3. Materials and Methods

3.1 Collection of Plant Materials

Curcuma zanthorrhiza Roxb. (CZ) and *Curcuma aromatica* Salib. (CA) rhizomes were collected from the original germplasm collection of NBPGR, Thrissur. The procured plant rhizomes were multiplied in experimental plots for the present investigation. The plant rhizomes were authenticated by Dr. Vimala Jose, Assistant Professsor at the Research and Post Graduate Department of Botany, St. Thomas' College (Autonomous), Thrissur, Kerala, India.

3.2 Preparation of Rhizome Extract

The rhizomes of both CZ and CA were cleaned thoroughly with tap water, cut into small pieces, shade dried, coarsely powdered and stored for future experimental purposes. 10 g of this powder was boiled in 100 ml of deionized water for 30 min. The crude solution was filtered using Whatman No.1 paper. The filtrate was further centrifuged at 5000 rpm and the supernatant was stored at 4 ^{0}C for further experiments.

3.3 Phytochemical analysis of Rhizome extract

3.3.1 Qualitative Analysis of Phytoconstituents

3.3.1.1 Test for carbohydrates

Molisch's Test: 2-3 drops of Molisch reagent (1 % alpha naphthol reagent) was added to the filtered rhizome extract taken in the test tubes. Along the sides of the test tubes 2 ml of sulphuric acid was added. The appearance of brown ring at the junction of the two liquid indicates the presence of carbohydrates.

3.3.1.2 Test for proteins

Biuret test: A volume of 2 ml, 5 % sodium hydroxide and 1 % copper sulphate was added to 1 ml of rhizome extract. The presence of violet colour indicates the presence of peptide linkage in protein and aminoacids.

3.3.1.3 Test for tannins and phenolic acids

Ferric chloride test: Few drops of ferric chloride (5 %) were added to 2 ml of rhizome extract. Appearance of bluish black precipitate indicates the presence of tannins and phenolic acids.

3.3.1.4 Test for terpenoids

Salkowski test: The rhizome extract (2 ml) was taken in the test tube. A volume of 2 ml of chloroform was added. Then 3 ml of concentrated sulphuric acid was added carefully to the test tubes. Formation of reddish brown colouration suggests the presence of terpenoids.

3.3.1.5 Test for flavonoids

Sodium hydroxide test: To 10 % sodium hydroxide solution, about 2 ml of rhizome extract was added. A change in colour of the reaction mixture from yellow to colourless on addition of few drops of dilute hydrochloric acid suggests the presence of flavonoids.

3.3.1.6 Test for steroids

Libermann Buchard test: A mixture of chloroform and acetic anhydride was cooled to 0 °C. Then one drop of sulphuric acid was added to this mixture, followed by the

addition of the rhizome extract. The change in colour of the reaction mixture including blue, green, red or orange with time indicates the presence of steroids.

3.3.1.7 Test for alkaloids

A volume of 5 ml rhizome extract and 5 ml of 1 % hydrochloric acid was heated together in a water bath for 20 min. The reaction mixture was divided into 3 portions.

Mayer's test: Formation of cream coloured precipitate upon addition of 1 ml of Mayer's reagent to the first portion indicates the presence of alkaloids.

Wagner's test: One drop of Wagner's reagent was added to the second portion. The presence of alkaloids was identified by the appearance of brown or reddish brown precipitate.

Dragendorff test: To the third portion of the reaction mixture, freshly prepared Dragendorrf reagent was added. The formation of orange brown precipitate indicates the presence of alkaloids.

3.3.1.8 Test for glycosides

Keller-Killani test: About 2 ml of rhizome extract was taken in a test tube. To the rhizome extract, 2 ml of glacial acetic acid, a drop of 5 % ferric chloride solution and few drops of concentrated suphuric acid were added. Appearance of reddish brown colour at the junction of two liquid layers and upper layer appears bluish green confirms the presence of glycosides.

3.3.1.9 Test for anthraquinones

To 2 ml of rhizome extract, approximately 10 ml of benzene was added, shaken thoroughly for few minutes and filtered. To the filtrate 5 ml of 5 % ammonia solution

was added. The appearance of pink colour in the ammonia layer indicated the presence of anthraquinones.

