MATERIALS AND METHODS

3.1. Plants

The medicinal plants *Mallotus tetracoccus* (Roxb.) Kurz and *Tabebuia rosea* (Bertol.) DC have been selected for the present investigation based on the Ayurvedic literature [Indian medicinal plants (Vol 1-5) of Warrier *et al*. (1996)] (Plate 3.1).

3.1.2. *Mallotus tetracoccus* (Roxb.) Kurz

Family : Euphorbiaceae

Common name : Mullu polavu, Vatta (Tamil), Thavatta, Vatta, Vatta kumbil, Vetta kumbil (Malayalam) and Uppale mara (Kannada)

Distribution : Evergreen forests up to altitude of 1600m

Plant nature : Trees grow up to 5-15 m tall

Parts used : Leaves

Leaves : Leaves simple, alternate, spiral; stipule caducous; petiole 3-12.5 cm long, terete, stellately tomentose, swollen at both ends; lamina 8-25 x 6.5-20 cm, broadly ovate to orbiculate, apex acute to acuminate, base peltate or subpeltate, usually entire or trilobed when young, coriaceous, densely white stellate tomentose beneath; 3-5 nerved at base; tertiary nerves nearly horizontal percurrent.

Properties and Uses : The reported bioactivities of the extracts or the individual chemical constituents isolated from this genus include antipyretic (Chattopadhyay *et al*., 2002), anti-inflammatory, hepatoprotective (Kim *et al*., 2000), antioxidant and radical scavenging activities (Arfan *et al*., 2007) (www.biotik.org).

3.1.2. *Tabebuia rosea* (Bertol.) DC

Family : Bignoniaceae

Common name : Pink trumpet tree

Distribution : Throughout India

Plant nature : Trees grow up to 15 meter and well known for its beautiful flowers

Parts used : Leaves

Properties and Uses : The herbal products obtained from the bark of *Tabebuia* trees are called "taheebo", "lapacho", "pau d'arco", and "ipe roxo". Tea made from the leaves and bark is known to have a fever-reducing effect (Gentry, 1992). *Tabebuia rosea* has various medicinal applications traditionally; as astringent, anti-inflammatory, antibacterial, antifungal, and laxative (Abbott *et al*., 1967; Hartwell, 1968; Bastein, 1983; Arenas, 1987; Almedia *et al*., 1990). It is also used to treat ulcers, syphilis, urinary tract infections, gastrointestinal problems, candida and yeast infections, cancer, diabetes, prostatitis, constipation and allergies. Pau d'arco and its chemicals have also demonstrated *in vitro* antimicrobial and antiviral properties (Oloyede *et al*., 2010).

3.1.3. Collection of Plant Material

Fresh and intermediate leaves of *Mallotus tetracoccus* (Roxb.) Kurz and *Tabebuia rosea* (Bertol.) DC was collected from Agasthiar Malai Biosphere Forest, Western Ghats and Centre for Biodiversity and Forest Studies (CBFS), Madurai Kamaraj University, Madurai, Tamilnadu, India respectively. The collected plant materials were identified (Matthews *et al*., 1999), authenticated by the Director, CBFS, MKU, and voucher specimens were deposited in the herbarium of CBFS of our university (No. AM-02, 03).

3.2. Preparation of Extracts

- The collected plant materials were washed under running tap water to remove the surface pollutants.
- The fresh plant leaves of *Mallotus tetracoccus* and *Tabebuia rosea* were shade dried and powdered in a mechanical grinder.
- The powdered plant materials were packed in small packets and extracted successively with different solvents such as petroleum ether, chloroform, ethyl acetate, acetone and 70 % ethanol in the increasing order of polarity using

Mallotus tetracoccus **(Roxb.) Kurz**

Tabebuia rosea **(Bertol.) DC**

Plate 3.1 Medicinal plants chosen for study

soxhlet apparatus for 72 h. Each time before extracting with the next solvent, the thimble was air dried.

- The different solvent fractions were filtered using Whattman filter paper # 1 and then concentrated by the solvent evaporation and vacuum dried at 45 °C for solvent removal, and the extracts were kept in sterile bottles under refrigerated conditions until use.

- The dried viscous plant residue fractions of *Mallotus tetracoccus* and *Tabebuia rosea* were used for various *in vitro* antioxidant, antimicrobial, toxicity studies and IR, GC-MS analysis.

