Materials & Methods

3. Materials and Methods

3.1 Collection and preparation of extracts

Piper longum fruit, *Acorus calamus* rhizome were collected from the Puducherry local market while the *Costus pictus* leaves and the *Ficus religiosa* leaves were collected from Pondicherry University campus, Puducherry, India. The *Piper longum* fruit, the *Acorus calamus* rhizome, *Costus pictus* leaves, and the *Ficus religiosa* leaves were dried at room temperature and further powdered finely and were stored in an air-tight bottle in dark for further use.

The dried powders were separately mixed with deionized water and the mixture was filtered through Whatman No. 1 filter paper. The water from the filtered solution was evaporated using a rotary evaporator and lyophilizer in order to obtain the residue. The phytochemical screening was performed using the residue.

3.2 Qualitative analysis of compounds

The phytochemical screening is an important procedure which helps in the identification of the various phytoconstituents present in a plant extract which helps in asserting the role of medicinal plants. Hence, the qualitative estimation of phytoconstituents present in the aqueous extracts of the *Piper longum* fruit (PLFE), *Acorus calamus* rhizome (ACRE), *Costus pictus* leaves (CPLE), and *Ficus religiosa* leaves (FRLE) were identified using standard procedures.

The lyophilized PLFE, CPLE, ACRE and FRLE were dissolved in water (1mg/mL) and the following qualitative phytochemical investigations were performed.

Phenolic compounds – To 0.1 mL of alcoholic ferric chloride solution, 0.5 mL of the sample was added. Occurrence of a bluish green coloration indicates the presence of phenols.

Flavonoids – 0.5 mL of the sample was treated with 0.1 mL of 30% ammonia and the formation of dark yellow coloration indicates the presence of flavonoids.

Alkaloids – To 0.5 mL of sample solution, few drops of Dragendorff's reagent (Potassium bismuth iodide solution) followed by 3 – 4 drops of acetic acid was added and agitated. The appearance of reddish brown color confirms the presence of alkaloids.

Saponins – 0.5 mL of the sample solution was shaken with water and a persistent froth formation indicates the presence of saponins.

Tannins – 3 to 4 drops of (0.1%) ferric chloride solution was added to 0.5 mL of the sample solution. The appearance of a brownish green color indicates the presence of tannins.

Anthraquinones – 0.5 g of the dried powder was suspended in 10 mL of benzene. The 0.5 mL of the suspension was taken and agitated after addition of 0.5 mL of 10% ammonia. The occurrence of violet coloration in the aqueous indicated the presence of anthraquinones.

Glycosides – 0.5 mL sample solution was treated with 2-3 drops of anthrone and concentrated hydrochloric acid followed by heating in water both. Glycosides were confirmed from the appearance of a dark green coloration.

Terpenoids – 2 mL of the sample solution was dissolved in methanol and to it was added 1 mL of HCl solution, 2, 4-dinitrophenyl hydrazine. The appearance of a yellow orange color indicates the presence of terpenoids.

Proteins – 0.5 mL of sample was treated with 1.0 mL of biuret reagent. The appearance of a purple coloration indicated the presence of proteins.

3.3 GC-MS analysis

The phytoconstituents of PLFE, CPLE, ACRE and FRLE were analysed using GC-MS (GC and MS JEOL GC mate supplied with the secondary electron multiplier). The interpretation of GC-MS spectra was performed by comparing the data of National Institute Standard and Technology (NIST) database.

3.4 Synthesis of AgNPs

The extracts were prepared separately by mixing the 1 g of the dry powders of PLFE, CPLE and ACRE respectively in100 mL of double distilled water and then kept in boiling water bath for 15 min. The Whatman no 1 filter paper were used for filtering the extracts which were then stored at 4 ^{o}C for further use. The nanoparticles were synthesized by mixing different ratios of 1 mM silver nitrate and prepared extracts at room temperature. Similarly, the 1 g of lyophilized FRLE was added to the silver nitrate solution in water bath at 50 ^{o}C. The color changes were observed in the mixtures which were indicative of the formation of the nanoparticles. The formations of AgNPs were confirmed through periodical observation of absorption of the solution using UV- visible spectroscopy in the range of 300-700 nm. The synthesized *Piper longum* silver nanoparticles (PLAgNPs), *Costus pictus* silver nanoparticles (CPAgNPs), *Acorus calamus* silver nanoparticles (ACAgNPs) and *Ficus religiosa* silver nanoparticles (FRAgNPs) were collected by using repeated centrifugation at

18000 rpm for 25 min. The obtained pellet was further repeatedly washed three times using double distilled water in order to remove the unbounded biomolecules which may be the secondary metabolites or the proteins. The purified AgNP pellets were dried at room temperature and their characterization was performed by using different techniques.