3.3.1.10 Test for coumarins

A volume of 2 ml rhizome extract was thoroughly mixed with 3 ml of 10 % sodium hydroxide solution. The appearance of yellow colour shows the presence of coumarins.

3.3.1.11 Test for phenols

Lead acetate test: To the rhizome extract taken in the test tubes, few drops of 10 % lead acetate were added. The appearance of white precipitate suggests the presence of phenols.

3.3.1.12 Test for saponins

About 5 ml of distilled water and 2 ml of rhizome extract was shaken vigourously in a test tube. The presence of persistent frothing while shaking indicates the presence of saponins.

3.3.2 Quantitative Analysis - Total phenolic content

Phenolic Content by Folins'-Ciocalteau Reagent (FCR) Assay (Tezcan, Gültekin-Özgüven, Diken, Özçelik, & Erim, 2009).

Reagents

10 % FCR, 7.5 % Sodium carbonate, Gallic acid (Sigma)

Procedure

The rhizome extract was diluted with distilled water in 1: 4 ratio by volume. To the diluted rhizome extract 2 ml of FCR was added. The mixture was incubated for 6 min

in dark condition. After incubation, 4 ml of sodium carbonate was added. Again incubated the mixture at room temperature for 30 min. The absorbance of the mixture was read at OD 765. The total phenolic content of the rhizome extract was expressed as mg / g equivalent gallic acid. The standard used was gallic acid (10 mg / ml)

3.3.3 Identification of bioactive compounds by HR-LCMS

The HR- LCMS was carried out on 1290 Infinity UPHLC System, 6550 iFunnel QTOFs (HR –LCMS, Aligent Technologies, USA). The liquid chromatographic conditions employed in the study include column aligent 1290 Infinity UHPLC (Hypersil gold 3 micron, 100 x 2.1 mm). Mass spectrometer was carried out in positive ionization mode with m / z ratio ranging from 50 to 1000. The eluent used was 90 % acetonitrile, 10 % water and 0.1 % Formic Acid. The flow rate was 0.3 ml / min. The nebulizer pressure and column temperature employed was 35 psi and 25 ºC respectively. The flow rate of dry gas was 13 l /min and the dry temperature being 250 ºC. The vapourizing temperature was 300 ºC. The bioactive compounds present in both the plant rhizome extracts were identified based on the spectral similarity with the MS library (NIST MS Search 2.0) and Human metabolome database. The relative percentage of each compound was calculated by comparing its average peak area to the total area of all the peaks.

3.4 Synthesis and Characterization of Silver nanoparticles

3.4.1 Synthesis of AgNPs using rhizome extracts

Silver nitrate stock solution (1mM) was prepared by dissolving 0.08g of $AgNO_3$ in 500 ml deionised water. The stock solution of silver nitrate was refrigerated in 500 ml dark bottle to avoid reduction. The aqueous rhizome extract was added to 1 mM silver nitrate solution in the ratio 1:10. The reaction was allowed to proceed

under the irradiation of sunlight with continuous stirring for 1 h in Erlenmeyer flask. The synthesized AgNPs were purified by repeated centrifugation at 12000 rpm for 20 min followed by dispersion of pellet in deionized water to remove water soluble biomolecules. The water suspended nanoparticles were lyophilized and were used for further studies.

3.4.2 Characterization of Silver Nanoparticles

3.4.2.1 UV-Visible Spectroscopy

The bioreduction of silver ions in the solution was monitored by measuring the UV-Visible spectra of the solution using UV-Visible spectrophotometer (Shimadzu UV probe 1800), at a resolution of 1nm in the scanning range 600 – 300 nm.

3.4.2.2 Fourier Transform Infra-Red (FTIR) spectroscopy

The FTIR studies were done to investigate the biomolecules responsible for the reduction and stabilization of silver ions in the aqueous medium, using FTIR Spectrophotometer (Thermo Nicolet, Avatar 370). The lyophilized nanoparticle powder was treated with KBr pellets and analysed in FTIR with spectral range 4000 – 400 cm^{-1} and resolution 4 cm^{-1}.