- MT1 - Petroleum ether fraction of *Mallotus tetracoccus* (MT).
- MT2 - Chloroform fraction of *Mallotus tetracoccus.*
- MT3 - Ethyl acetate fraction of *Mallotus tetracoccus.*
- MT4 - Acetone fraction of *Mallotus tetracoccus.*
- MT5 - Aqueous ethanolic fraction of *Mallotus tetracoccus.*
- TR1 - Petroleum ether fraction of *Tabebuia rosea*.
- TR2 - Chloroform fraction of *Tabebuia rosea.*
- TR3 - Ethyl acetate fraction of *Tabebuia rosea.*
- TR4 - Acetone fraction of *Tabebuia rosea.*
- TR5 - Aqueous ethanolic fraction of *Tabebuia rosea.*

3.3. Physicochemical Analysis

3.3.1. Study on Organoleptic Characteristics

The ash values are useful to determine the purity and quality of the crude drug. Ash contains inorganic radicals like phosphate, carbonates and silicates of sodium, potassium, magnesium, calcium etc. Sometimes inorganic variables like calcium

oxalate, silica, carbonate content of the crude drug affects total ash value. Hence determination of ash values is also considered as important parameters in pharmacognostic evaluation of drug.

The procedure recommended in Lala (1993) and Indian Pharmacopoeia (1996) were followed for the determination of moisture content, total ash, acid insoluble ash and water insoluble ash.

A. Loss on Drying

The powdered samples (2 g) were taken in tarred china dish and dried in an electrical oven at 105 ^{o}C, cooled in a dessicator and watched for loss on drying.

B. Determination of pH of Aqueous Solution

The powdered materials (80 µm mesh) were suspended in glass distilled water. After 2 h, filtered and the clear solution was measured for pH.

C. Total Ash Value

About 2 g of plant powder were ignited in an electric furnace at 500 – 550 ^{o}C in silica crucible until the sample reached a constant weight.

D. Water Insoluble Ash Value

About 2 g of powdered sample was taken in a silica crucible and 25 ml of distilled water was added, mixed well and boiled. The insoluble matter was filtered on ash less filter paper (grade 4T SDS clear drop, 90 mm code F0401 C10, circular 100). The residue obtained was ignited in crucible and cooled. The residue was weighed and calculated for water insoluble ash value.

E. Acid Insoluble Ash Value

About 2 g of powdered sample was taken in a silica crucible and 25 ml of hydrochloric acid was added. Total ash was boiled for 5 min and diluted with 25 ml hydrochloric acid. The residue was ignited in crucible and cooled. The residue was weighed and calculated for acid insoluble ash value.

3.4. Qualitative Phytochemical Screening

The different qualitative chemical tests were performed for establishing profile of the given extract for its chemical composition. Qualitative phytochemical analyses were done using the procedures of Brindha *et al*. (1981). The following tests were performed on extracts to detect various phytochemical constituents present in them.

3.4.1. Detection of Alkaloids (Dragendorff's test)

50 mg of solvent free extract was stirred with 5 ml of dilute hydrochloric acid and filtered. To a 3 ml of filtrate, 1 – 2 ml of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive for the presence of alkaloids.

3.4.2. Detection of Terpenoids

0.5 g of each plant extract was taken and mixed with 2 ml of chloroform. To this concentrated sulphuric acid was added carefully to form a layer and observed for the presence of reddish brown colour interface which indicates positive results for the presence of terpenoids.

3.4.3. Detection of Reducing Sugars

Three test tubes were taken separately for each plant material and 2 ml of each crude plant extract was added in separate test tubes with 5 ml of distilled water and filtered. The filtrates were boiled with 3-4 drops of Fehling's solution A and B for 2 minutes. The orange red precipitate indicates the presence of reducing sugars.

3.4.4. Detection of Glycosides (Borntrager's test)

For the detection of glycosides, 50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hrs on a water bath, filtered and the hydrolysates were collected. To 2 ml of the filtered hydrolysate, 3 ml of chloroform was added and shaken; the chloroform layer was separated and 10 % ammonia solution was added to it. The pink colour indicated the presence of glycosides.

3.4.5. Detection of Saponins by Foam Test

50 mg of plant extract was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min, where two cm layer of foam indicates the presence of saponins.

3.4.6. Detection of Flavonoids (Alkaline Reagent Test)

0.2 g of plant extract dissolved in diluted sodium hydroxide, diluted hydrochloride were added and observed for yellow solutions that turns colorless which indicates the presence of flavonoids.