3.5 Characterization of AgNPs

3.5.1 UV–visible spectral analysis

The formation of PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were determined using UV–visible spectrophotometer (UV–1700 Shimadzu), with absorption measurements performed at different time intervals between the wavelength ranges of 300 – 700 nm. The baseline was corrected using double distilled water.

3.5.2 TEM-EDAX analysis

The micrograph image and elemental composition of the PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs was determined by TEM along with EDAX (JEOL 3010). The sample was prepared by placing dry powdered AgNPs on the carbon coated copper grid which have been dried under mercury lamp for 5 min. The excess powder was removed by using tissue paper.

3.5.3 FTIR analysis

The functional groups present in the PLFE, CPLE, ACRE, FRLE and in their respective AgNPs *viz.* PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were identified by FTIR spectroscopy (Thermo Nicolet Nexus 670 equipped with KBr optics and a DTGS detector). The instrument was run with a resolution 4 cm^{-1} and scanned between the frequency ranges of 500 - 4000 cm^{-1}.

3.5.4 DLS particle size and zeta potential analysis

The average hydrodynamic diameter and polydispersity nature of PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were determined by DLS particle size analyzer (ZETA Seizers Nanoseries). The zeta potential of the PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs respectively in pure water was further analyzed by using electrophoretic light scattering at 25 ^{o}C in 150 V (Malvern Instruments Nano ZS).

3.5.5 TG–DSC analysis

The thermal analysis of PLFE, CPLE, ACRE, FRLE and of their respective AgNPs were analysed by increasing the temperature of the dry powders of the samples

between the temperature range of 0 – 800 ^{o}C in an SDT Q600 and Q20 DSC thermal gravimetric analyzer.

3.6 *In vitro* free radical scavenging assays

3.6.1 DPPH free radical quenching activity

The DPPH radical quenching activities of PLFE, CPLE, ACRE, FRLE and of their respective AgNPs were assessed as stated in the method developed by Chang *et al*. 2002. Herein, different concentrations of PLFE and PLAgNPs (100 – 600 µg/mL), CPLE and CPAgNPs (0.1 - 5.0 mg/mL), ACRE and ACAgNPs (0.1 - 3.0 mg/mL), FRLE and FRAgNPs (0.1 - 4.0 mg/mL) were distinctly added to the 3 mL of DPPH (0.1 mM) solution and the system is kept for 15 min in dark. After incubation, the absorbance of the sample at 517 nm was measured using methanol as a blank. This assay also included a control and standard respectively, DPPH in methanol and the familiar antioxidant rutin. The DPPH radical inhibition percentage was intended as stated in the below formula

$$I = (OD_c - OD_t)/OD_c \times 100$$

Herein, OD_c is control optical density and OD_t is test optical density

3.6.2 Reducing power assay

The total antioxidant capacity of the PLFE, CPLE, ACRE, FRLE and of their respective AgNPs was detected by performing the reducing power assay and the experiment was performed according to procedures designed by Makari *et al*. 2008. 0.5 mL of various concentrations of PLFE and PLAgNPs (100 – 600 µg/mL), CPLE and CPAgNPs (0.1 - 5.0 mg/mL), ACRE and ACAgNPs (0.1 - 3.0 mg/mL), FRLE and FRAgNPs (0.1 – 5 .0 mg/mL) were separately added to 2.5 mL of 0.2 M phosphate-buffered saline (PBS) along with 2.5 mL of 1% potassium ferricyanide and then kept at 50 ^{o}C for 20 min. To the above mixture, the 2.5 mL of 10% trichloroacetic acid (TCA) was added and then centrifuged at 3000 rpm for 10 min. Finally, the 2.5 mL of supernatant were collected to a new centrifuge tube and to it 2.5 mL of double distilled water was added followed by 0.5 mL of aqueous ferric chloride solution (0.01%). The UV – visible spectrophotometer was used to estimate the optical density of the samples at 700 nm against phosphate buffer blank and rutin as a standard. The reducing power activity is directly proportional to the increase in the absorption of sample with increasing concentrations.