3.4.2.3 Powder X-Ray Diffraction Studies

Powder X-ray diffraction (XRD) pattern of the both CZAgNPs and CAAgNPs was collected on a Malvern Panalytical Aeris diffractometer using Ni-filtered Cu Ka radiation. The data was collected with a step size of 0.02° and count time of 2 s per step over the range $10° < 2\theta < 80°$. The average crystallite size of CZAgNPs was calculated using the Scherrer equation, where, $D = k / \cos \theta/2$, where, D = average

crystallite size, k = Scherrer's constant (0.94), λ = X-ray wavelength, β1/2 = pure diffraction broadening peak at half height and cos θ = diffraction angle.

3.4.2.4 High Resolution Transmission Electron Microscopy

High Resolution Transmission Electron Microscope (HR - TEM, Tecnai G2, F30) was used to analyze the size, morphology, diffraction ring pattern (SAED), lattice fringes and d spacing of the green synthesized silver nanoparticles. In order to obtain the HRTEM images a drop of filtered silver nanoparticle solution was placed on a carbon coated standard copper grid and the instrument was operated at a voltage of 300 kV.

3.4.2.5 Field Emission Scanning Electron Microscopy

The topography of the nanoparticles was studied by Field Emission Scanning Electron Microscope (FESEM - Supra 55, Karl Zeiss) with accelerating voltage of 20 kV. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample (AgNP solution) on the grid. The extra solution was removed using a blotting paper and then the film on the SEM grid was allowed to dry by placing it under a mercury lamp for 5 min. The elemental composition analysis of synthesized silver nanoparticles was carried out by Energy Dispersive Analysis of X-rays (EDAX) attached with FESEM.

3.4.2.6 Dynamic Light Scattering Analysis

The hydrodynamic particle size of both CZAgNPs and CAAgNPs was measured using Dynamic Light Scattering (DLS) technique (Horiba Scientific SZ-100). The measurement parameters included 633 nm wavelength, scattering angle of 90°, temperature of 25 °C, and dispersion medium viscosity of 0.894 m Pa. s.

3.4.2.7 BET Surface Analysis

The specific area of the AgNPs was determined using the surface area and porosity analyzer (BET), Belsorp Mini 11, BEL Japan. The porosity was measured using a nitrogen adsorbent isotherm at 77 °C. Total pore volume and pore size were estimated by Barrett-Joyner-Halenda (BJH) method.

3.4.2.8 High Resolution Liquid Chromatography Mass Spectroscopy

The similar conditions mentioned in section 3.3.3 was employed to identify the metabolites of the plant rhizome involved in the capping and providing stability for the green synthesized silver nanoparticles.

The aqueous nanoparticle solution was used as the sample for analysis.

3.5 Determination of catalytic property of AgNPs

3.5.1 Photocatalytic Dye Degradation of ionic dyes

The photocatalytic activity of synthesized silver nanoparticles was evaluated by investigating the degradation of Malachite green (MG) and Coomassie Brilliant Blue (CBB) in aqueous solution as a model system for cationic and anionic dyes respectively. The light source used for the irradiation was sunlight and UV lamp (30 W, 253 nm), Philips Holland. An amount of 0.1 mg biosynthesized nanoparticles were added to 50 ml MG (100 mg / l) and tested for photocatalytic activity. The suspension was stirred under dark condition to ensure the equilibrium of working solution, prior to irradiation. At given time intervals, 5 ml suspensions were taken and centrifuged at 10000 rpm for 10 min. Subsequently the absorbance spectra of the supernatant were

measured using UV-Visible spectrophotometer for regular time intervals. The percentage of degradation was calculated using,

$$\% \text{ degradation} = [(C_0 - C) / C_o] \times 100$$

Where, C_0- concentration of dye prior to reaction, C- concentration of dye after reaction.

The above procedure was followed to study the degradation potential of both CZAgNPs and CAAgNPs to eliminate CBB (100 mg / l) dye also.