3.4.7. Detection of Phenolic Compounds (Ferric Chloride Test)

About 50 mg of plant extract was dissolved in 5 ml of distilled water. To this, a few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

3.4.8. Detection of Steroids

To the plant extract, 2 ml of acetic anhydride and 2 ml of sulphuric acid were added and observed for the color change from violet to blue or green in samples indicating the presence of steroids.

3.5. Quantitative Phytochemical Analysis

3.5.1. Determination of Total Phenols

The total phenolic contents of various fractions were determined according to the method described by Siddhuraja and Becker (2003). Aliquots of each extracts were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20 %) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbances of the samples were recorded at 725 nm against the reagent blank. Total phenol contents were expressed in terms of gallic acid equivalent (mg/g of dry mass), which is used as a reference compound.

3.5.2. Determination of Flavonoids

Flavonoid contents of plant fractions were determined according to the aluminum chloride colorimetric method by Chang *et al*. (2002) with some modifications. Quercetin compound was used as a standard to make the calibration curve. The sample solution (0.5 ml) were mixed with 1.5 ml of 95 % ethanol, 0.1 ml of 10 % aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 40 min the absorbance of the reaction mixture was measured at 415 nm. The same amount (0.1 ml) of distilled water substituted for the amount of 10 % aluminum chloride in the blank.

3.5.3. Determination of Carotenoids

Total carotenoids were determined by the method of Jensen (1978). One gram of samples was extracted with 100 ml of 80 % methanol solution and centrifuged at 4000 rpm for 30 min. The supernatants were concentrated to dryness. The residues were dissolved in 15 ml of diethyl ether and after addition of 15 ml of 10 % methanolic KOH, the mixtures were washed with 5 % ice-cold saline water to remove alkali. The free ether extracts were dried over anhydrous sodium sulphate for 2 h. The ether extracts were filtered and absorbances of various fractions were measured at 450 nm using ether as blank.

3.6. Spectral Analysis

3.6.1. UV-Vis Spectroscopy Analysis

The fractions of *M. tetracoccus* (1-5) and *T. rosea* (1-5) were subjected to spectral analysis under the ultraviolet and visible spectrum using UV-Vis spectrophotometer.

3.6.2. Volatile Profile analysis by GC –MS

3.6.2.1. Instruments and Chromatographic Conditions

GC-MS analysis were carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass

spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100 % Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999 %) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) with injector temperature 250 °C and ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C/min, then 5 °C/min to 280 °C/min, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Interpretations on mass spectrums of GC-MS were done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectra of the unknown components were compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

3.7. *In vitro* Antioxidant Studies

3.7.1. DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 2, 2-diphenyl-2- picrylhydrazyl (DPPH) free radicals were determined by the method described by Braca *et al.* (2002). Plant extract (0.1 ml) was added to 3 ml of a 0.004 % methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage inhibition activity was calculated from $[(A_0–A_1)/A_0] \times 100$,

Where, A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract/ standard.

A percent inhibition versus concentration curves were plotted and the concentration of sample required for 50 % inhibition was determined and represented as IC_{50} value for each of the test solutions.

3.7.2. Reducing Power Assay (Iron (III) to Iron (II) Reduction)

Aliquots of each fraction were taken in test tubes and dissolved in 1 ml of 0.2 M phosphate buffer to which 5 ml of 0.1 % solution of potassium ferric cyanide were added (Siddhuraju *et al*., 2002). The mixtures were incubated 50 °C for 20 min. Following this, 5 ml of trichloroacetic acid (10 %) (w/v) solution was added to samples and the mixtures were then centrifuged at 7000 rpm for 10 min. A 5 ml of aliquot of the upper layer combined with 5 ml of distilled water and 1 ml of ferric chloride solution (0.1 %), were used for measuring the absorbance at 700 nm against reagent blank.

3.7.3. Metal Chelating Activity

The chelating of ferrous ions of various sample were estimated by the method of Dinis *et al*. (1994). The extract samples (250 µl) were added to a solution of 2 mmol/l $FeCl_2$ (0.05 ml). The reaction of test samples were initiated by the addition of 5 mmol/l ferrozine (0.2 ml) and the mixtures were shaken vigorously and left standing at room temperature for 10 min, after which the absorbance was measured spectrophotometrically at 562 nm. The chelating activities of the extracts were evaluated using EDTA as standard. The metal chelating activity of the extract is expressed as mg EDTA equivalent/g extract.