3.6.3 Superoxide radical scavenging activity

Superoxide radical quenching activities of the PLFE, CPLE, ACRE, FRLE and their respective AgNPs such as PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were studied by following the previously reported method of Nishikimi *et al*. 1972. In this assay, the free radicals are generated by phenazinemethosulfate nicotinamide adenine dinucleotide (PMS-NADH) non-enzymatic system, which reacts with nitrobluetetrazolium (NBT) to form purple colored formazan. In this test, 1 mL of test solution containing different concentrations of PLFE and PLAgNPs (100 – 600 µg/mL), CPLE and CPAgNPs (0.1 - 5.0 mg/mL), ACRE and ACAgNPs (0.1 - 3.0 mg/mL), FRLE and FRAgNPs (0.1 – 5 .0 mg/mL) of samples, phosphate buffer (pH 7.4, 100 mM), NADH (468 µM), NBT (156 µM), and PMS (60 µM). After 5 min of incubation, the absorbance of the generated formazan was detected against appropriate blank at 560 nm in a UV – visible spectrophotometer using rutin as standard. The superoxide radical inhibition percentage was calculated based on the following equation.

$$I = (OD_c - OD_t)/OD_c \times 100$$

Herein, OD_c is control optical density and OD_t is test optical density

3.6.4 Nitric oxide radical quenching activity

The nitric oxide radical scavenging ability of the PLFE, CPLE, ACRE, FRLE and their respective AgNPs such as PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were assessed based on the Griess Illosvoy reaction (Garatt 1964). In this reaction, the sodium nitroprusside generates nitric oxide, which reacts with the oxygen molecules and the resultant amount of nitrite ions are measured. Herein, 3 mL of the reaction solution is prepared by adding different concentrations of PLFE and PLAgNPs (100 – 600 µg/mL), CPLE and CPAgNPs (0.1 - 5.0 mg/mL), ACRE and ACAgNPs (0.1 - 3.0 mg/mL), FRLE and FRAgNPs (0.1 – 5 .0 mg/mL), PBS (pH 7.4), 10 mM of sodium nitroprusside and then the solution is incubated for 90 min at room temperature. After incubation, the 0.5 mL of the above mixture was taken and then mixed with 1 mL of sulfonamide (0.33% in 22% glacial acetic acid) as well as 1 mL of N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) (0.1% w/v). The formed pink chromophore was estimated by using UV–visible spectrophotometer at 540 nm and the rutin was used as a standard. The percentage of nitric oxide radical inhibition was assessed using the below formula

$$I = (OD_c - OD_t)/OD_c \times 100$$

Herein, OD_c is control optical density and OD_t is test optical density

3.6.5 Hydrogen peroxide quenching activity

The hydrogen peroxide quenching ability of PLFE, CPLE, ACRE, FRLE and their respective AgNPs such as PLAgNPs, CPAgNPs, ACAgNPs and FRAgNPs were assessed based on the previously reported method with slight modifications (Patel *et al*. 2010). In this experiment, freshly prepared 600 µL hydrogen peroxide (100 mM) is separately added to various concentrations of PLFE and PLAgNPs (100 – 600 µg/mL), CPLE and CPAgNPs (0.1 – 5.0 mg/mL), ACRE and ACAgNPs (0.1 – 3.0 mg/mL), FRLE and FRAgNPs (0.1 – 5 .0 mg/mL) solutions and then made up to 1 mL volume by addition of PBS. The UV–visible spectrophotometer was used to measure the absorbance of the above mixture against appropriate reagent blank at 230 nm and the quercetin was used as a standard. The hydrogen peroxide inhibition percentage was intended based on the below formula

$$I = (OD_c - OD_t)/OD_c \times 100$$

Herein, OD_c is control optical density and OD_t is test optical density

3.6 Antimicrobial activity

3.6.1 Kirby-Bauer Disk diffusion method

The antibacterial ability of the ACRE, ACAgNPs, FRLE and FRAgNPs was analysed by Kirby-Bauer Method (Cormican *et al*. 1996). Different species of bacteria such as *E. coli, B. subtilis, B. cereus* and *S. aureus, Pseudomonous fluorescence* and *Salmonella typhi* were cultured in nutrient broth/ agar (1 g beef extract, 1 g peptone, 0.5 g sodium chloride (NaCl) dissolved in 100 mL of deionized water) and incubated overnight at 30 °C. About 5 mm of sterile discs of Whatman filter papers were infused with ACRE and ACAgNPs and the discs were then placed on the *B. subtilis, B. cereus* and *S. aureus* bacterial lawn in agar plates. Similarly, the sterile Whatman filter paper discs (5 mm diameter) were infused with FRLE and FRAgNPs, and then placed on the *E. coli, B. subtilis, P. fluorescence* and *S. typhi* bacterial lawn in agar plates. These cultured plates were incubated for 24 h at 37 °C and then the zone of inhibition was measured using Hi-Media measuring scale. The standard streptomycin (30 µg) antibiotic discs were used as a reference drug.