3.5.2 Non-Photocatalytic Dye Degradation of Azo dyes

The non - photocatalytic activity of CZAgNPs and CAAgNPs was evaluated by analyzing the degradation of azo dyes in an aqueous system using $NaBH_4$ as a substrate. The azo dyes used in this study were Orange G (OG), Congo Red (CR), Eriochrome Black T (EBT) and Methyl orange (MO). Three experimental set up was made, which contained mixture of dye and $NaBH_4$, dye along with CZAgNPs and dye along with CZAgNPs, and $NaBH_4$. Dye alone served as a control system. To 30 µl of 0.01 M dye, 500 µg / ml of CZAgNPs were added, made up to 6 ml with deionized water. The reaction system was stirred in dark to ensure the equilibrium of the solution. To this reaction system 200 µl of 0.1 M $NaBH_4$ was added, which served as the first experimental setup. 30 µl of 0.01 M dye and 500 µg / ml of CZAgNPs; 30 µl of 0.01 M dye and 200 µl of 0.1 M $NaBH_4$ served as the second and third experimental set up respectively, which were made up to a final volume of 6 ml with deionized water. The control and experimental systems were incubated in dark for 2 h. After incubation, the degradation of dyes was monitored using UV – Visible Spectrophotometer in the range 200 -700 nm. The values of the absorption band

maxima were measured and compared. The percentage of degradation was calculated using,

$$\% \text{ degradation} = (C_0 - C) / C_o \times 100$$

Where, C_0- concentration of dye prior to reaction, C- concentration of dye after reaction.

The above given procedure was followed using CAAgNPs also to study its potential in degrading the azo dyes Orange G (OG), Congo Red (CR), Eriochrome Black T (EBT) and Methyl orange (MO).

3.6 Determination of Antibacterial Activity of AgNPs

3.6.1 Disc diffusion Assay

Antibacterial activity of silver nanoparticles was analyzed using Kirby – Bauer disc-diffusion method (Bauer, Kirby, Sherris, Turck, & Graevenitz, 1978) against selected bacterial strains *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (ATCC 25619), *Klebsiella pneumoniae* (MTCC 3040), *Proteus mirabilis* (ATCC 7002), *Serratia marcescens* (MTCC 2645), *Bacillus subtilis* (MTCC 736) and *Staphylococcus aureus* (MTCC 96). The pure cultures obtained from Institute of Microbial Technology, Chandigarh, India were used for the experiment. Molten nutrient agar bacteriological medium (Sigma – Aldrich, 28 g / l) was poured into sterile petriplates under asceptic condition and allowed to solidify. The solidified medium of each plate was then smeared with a sterile cotton swab of the selected bacterial strain. Subsequently, the sterile discs (Himedia) impregnated with 20 µl of various concentrations (10, 25, 50, 75, and 100 µg / ml) of CZAgNPs and CAAgNPs respectively were allowed to dry in sterile conditions. Later they were placed on

solidified agar plates at equidistantly along with control. Tetracyclin disc (Sigma - Aldrich) was used as the positive control and a disc with deionised water served as the negative control. In addition, discs with 20 µl of 1 mM silver nitrate solution and 0.1 g / ml plant rhizome extract (CZ and CA) were also maintained. The plates (in triplicate) were then incubated at 37 ºC, and the zone of inhibition (ZOI) was measured in mm after 24 h.

3.6.2 Broth Microdilution Assay

Six serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} ml) of clinical isolates of *E. coli* and *S. aureus* were made using the Luria Bertani (LB) broth (Sigma – Aldrich). Thereafter 1 ml each of bacterial suspension from 10^{-5} dilution was transferred to the sterile petriplates and molten nutrient agar was added to it. The petriplates were incubated overnight, after thorough rotation. Subsequently, 4 to 5 colonies from the nutrient agar plate were inoculated into the LB broth. The culture was read at 600 nm to adjust the final inoculum to 5 x 10^5 cfu / ml.

For the broth microdilution test, 50 µl of each bacterial suspension (*E. coli, S. aureus*) in LB broth was added to the wells of a sterile 96-well microtitre plate which already contained 50 µl of two-fold serially diluted antibiotic (Ampicillin), CZAgNPs, CZ and silver nitrate solution in LB broth medium respectively. The highest concentration of antibiotic and CZAgNPs was 10 mg / l. A volume of 10 mg / l solution was further diluted to 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 µg / ml and the end volume in each well was maintained at 100 µl. The wells 11 and 12 served as the growth control and sterile control, respectively. The contents of each well were mixed up and down using micropipettes before incubation for 24 h. The MIC (Minimum Inhibitory Concentration) was the lowest concentration of inoculum where no growth

was observed after 24 h incubation with test solutions. The respiratory activity was determined by adding 10 µl / well of TTC (2,3,5- triphenyl tetrazolium chloride, Sigma - Aldrich) dissolved in water (TTC 20 mg / ml) and incubated for 30 min in the dark. The plates were read with a microplate reader (BIO-Base) at 600 nm. The wells with bacterial suspension in an appropriate growth medium served as positive control. The wells with growth medium containing plant rhizome extract served as negative control. All measurements of MIC values were repeated in triplicates. Similar conditions were employed using CAAgNPs and CA.