3.7.4. Total Antioxidant Activity

The antioxidant activities of fractions were evaluated by the green phosphomolybdenum complex formation according to the method of Prieto *et al*. (1999). An aliquot of 100 µl of sample solution was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in a 4 ml vial. The vials were canned and incubated in a water bath at 95 °C for 90 min. After the samples have cooled to room temperature, the absorbances of the reaction mixtures were measured at 695 nm against a blank. The results reported (ascorbic acid equivalent antioxidant activity) are mean values expressed as g of ascorbic acid equivalents/100 g extract.

3.7.5. Ferric Thiocyanate (FTC) Method

The antioxidant activity of the plant fractions were also tested using two methods: ferric thiocyanate (FTC) and thiobarbituric acid (TBA).

The standard method described by Kikuzaki and Nakatani (1993) was used. A mixture of 4.0 mg plant extract in 4 ml absolute ethanol, 4.1 ml of 2.5 % linolenic acid in absolute ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water were placed in a vial with a screw cap and then placed in an oven at 40 ˚C in the dark. To 0.1 ml of this solution, 9.7 ml of 75 % ethanol and 0.1 ml of 30 % ammonium thiocyanate were added. Precisely 3 min after addition of 0.1 ml of 0.02 M ferrous chloride in 3.5 % HCl to the reaction mixture, the absorbance of red color was measured at 500 nm each 24 h until the day after absorbance of control reached maximum. BHT and α- tocopherol were used as the positive controls while the mixture without plant sample was used as the negative control.

3.7.6. Thiobarbituric acid (TBA) Method

The method of Ottolenghi (1959) was referred. 2 ml of 20 % trichloroacetic acid and 2 ml of 0.67 % 2-thiobarbituric acid were added to 1 ml of sample solutions, as prepared in FTC method. The mixtures were placed in a boiling water bath for 45 min, cooled and centrifuged at 3000 rpm for 20 min. Absorbance of supernatants were measured at 552 nm. Antioxidant activities were recorded based on the absorbance on the final day of FTC method.

3.8. Antibacterial Studies

3.8.1. Disc Diffusion Method

The various fractions of *M. tetracoccus* and *T. rosea* were screened against six bacterial pathogens. The clinical isolates used for study are *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa.* The different concentrations of the fractions (5, 10, 50, 100, 250 and 500 µg/ml) were tested for antibacterial activity using agar disc diffusion assay according to the method of Bauer *et al*. (1966). The strains of microorganisms obtained

were inoculated in conical flask containing 100 ml of nutrient broth. These conical flasks were incubated at 37 ºC for 24 h and were referred to as seeded broth. Media were prepared using Muller Hinton Agar (Himedia, Mumbai, India), poured on Petri dishes and inoculated with the test organisms from the seeded broth using cotton swabs. Sterile discs of six millimeter width had been impregnated with test extract and introduced onto the upper layer of the seeded agar plate. The plates were incubated overnight at 37 ºC. Antibacterial activities were measured as inhibition zones formed around the discs. The experiments were done three times and the mean values were presented. Streptomycin (10 µg/disc) and penicillin (10 µg/disc) were used as standards.

3.9. Cytotoxicity Bioassay using Brine Shrimps

Brine shrimp cytotoxicity assay were performed according to the standard procedure described by McLaughlin (1991). Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 48 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Twenty nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light and surviving larvae were counted with a hand lens. Experiments were conducted along with control (vehicle treated), different concentrations (1-1000 µg/ml) of the test substances in a set of three tubes per dose. Based on the percent mortality, the LD_{50} of the test compound was determined using probit scale (Wardlaw, 1985).

3.10. Phytotoxicity Studies using Radish Seeds

The phytotoxic properties of the various fractions of plant extracts were evaluated using radish seed phytotoxicity assay (Turker and Camper, 2002; Islam *et al*., 2009). Three types of determinations were done for this purpose:

3.10.1. Root Length Determination

Radish seeds were washed with distilled water and with 1 % mercuric chloride. Whatman # 1 filter papers were kept on Petri dish and 5 ml extracts (100 ppm, 1000 ppm and 10,000 ppm) were added separately. Filter papers were dried at room temperature for reducing extra solvent. 5 ml double distilled water was added and then 20 radish seeds were placed on Petri dishes followed by tightly sealed and incubation at 23 ± 2 °C. Root lengths were measured after 1, 3 and 5 days of interval. Petri dishes containing double distilled water were used as control. Each of the assays was carried out three times. Similar process was carried out for shoot length determination.