3.6.2 Growth kinetic studies

The growth inhibition curve of *E. coli* was examined after the exposureof ACAgNPs and FRAgNPs. In this assay, 40 µg/mL concentration of ACAgNPs and different concentrations of (10, 30, 60, 100 µg/mL) of FRAgNPs were added to the overnight

E. coli culture and kept in the shaker at 27 ^{o}C. The absorbance of the bacterial cultures were measured using UV–visible spectrophotometer at 600 nm for every one hour intervals up to 24 h and the growth curve plot was made by using absorbance vs time.

3.7 Catalytic activity

The catalytic role of ACAgNPs and FRAgNPs was assessed using 4–NP, 3–nitophenol (3-NP), 2, 4, 6–TNT or PA, CBB, CR, EY, RB, MB, MR, MO, cresol red (CRR), AO, eriochrome black T (EBT), and PR. Briefly, the experiment was performed by mixing 10 mM of 0.3 mL of 4–NP, 1.7 mL of deionized water, 1 mL of 150 mM $NaBH_4$ and 15 µg/mL of ACAgNPs or FRAgNPs. The 10 mM of 30 µL picric acid (PA) or 2, 4, 6–TNT was mixed with 1.97 mL of deionized water, 1 mL of 150 mM $NaBH_4$ and 15 µg/mL of ACAgNPs or FRAgNPs. Similarly the 10 mM of 100 µL 3–NP, CBB, CR, EY, RB, MB, MR, MO, CRR, AO, EBT, and PR were individually mixed with 1.9 mL of deionized water, 1 mL of 150 mM $NaBH_4$ and 15 µg/mL of ACAgNPs or FRAgNPs. The absorption of the mixture was monitored periodically with different time intervals between the ranges of 200 to 800 nm using UV–visible spectrophotometer.

3.8 Cell culture

The cancer cells of A549 (human lung adenocarcinoma epithelial cells), HeLa (human cervical carcinoma cells), Hep2 (human epidermoid carcinoma cell line), COLO 205 (human colon cancer cells), and normal cells of HaCaT (human keratinocyte cell line) were cultured in Dulbecco's modified of Eagle medium with L-glutamine & 1000 mg/L glucose (DMEM) which was supplemented with 10 % fetal bovine serum, streptomycin sulfate (0.1 mg/mL) and penicillin G (100 units/mL) in the humidified environment consisting of 5% CO_2 at 37 °C. Similarly, the SHSY-5Y (human neuroblastoma cells) cancer cells were cultured in 1:1 ratio of Dulbecco's modified Eagle medium and Hams F12 medium owning L-glutamine & 1000 mg/L glucose with accompanied 10% fetal bovine serum, streptomycin sulfate (0.1 mg/mL) and penicillin G (100 units/mL) in a humidified environment consisting of 5% CO_2 at 37 °C.

3.8.1 MTT [(4, 5–dimethylthiazol–2–yl) –2, 5-diphenyl tetrazolium Bromide] assay

The anti–proliferative ability of ACAgNPs and FRAgNPs on different cancer cell lines (A549, HeLa, Hep2, COLO 205, and SHSY-5Y) was tested by MTT assay. Briefly, the cultured cells were trypsinized using trypsin –ethylene diamine tetraacetic

acid (EDTA) solution and then few mL of complete medium was added in order to neutralize the trypsin. Then the cells were harvested by centrifuging for 5 min at 1500 rpm. The cells were again mixed with the complete medium and seeded in to separate 96 well plates at 1 x 10^4cells/well (200 µL/well) and then placed in an incubator for 12 h. The cells were then exposed to the various concentrations of ACAgNPs and FRAgNPs and were incubated for 24 and 48 h. After incubation, the 20 µL of MTT (5 mg/mL) was mixed to the cells and again incubated for 4 h. Finally, the 150 µL of dimethyl sulfoxide (DMSO) was used to dissolve the formed formazan crystals and the plate's optical densities were recorded at 570 nm in an ELISA plate reader. The cell viability was determined by using the following equation and then calculated IC_{50} values.