3.6.3 In silico Molecular docking

In-silico molecular docking was performed to further substantiate the antibacterial property of the bioactive compounds capped in the CAAgNPs and CZAgNPs against gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria. The protein targets identified for *S. aureus* and *E. coli* were α Glutamine Amidotransferase and Diaminopimelate Epimerase respectively. The three dimensional structure of the target proteins were retrieved from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) and active site identified using the tool Castp. The PDB ID of α glutamine amidotransferase and diaminopimelate epimerase are 5N9M and 4IJZ respectively.

Thirteen ligand molecules namely curculone, curcumenolactone C, curdione, curzene, dihydrosphingosine, epicurzerenone, furanogermenone, glecomafuran, glycerolpalmitate, glycerolmonostearate, procurcumadiol, zedoarolideb and zeroarondiol were identified from the HRLCMS analysis of CZAgNP and CAAgNP samples. Their structure was retrieved from Corina tool using SMILES as input. The molecular properties of the selected ligands including molecular weight, molecular

formula, logp, H bond acceptor and donors were identified from PubChem and are represented in Table 4.11.

The interaction between the selected protein target molecules and the thirteen ligands were studied using the tool Auto Dock 4.2. Here rigid protein flexible ligand docking was employed to study the interactions. Prior to analysis the water molecules and other unwanted residues were removed from the target protein molecule and Gasteiger charge was calculated.

The number of torsion was set for all the 13 ligands molecules and the structure of ligand molecules were saved in pdbqt format (extended file for PDB). After the preliminary preparation of both target molecule and ligand, autogrid programme was started. Autogrid was performed to pre-calculate the grid maps of interaction energies for ligand molecues with the protein targets. These grid maps were used to determine the total interaction energy for a ligand with a protein. The final step involved in docking was the preparation and running of the docking parameter file. Further the protein ligand bound complex was visualized using Pymol.

3.7 Determination of Antifungal Activity of AgNPs

3.7.1 Poisoned Food Technique

For evaluating the antifungal activity of CZAgNPs and CAAgNPs, poisoned food technique was followed. The Potato Dextrose Agar (PDA) was prepared (HiMedia – 39 g / l) and kept in molten state at 70 - 80 ºC. The various concentrations of CZAgNPs and CAAgNPs solution (10, 25, 50, 75 and 100 µg / ml) were prepared; 2 ml of these samples were pipetted into each petriplate, mixed well with 20 ml of molten agar and allowed to solidify. A PDA plate with 2 ml each of sterile distilled water and fluconazole (100 µg / ml) served as negative and positive control

respectively. Additionally, PDA plates with 2 ml of 1 mM silver nitrate solution and plant rhizome extract (0.1 g / ml) were also maintained. Inoculum discs were cut with a potato borer (4 mm diameter) from a pure culture plate of *Aspergillus niger*, obtained from Pathology division, Kerala Agricultural University, Thrissur, Kerala, India. The plug of *A. niger* with the mycelial surface facing downwards was inoculated in the centre of each agar plate. The plates in triplicate were incubated at 37 °C in dark condition for 7 days and the colony diameter was measured in mm. The fungi toxicity of the CZAgNPs and CAAgNPs solution in terms of percentage of inhibition of mycelial growth was calculated using the formula:

$$\text{Percentage of inhibition} = [(C- T) / C] \times 100$$

Where, C = Average increase in mycelial growth in the control plate (negative control), T = Average increase in the mycelial growth in the treatment plate.

3.7.2 In silico Molecular docking

In-silico molecular docking was performed to further understand the antifungal property of the bound bioactive compounds of both the nanoparticles (CZAgNPs and CAAgNPs) against *Aspergillus niger*. The protein target identified for *A. niger* was Isocitrate lyase (PDB ID: 1DQU). The ligand molecules selected and the docking procedure is described in 3.5.1.