3.10.2. Seed Germination Determination

This part of the determination is similar to that of earlier determination except for the extract concentrations and number of seeds. Here two different concentrations (1000 ppm and 10,000 ppm) and 100 radish seeds were used. Germinated seeds were counted after every day up to 5 days. Each experiment was carried out three times.

3.10.3. Data Analysis

The following parameters were adopted in this analysis to evaluate the conditions of seed germination: Relative germination rate and Germination Index. They were calculated based on the following equations according to previous reports (Thompson *et al*., 2001):

Relative germination rate = [Seeds germinated in test sample/Seeds germinated in control]*100

Relative root elongation = [Mean root length in test sample/Mean root length in control]*100

Germination Index = [Relative germination rate* Relative root elongation]/100.

3.11. Genotoxicity Studies

3.11.1. Cells Culture

Human T-cell leukemia (MOLT-4) cells procured from National Centre for Cell Sciences, Pune, India were used for evaluating anticancer potential. Peripheral whole blood (15 ml) from 3 healthy, 22-25 years old female volunteers was collected with informed consent in heparinized vacutainer tubes (Becton Dickinson Lab ware, USA). Donors were nonsmokers and had not been exposed to chemicals, drugs or X rays in the last 6 months before blood sampling. Lymphocytes were separated using histopaque-1077 (a leukocyte separation technique by Sigma Diagnostics) by density gradient centrifugation at 500 g for 10 min. After recovering the buffy coat, lymphocytes were washed twice with phosphate buffered saline and resuspended in complete media. Lymphocytes were counted using heamocytometer and adjusted to a density of 1×10^6 cells/ml for cell culture studies. Both the cells were maintained in RPMI-1640 media supplemented with 10 % heat inactivated fetal calf serum, 2 mM L-glutamine, 0.4 % sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 % phytohemeagglutinin in a humidified atmosphere containing 5 % CO_2.

3.11.2. Cytokinesis-Block Micronucleus Assay

The micronucleus assay was performed according to Matsuoka *et al*. (1993) with modifications (Bonacker *et al*., 2004). About 1×10^6 cells/ml medium were exposed to increasing concentrations of different extracts in 5 ml culture medium and incubated at 37 °C in an atmosphere of 5 % CO_2. Mitomycin-C (6 µg/ml medium) and complete media were treated as positive and negative controls respectively. After 44 h, cytochalasin-B (6 µg/ml) was added and further incubated for 28 h. At the end of the incubation the cells were harvested by low centrifugation, treated with a hypotonic solution of KCl (0.075 M) and fixed in methanol: acetic acid (3:1) for 3-4 h. Two to three drops of the fixed cell suspension were dispensed onto the surface of cold microslides, air dried and stained with 3 % Giemsa solution in Sorenson phosphate buffer (pH 6.8) for 5-7 min. The slides were coded and for each treatment at least 2000 binucleated cells (BNC) per concentration were scored for micronucleus frequency in

each treatment. The criteria employed for the analysis of micronuclei and binucleated cells were established by Fenech (2000).

3.12. Preparation, Purification and Characterization of Silver Nanoparticles

3.12.1. Synthesis of Silver Nanoparticles

Silver nitrate ($AgNO_3$) procured from Sigma-Aldrich (Bangalore, India) was used. 10 ml of aqueous plant extracts were added separately into 90 ml of aqueous solution of 1 mM silver nitrate for reduction of Ag^+ ions and incubated overnight at room temperature in dark. The resultant yellowish brown solution indicated the formation of silver nanoparticles.

3.12.2. Purification of Silver Nanoparticles

The broth containing nanoparticles were centrifuged at 15,000 rpm for 15 min to obtain the pellet to redisperse in sterile deionized water for getting rid of any biological molecule. The process of centrifugation and redispersion in sterile deionized distilled water were repeated thrice to obtain better separation of entities from the metal nanoparticles. The purified pellets were then freeze dried using Lyophillizer (Micro Modulyo 230 freeze dryer, Thermo Electron Corporation, India).