$$\text{Percentage of viability} = \frac{\text{Absorption (test)} * 100}{\text{Absorption (control)}}$$

3.8.2 Cytomorphological analysis

The cultured A549 and Hep2 cells were separately seeded (1x10^5 cells/well) in 12 well chamber plates and kept 12 h in a humidified environment consisting of 5% CO_2 at 37 °C. The seeded cells were then treated with ACAgNPs and FRAgNPs with their respective IC_{50} values and then maintained for 24 h. The cytomorphology of the treated and untreated cells was examined by in a OPTIKA (Italia) inverted phase-contrast microscope.

3.8.3 Acridine orange/ethidium bromide (AO – EB) staining

The apoptosis induction of ACAgNPs and FRAgNPs in A549 and Hep2 cells was examined by AO/EB double staining technique. The cultured A549 and Hep2 cells were separately seeded (1x10^5 cells/well) in 12 well chamber plates and kept 12 h in a humidified environment consisting of 5% CO_2 at 37 °C. Then the seeded cells were treated with respective IC_{50} values of ACAgNPs and FRAgNPs and incubated at 37 ^oC for 24 h. Finally, the 50 µL of dye mixture (AO-EB) was added to the cells and then observed under fluorescence microscopy (Nikon Eclipse Ti Japan) to detect the apoptotic cell death.

3.8.4 Detection of intracellular ROS

Herein, the 2′, 7′- dichlorofluorescein diacetate (DCFH-DA) was used to detect the generation of ROS in A549 and Hep2 cells. DCFH-DA easily enter into cells and the cellular esterase's deacetylates to produce an intermediate non-fluorescent compound, which then gets oxidized by ROS to generate highly green fluorescent compound 2',

7'- dichlorofluorescein. Herein, the $1x10^5$ cells/well were plated separately in 6 well plates and treated with respective IC_{50} values of ACAgNPs and FRAgNPs for 24 h. Following the treatment, the 10 µM of DCFH-DA were addedand then kept for 30 min incubation at 37 ^oC. The treated and untreated cells were observed under the fluorescence microscopy (Nikon Eclipse Ti Japan) to detect the ROS production.

3.8.5 Quantification of ROS

In this assay, the cultured A549 and Hep2 cells were separately seeded ($1x10^5$ cells/well) in 12 well chamber plates and kept for 12 h in a humidified environment consisting of 5% CO_2 at 37 °C. The cells were then exposed to the IC_{50} concentrations of ACAgNPs and FRAgNPs for 24 h. After the treatment, the cells were then trypsinized and separately collected in aluminium foil wrapped eppendorf tubes. The DCFH-DA (25 µM) solution was added to cells followed by incubation for 45 min at 37 °C. The intensity of fluorescence was noted by using the Fluorolog-FL3-11 spectrofluorometer (HORIBA JobinYvon, NJ, USA) with respective wavelengths of excitation and emission.

3.8.6 Oxidative stress parameters

The A549 and Hep2 cells were cultured in 75 cm^2 flasks and separately treated with ACAgNPs as well as with FRAgNPs with respective IC_{50} concentrations for 24 h. After the treatment, the cells were washed with PBS and collected in ice-cold PBS at 4 ^oC. The collected cell pellets were then lysed by using lysis buffer and centrifuged at 10000 g for 10 min at 4 ^oC. The supernatant was then assembled and maintained at 4 ^oC until all the oxidative biomarkers assays were performed.

3.8.6.1 Estimation of Total protein

The lysed samples total protein contents were assessed by following the Lowry method (Lowry *et al.*, 1951).

Reagents

Sodium carbonate ($Na_2 CO_3$) 2% in 0.1N sodium hydroxide (NaOH) (Reagent A)

Copper sulphate (0.5%) in 1% potassium sodium tartrate (Reagent B)

Alkaline copper sulphate: 50 mL of reagent A & 1.0 mL of reagent B (Reagent C)

Folin – Ciocalteu reagent (Reagent D): Commercially available (1:2 dilution)

Stock protein solution: mg/mL concentration was prepared from bovine serum

albumin (fraction V), working standard: 0.2 mg/mL concentration of stock solution (1:5 dilutions)

Procedure

To 50 µL of tissue homogenate 950 µL of deionized water and 1.0 mL of reagent C were added and assembly was kept aside for 10 minutes. Blank and standards were also performed by an appropriate method. To the above solution, 0.1 mL of reagent D was added and left undisturbed for 30 minutes. Absorbance was measured using UV-visible spectrophotometer at 660 nm. A standard graph for the protein absorbance was plotted from which protein concentration in the tissue was determined.