3.8 In Vitro Antioxidant Assay of AgNPs

3.8.1 DPPH free radical scavenging activity

The electron donating ability of CZ, CA, CZAgNPs and CAAgNPs was determined using the synthetic stable free radical, 2,2-diphenyl -1-picrylhydrazyl (DPPH) (Aquino *et al*., 2001).

Principle

The DPPH radical (DPPH$^{\bullet}$) produces purple colour when dissolved in methanol. It has maximum absorption at 517 nm. In the presence of antioxidants, the purple colour fades to yellow colour.

Procedure

Different volumes of CZ, CA, CZAgNPs and CAAgNPs (20 -100 µl / ml) were mixed with 187 µl of freshly prepared DPPH solution (3 mg / 25 ml methanol). The reaction mixture was incubated in dark for 20 min. After incubation, the absorbance of the reaction mixture was taken at 517 nm. Methanol was used as blank. The inhibition percentage of DPPH radical was calculated using the formula,

Percentage inhibition (%) = [(OD of Control – OD of sample) / OD of control] x 100.

Percentage inhibition of DPPH radical was plotted and volume of CZ, CA, CZAgNPs and CAAgNPs required to scavenge 50 % of DPPH radical was calculated.

3.9 In Vitro Cytotoxicity Analysis of AgNPs

3.9.1 Trypan Blue Exclusion Method

The Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines collected from the peritoneal cavity of mice were washed with Phosphate Buffer Saline (PBS). It was centrifuged at 1500 rpm for 3 min to remove the traces of blood. The DLA and EAC cell pellets were re-suspended in PBS to get a concentration of 1 x 10^7 cells / ml and used for cytotoxicity analysis. Trypan Blue Exclusion method was performed to evaluate the *in vitro* cytotoxicity assay. The assay was also conducted in normal cells. Approximately 1 x 10^6 DLA cells were

distributed into test tubes containing different concentrations of CZAgNPs and CAAgNPs (0.2, 0.6, 0.8, 1, 1.6 and 2.5 µg / ml), CZ and CA (10, 25, 55, 75, 100 µg / ml) and silver nitrate solution (0.2, 0.4, 0.5, 0.6, 0.8, 1 µg / ml) in 1 ml PBS. Various concentrations of CZAgNPs and CAAgNPs (1, 1.6, 1.8, 2, 2.5 and 3 µg / ml), CZ and CA (10, 25, 55, 75, 100 µg / ml) and silver nitrate solution (0.2, 0.4, 0.5, 0.6, 0.8, 1 µg / ml) were added to test tubes with 1 x 10^6 EAC cells and were made upto 1 ml with PBS. The test tubes with cancer cell lines and various drugs were incubated for 3 h at 37 °C. After the incubation period, 100 µl of 1 % trypan blue was added to the test tubes and allowed to stand for 2 min. The live (unstained) and dead (stained) cells were counted using haemocytometer to determine the percentage of toxicity and the IC_{50} values. The percentage of toxicity was calculated using the formula,

$$\text{Percentage of cytotoxicity} = \frac{\text{No.of dead cells}}{\text{No.of live cells+No.of dead cells}} \times 100.$$

3.9.2 MTT Assay

Cell line and culture:

The human breast cancer cell line Michigan Cancer Foundation-7 (MCF - 7) was grown in Roswell Park Memorial Institute -1640 medium (RPMI-1640 medium) containing 10 % fetal bovine serum (FBS). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS), streptomycin (100 µg / ml) and penicillin (100 U / ml). The cells were kept in humidified atmosphere of 5 % CO_2 at 37 °C. Maintenance cultures were passaged weekly, and the culture medium was changed weekly.

Reagents

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Hi Media Laboratories; FBS was purchased from Cistron laboratories; Trypsin, Dimethyl Sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) were purchased from Sisco research laboratory chemicals, Mumbai. All other chemicals and reagents were obtained from Sigma Aldrich, Mumbai.