3.12.3. UV–Visible Spectral Analysis

The brown colour change observed in the silver nitrate solution incubated with aqueous plant extract shows the bioreduction of silver to form Ag nanoparticles, which were monitored by periodic sampling of aliquots (0.2 ml) of aqueous component and measuring the UV–vis spectrum of the solution in 10-mm- optical-path-length quartz cuvettes with an UV spectrophotometer (Shimadzu, UV 2500, Japan), at a resolution of 1 nm between 300 and 600 nm. The nanoparticle solutions were diluted 20 times with deionized water to avoid errors due to high optical density of the solution. The above mentioned procedure was repeated for optimization of temperature and pH. The reaction temperatures of the solutions were maintained at 30 °C to 70 °C. The pH was maintained in the range of 5, 6, 7, 8 and 9 using 0.1 N HCl and 0.1 N NaOH

respectively. The absorbances of resulting solutions were measured spectrophotometrically.

3.12.4. Atomic Force Microscopy

A thin film of the samples was prepared on a cover slip by droping 0.1 ml of the sample on the slide, and allowed to dry for 30 min. The slides were then scanned with AFM (APE Research- model no: A100SGS). The AFM characterizations were carried out in ambient temperature in non contact mode using silicon nitrate tips with varying resonance frequencies. These tips have spring constants approximately 0.15 nm^{-1} and were conical in shape with a cone angle of 20° and an effective radius of curvature at the tip of 10 nm.

3.12.5. Fourier Transform Infra-Red (FTIR) Spectroscopy

For FTIR measurements, bioreduced silver NP dried powder was analyzed using FTIR. The samples were dried and ground with KBr pellets and analyzed in a SHIZAMAZU model no 8400S spectrum instrument. A disk of 50 mg of KBr were prepared with a mixture of 2 % finely dried sample and then examined under IR-Spectrometer. Infrared spectra were recorded in the region of 500 to 4500 cm^{-1}.

3.12.6. Scanning Electron Microscopy (SEM) & Energy Dispersive X-Ray Analysis (EDX)

The lyophilized silver NPs were mounted on the copper stubs and the images were studied and observations were carried out on a ZEISS EVO 40 EP Electron microscope. EDX analyses were carried with (JEOL model-L6390) secondary electron detectors at an operating voltage of 30 kV.

3.13. Cytotoxic Activity

3.13.1. Cell Culture

Human ductal breast carcinoma cells (T47D) were obtained from the National Centre for Cell Science (NCCS), Pune, and maintained in Roswell Park Memorial Institute (RPMI) medium containing 10 % fetal bovine serum (FBS). For evaluation of

the cytotoxicity, cells were seeded on a 96 well plate with a density of 1×10^4 cells/cm^2. Normal cells of L929 (human fibroblast cell line) were maintained in MEM (Minimum essential Medium) containing 10% fetal bovine serum (FBS) provided by Promo Cell Germany.

3.13.2. Assessment of Cytotoxicity

Cytotoxicity of the MT, TR and nanosamples of MT and TR were evaluated using the MTT [3- (4,5-dimethylthiazole-2-yl)- 2,5-diphenyltetrazolium] assay. Stock solutions of the samples were freshly prepared (1 mg/1 ml) and diluted with cell culture medium to the desired concentrations (20, 50 and 100 μg). The compounds with different concentrations were added and incubated with phosphate buffer saline (PBS) resuspended cells, after attaining 90 % confluency. Cells in media devoid of compound acted as the negative control. Paclitaxel, the positive control, was prepared in DMSO and then diluted with a medium containing all the supplements and gentamicin to obtain desired concentration of 1 x 10^{-3} M. About 5 mg of MTT (Sigma) was dissolved in 1 ml of PBS and filter sterilized. 10 μl of the MTT solutions were further diluted to 100 μl with 90 μl of serum and phenol red free medium. The cells were incubated with 100 μl of the above solution for 4 h to form formazan crystals by mitochondrial dehydrogenates. 100 μl of the solubilisation solution (10 % Triton X-100, 0.1 N HCl and isopropanol) was added to each well and incubated at room temperature for 1 h to dissolve the formazan crystals. The absorbance of the solutions were measured at a wavelength = 570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment.

3.14. Statistical Analysis

The values are presented as mean ± SD (standard deviation) of triplicate measurements. Multiple comparisons between more than two groups were performed by one way ANOVA supplemented with Duncan's multiple range post hoc tests using SPSS software. The Pearson correlation analysis was performed to determine the correlation between various factors. Statistical significance was set at $p < 0.05$.