3.8.6.2 Assay for Lipid peroxidase (LPO)

The LPO activity was assessed based on the malondialdehyde (MDA) production in the method of Ohkawa *et al*. 1979.

Reagents

Butylated Hydroxyl Toluene (BHT) 0.05% in methanol

Saline

Thiobarbituric acid (TBA) 0.8% in 0.5N hydrochloric acid

In this experiment a reaction mixture of 5 mL volume containing 200 µL of cell extract, 0.5 mL of BHT, 0.8 mL of saline, and 3.5 mL of TBA reagent was prepared. The above mixture was boiled for 90 min and then centrifuged at 3500 rpm for 10 min to remove the formed precipitate. Finally, the optical density of the supernatant was measured at 532 nm against a blank using UV–visible spectrophotometer. The pure MDA (1 to 5 µg) was treated in a similar way for obtaining the calibration graph and the test results were obtained from the interpolation with respect to the standard graph. The results were expressed in mg of TBARS produced/mg of protein.

3.8.6.3 Assay for reduced glutathione (GSH)

The reduced GSH levels in the treated and untreated cells were estimated by method described by using Ellmans reagent (Ellman 1959).

Reagent

Cell extract

Phosphate Buffer (pH 8, 0.2 M)

TCA (5%)

Ellman’s reagent: 19.8 mg of Dintrobenzine (DTNB) in 100 mL of 0.1 % sodium citrate.

In this assay, the 1 mL of TCA solution was used to precipitate 1 mL of cell lysate and then centrifuged for 15 min at 5000 rpm in order to collect the supernatant. To 1 mL of supernatant 0.5 mL of Ellman's reagent, 3 mL of PBS was added. Finally, the optical density of the solution was measured in a UV–visible spectrophotometer at 412 nm. In a similar manner, a set of standards were performed using 3.5 mL of PBS as a blank. The total level of GSH was stated in terms of nmol/mg protein.

3.8.6.4 Catalase assay

The catalase activity level was examined in accordance with the previously reported method by Sinha *et al*. 1972.

Reagents

Hydrogen peroxide (0.02 M)

Phosphate buffer (50 mM, pH 7.0)

In this experiment, a reaction mixture of 3 mL volume containing 50 µL of sample, 2.94 mL of phosphate buffer and 10 µL of hydrogen peroxide was prepared. The UV-visible spectrophotometer was used to measure the absorbance of the sample at an interval of 10 seconds up to 1 min, against the reagent blank at 240 nm. The activity of the catalase was stated in µmol of hydrogen peroxide degraded/min/mg/protein.

3.8.6.5 Superoxide dismutase (SOD) assay

The experiment was implemented by following the method proposed by Marklund and Marklund 1974.

Reagents

Tris - hydrochloric acid buffer (50 mM) prepared in EDTA solution (1 mM; pH 8.2)

Pyrogallol (0.2 mM) in hydrochloric acid (10 mM)

In this method, analyte contains 100 µL cell extract, 2.4 mL tris-hydrochloric acid buffer and 300 µL pyrogallol was prepared. The UV–visible spectrophotometer was used immediately to measure the change in absorbance at 420 nm against blank at an interval of 10 seconds for 3 min. The SOD activity was indicated in nmol pyrogallol oxisized/min/mg protein.

3.8.6.6 Glutathione peroxidase (GPx) assay

The assay was executed based on the method noted by Rotruck *et al.* 1973.

Reagents

2- Dinitrobenzoic acid (DTNB, 0.6 mM)

Potassium EDTA (0.4 mM)

Sodium azide (10 mM)

Hydrogen peroxide (1 mM)

Glutathione (2 mM)

10% TCA

Tris-hydrochloric acid buffer (0.4 mM)

A reaction mixture was prepared containing 200 µL of cell extract, 200 µL of tris-hydrochloric acid buffer, 100 µL of potassium EDTA, 100 µL of sodium azide, 200 µL of glutathione solution and 100 µL of hydrogen peroxide solution. The reaction mixture was incubated for 10 min and further 0.5 mL of 10% TCA was added to stop the reaction. The precipitate formed in the reaction mixture was separated by centrifugation at 3500 rpm for 10 min, and optical density of the supernatant was evaluated using a UV–visible spectrophotometer at 412 nm. Similarly, the pure glutathione (4 to 20 µg) was used instead of the cell extract and mixed in the same manner in order to obtain the results for standard graph. The test results were attained by interpolation with the standard graph and the values were represented in nmol of GSH utilized/mg of protein.