Cytotoxic assay:

Cytotoxic assay was evaluated by the MTT reduction assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium] (Monks *et al*., 1991). The monolayer cells were detached using trypsin and single cell suspensions were made. The viable cells were counted using trypan blue on haemocytometer. Further, the cell suspension was diluted with growth medium containing 5 % FBS in order to obtain final density of 1 x 10^5 cells/ml. MCF - 7 cells (1 x 10^5 / ml) were seeded on 48 well plates. After reaching 70% confluency, cells were exposed to various concentrations of drug [CA, CZ, CAAgNPs and CZAgNPs (12.5–100 µg / ml)] over period of 24 h to test their toxicity. The untreated cells were kept as control. The cell viability was assessed using 3-(4, 5- dimethyl thiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide (MTT). For this, following incubation with additives, cells were washed with PBS and the media was replaced by fresh growth media. MTT (5 mg / ml) was added and incubated further for 4 h. After the incubation, 500 µl of solubilization reagent (DMSO) was added and mixed well. Absorbance was taken at 570 nm. The percentage death was calculated according to the following formula,

$$\% \text{ Cytotoxicity} = 100 - [\text{Abs (sample)} / \text{Abs (control)}] \times 100.$$

3.9.2.1 *In silico* Molecular docking

The interaction of the bioactive compounds of CZAgNPs and CAAgNPs binding with the receptors of cancer cell line (MCF -7) to assess the binding efficacy was performed using *in-silico* molecular docking. Human tyrosine protein kinase C-SRC and CDK 2 with EGFR inhibitor compound 8 was used as the protein targets of MCF - 7 cell lines. The PDB ID of the target proteins are 2SRC and 4RJ3 respectively. The steps involved in docking were performed according to the method described in the section 3.5.3.

3.10 In Vivo Toxicity Analysis of AgNPs

3.10.1 Acute toxicity

Healthy Swiss albino mice (25 – 30 g) were divided into two groups of 6 animals (3 males and 3 females) each and were kept in separate cages based on their sex. Animals having similar weight and sex were grouped together. Group I that received no treatment was considered as normal and group II was administered with a single oral dose (500 mg / kg b. wt) of CZAgNPs. The feed was removed 4 h before drug administration and was replaced after 1 h. The changes in the general behavior, water and feed consumption, change in body weight and adverse effects any was recorded daily for a total of 14 days. At the end of the experimental period the animals were euthanized to examine the changes in the internal organs. In the absence of mortality, doses ranging between $1/5^{th}$ and $1/10^{th}$ of LD 50 documented in acute toxicity were selected for further studies (OECD, 425).

3.10.2 Sub – Acute toxicity

The sub – acute toxicity evaluation of CZAgNPs was performed according to the guidelines of OECD (OECD, 407). Healthy Swiss albino mice weighing 25 – 30 g were divided into 4 groups of 10 animals, wherein 5 males and 5 females were maintained in separate cages. Group I without any treatment served as normal group. Group II, III and IV were treated with 50 mg / kg b. wt (Low dose), 75 mg / kg b. wt (Medium dose) and 100 mg / kg b. wt (High dose) of CZAgNPs respectively. The CZAgNPs was administered orally once daily for 28 days. Water and feed consumption was determined in regular intervals. During the experimental period the animals were also monitored for any abnormal behavior symptoms. After 28 days, all the animals were euthanized and necropsy of animals was performed. The body weight of animal was monitored throughout the period of study to calculate the change in mean body weight.

Blood withdrawn by cardiac puncture was collected into tubes with and without anticoagulant (ethylene diamine tetra acetic acid, EDTA) and it was used for haematological and biochemical analysis, respectively. The blood collected in tubes without EDTA was centrifuged at 5000 rpm for 10 min to separate serum. This separated serum was used for the analysis of hepatic and renal function markers using assay kits of Agappe Diagnostics, India. Total cholesterol, triglycerides, HDL and LDL cholesterol was estimated using diagnostic kits of Agappe. VLDL was calculated by the Friedewald equation (VLDL = triglyceride / 5).

3.10.2.1 Relative Organ Weight

After sacrifice the vital organs including brain, heart, lungs, liver, kidney, stomach and sex organs (testes or ovary) were dissected out and washed with 0.9 % ice cold saline. The weight of these organs were taken using electronic weighing balance and relative organ weight was calculated using the formula;

Relative organ weight = (Organ weight / Body weight) x 100

3.10.2.2 Estimation of Hematological parameters

3.10.2.2.1 Estimation of Haemoglobin (Hb)

According to the method of Drabkin and Austin (Drabkin & Austin, 1935) the level of Hb was analyzed using Hb diagnostic kit supplied by Agappe.