3.8.7 Assessment of mitochondrial membrane potential (MMP or ΔΨm)

The MMP loss in ACAgNPs and FRAgNPs treated A549 and Hep2 cells were determined by Rhodamine 123 staining. Herein, the cultured A549 and Hep2 cells (5x 10^5 cells/well) were separately seeded in six well plates, which were incubated for 12 h in humidified atmosphere having 5% CO_2 at 37 °C. Then these cells were exposed to the respective IC_{50} concentrations of ACAgNPs and FRAgNPs for 24 h. Further, the cells were washed using PBS and fixed in 4% paraformaldehyde followed by 70% ethanol for 10 min. The 50 µL of rhodamine 123 (10 µg/mL) were added and then kept for 30 min. The excess dye was removed by using PBS and the cells were detected under a fluorescence microscope (Nikon Eclipse Ti Japan) at 20 X magnification.

3.8.8 Quantification of MMP or ΔΨmloss

Herein, the cultured A549 and Hep2 cells (5 x 10^5 cells/well) were separately plated in the six well plates and kept for 12 h in humidified atmosphere having 5% CO_2 at 37 °C. The seeded cells were treated with respective IC_{50} concentrations of ACAgNPs and FRAgNPs for 24 h. After 24 h, the trypsinized cells were collected in aluminum

foiled effendrof tube. Finally, the rhodamine 123 was added to effendrof tube and kept for 45 min incubation at 37 °C. The fluorescence intensity was noted by using a HORIBA JobinYvon, (NJ, USA) Fluorolog-FL3-11 spectrofluorometer with respective wavelengths of excitation/emission.

3.8.9 Propidium iodide (PI) staining

The apoptosis associated changes in the cell nucleus like condensation/fragmentation were identified using PI staining. Herein, the cultured A549 and Hep2 cells (1 x 10^5 cells/well) were separately plated in a six well plates and kept for 12 h in incubator. The cells were treated with an appropriate IC_{50} concentration of ACAgNPs and FRAgNPs. The treated cells were kept for 24 h at 37 ^oC with 5% CO_2. After the treatment, the cells were washed using PBS and fixed in 4% paraformaldehyde and then in 70% ethanol. Following the fixation, the cells were stained using the PI at the concentration 50 µg/mL and incubated for 20 min. The excess dye was removed and examined under fluorescence microscopy (Nikon Eclipse Ti Japan) at 20 X magnification.

3.8.10 4', 6 – Diamidino – 2-phenylindole dihydrochloride (DAPI) staining

The apoptosis associated changes in the cell nucleus like condensation/fragmentation were identified using DAPI staining. In this experiment, the cultured A549 and Hep2 cells (1 x 10^5 cells/well) were separately plated in a six well plates and kept for 12 h in incubator. The cells were treated with their appropriate IC_{50} concentrations of ACAgNPs and FRAgNPs and then kept for 24 h at 37 ^oC with 5% CO_2. After incubation, the cells in the six well plate were washed using PBS and were fixed in 4% paraformaldehyde followed by 70% ethanol. At the end of the experiment, the cells were stained using DAPI and observed under fluorescence microscopy (Nikon Eclipse Ti Japan) at 20 X magnification.

3.8.11 Western blot analysis

A549 and Hep2 cells were exposed to the appropriate IC_{50} concentrations of ACAgNPs and FRAgNPs and further incubated at 5% CO_2 at 37 °C. At different time intervals the cells were harvested and lysed with lysis buffer in the presence of protease inhibitor cocktail. The collected lysates were then centrifuged at the rate of 8000 rpm for 10 min at 4 °C and the concentration of the proteins in the supernatant was estimated by the Lowry method. Equal concentration of samples was separated by using SDS-PAGE electrophoresis. The separated proteins were then transferred

into the nitrocellulose membrane which was blocked with 5% non-fat milk solution for 1 h. After incubation, the primary antibody (active caspase 8, 9, 3, pro-lamin, cleaved lamin, cleaved PARP, β-actin) was added and then kept for overnight at 4 °C followed by the addition of enzyme horseradish peroxidase (HRP) linked secondary antibody for 2 h at room temperature (RT). The protein bands were noticed by using 3, 3', 5, 5'–Tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) solution or enhanced chemiluminescence (ECL) and were quantified by using Image J software.

3.9 *In vivo* studies

3.9.1 Experimental animals

The male Wistar rats with body weight 200-230 g (12-14 weeks) were purchased and maintained in the room temperature (22±2^{o}C) with 12:12 h lights and dark cycle according to the recommendations of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. The rats were kept two weeks for acclimatization and the work was performed with the permission from Institutional Animal Ethical Committee, Pondicherry University (PU/SLS/IAEC/2014/23).

3.9.2 Experimental Procedure

The rats were divided in to five groups each comprising of 6 rats (n= 6). The total experimental period was 88 days.

Group I rats were fed with normal pellet diet (NPD) and they were considered as a control group;

Group II rats were provided with 5 mg/kg b.w of ACAgNPs for 28 days orally on alternate days;

Group III rats were treated with 10 mg/kg b.w of ACAgNPs for 28 days orally on alternate days.

Group IV rats were treated with 5 mg/kg b.w of FRAgNPs for 28 days orally on alternate days;

Group V rats were administered with 10 mg/kg b.w of FRAgNPs for 28 days orally on alternate days.

At the termination of experimental period, from each group four rats were sacrificed and remaining two rats in each group were kept for 60 more days. At 89th day the remaining two rats were sacrificed and experiments were performed.

3.9.3 Body weight

Rats were weighed weekly once in order to check the change in their body weights throughout the experimental period of 28 days.

3.9.4 Serum markers

At the end of experimental period, the rats were sacrificed and the blood from their heart was collected. The serum was collected immediately from the blood. The serum levels of glucose, inorganic phosphorus (IP), aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and alkaline phophatase (ALP) were determined by using diagnostic kits (AGGAPE–diagnostics). Serum cholesterol was estimated by CHOD-PAP (Cholesterol Oxidase/Peroxidase Amino Phenazone) method (AGGAPE diagnostics Pvt. Ltd, Kerala, India). Serum TG was estimated by Glycerol-3-Phosphate Oxidase – Phenol & Amino Phenazone (GPO–PAP) method (AGGAPE–diagnostics Pvt. Ltd, Kerala, India).

3.9.5 Serum inflammatory markers

The inflammatory markers of TNF–α and IL–6 were estimated by ELISA kits as stated to the suppliers instructions (TNF–α – EASIA and IL–6–EASIA–CE, DIAsource Immuno Assays S.A. – Rue du Bosquet, 2 – B–1348 Louvain–la–Neuve–Belgium).

3.9.6 Oxidative stress markers estimation

Standard procedures were followed in order to estimate the liver oxidative stress markers, such as LPO (Ohkawa *et al*. 1979), SOD (Marklund *et al*. 1974), catalase (Sinha *et al*. 1972), and GPx (Rotruck *et al.* 1973) and non-enzymatic antioxidant GSH (Ellmans 1959).

3.9.7 Distribution of ACAgNPs and FRAgNPs in differenttissues

The distribution of ACAgNPs and FRAgNPs in different tissues such as liver, kidney, heart, brain, lungs, spleen and testis were detected by using an inductively coupled plasma optical emission spectrometry (ICP–OES) technique on 29^{th} and 89^{th} day. In this method, 0.5 g tissue were weighed and digested in 5 mL of nitric acid for 16 h at 90 ^{o}C in a water bath followed by addition of 1 mL hydrogen peroxide solution. Finally, the samples were diluted to a known volume using deionized water. Concentration of silver in different tissues was assessed by ICP–OES and the silver concentration was expressed as silver quantity/g of tissue.

3.9.8 Histopathology

In this procedure, the different tissues obtained from kidneys, spleen, liver, brain, heart, lungs, testis collected and was instantaneously fixed in 10 % of neutral buffered

formalin solution. The tissue samples were processes by using paraffin embedding bath system and thin slices were prepared. The sections were stained using hematoxylin and eosin (H & E) for being analyzed in a light microscope (Olympus BX40, Japan).

Statistical analysis

Statistical analysis was done to the all related experiments among the groups as compared to the control using SPSS software version 20 ANOVA followed Tukey's HSD or by LSD. Student't' test was used to analyze the statistical significance between the groups. Significance difference was set at *$P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq 0.001$.