Principle:

Haemoglobin and its derivates are oxidized into methaemoglobin in the presence of alkaline potassium ferrocyanide. Methaemoglobin eventually reacts with potassium cyanide and gives cyanmethaemoglobin. The absorbance of cyanmethaemoglobin is directly proportional to the concentration of haemoglobin. The maximum absorption of cyanmethaemoglobin is at 546 nm.

Procedure:

Drabkin's reagent (5 ml) was mixed with 20 µl of blood and incubated at room temperature for 5 min. Absorbance of this reaction mixture was recorded at 546 nm. The absorbance of the standard solution (60 mg / dl) provided along with kit was

also measured along with the sample. The level of haemoglobin in blood was calculated using the formula;

Hb concentration (g / dl) = (Absorbance of sample / Absorbance of standard) x (251/1000) x 60

Here, 251 = dilution factor; 1000 = multiplication factor to convert mg to g and 60 = concentration of the Hb standard in mg.

3.10.2.2.2 Determination of Red Blood Cells (RBCs)

The method of Cheesbrough and Mc Arthur (Cheesbrough M; McArthur J., 1976) was used to estimate the total count of RBC.

Principle:

The blood sample was diluted using Hayem's solution (RBC diluting fluid) in the ratio 1:200 to fix the RBC in the blood and to stop haemolysis. Then the RBC count was determined using haemocytometer and expressed as number of RBC / mm^3 of whole blood.

Procedure:

The blood was taken in the RBC pipette up to the mark of 0.5, then RBC diluting fluid was filled up to the mark of 101 and rotated the pipette gently for 2 -3 min between palms. The haemocytometer was kept flat on a table and cover slip was placed over it. About 4 – 5 drops of blood from RBC pipette, without air bubbles was added to the edge of the counting chamber. The cells were allowed to settle down for few minutes. The RBCs were counted under high power (40 X) of light microscope. The RBC count was determined by the formula;

RBC count (cells / mm^3) = Number of cells counted x dilution factor / Depth factor x area counted

Here, dilution factor = 200, area = 5 / 25 (out of the 25 squares of the larger central square, RBCs of 5 squares were counted) and depth factor = 0.1 mm.

Hence, total RBC count = Number of cells x 10,000 / mm^3.

3.10.2.2.3 Determination of Total White Blood Cells Counts (TC)

The total WBC count was estimated according to the method of Cheesbrough and Mc Arthur (Cheesbrough M; McArthur J., 1976).

Principle:

Blood was filled up to the mark of 0.5 followed by Turk's fluid up to the mark of 11 in WBC pipette. The pipette was rotated in horizontal position between the palms and allowed to settle the cells for 3 min. This mixture was poured over the haemocytometer and the number of WBCs in the large four corner squares was counted by using the low power (10 X) objective. The total WBC count was determined using the formula;

Total WBC (cells / mm^3) = Number of cells counted x 50.

3.10.2.2.4 Determination of Differential Leucocyte Count

Under specific pathological conditions, the relative number of leucocytes varies. The differential count of leucocytes is the determination of proportion of various kinds of leucocytes in blood. This was done according to the method of Wintrobe and Greer (Wintrobe, M. M., & Greer, 2009).

Principle:

By observing the cell size, nuclei shape and presence or absence of granules, various types of leucocytes can be distinguished easily from each other when stained with Leishman stain. The nuclei and granules in cytoplasm of WBCs stain differentially. After Leishman staining, agranulocytes including monocytes appear purple coloured with horseshoe shaped nucleus with pink cytoplasm. The lymphocytes have light blue cytoplasm with dark blue circular nucleus. Cells with pink cytoplasm and purple coloured nuclei with multilobes are identified as neutrophils. Eosinophils have dumbbell shaped purple nucleus, orange-red granules and pink cytoplasm whereas basophils have granules with dark blue colour and irregular purple nucleus.

Procedure:

Thin blood smear was prepared with a drop of blood on clean glass slide. The smear was air dried and stained with 1 ml of Leishman stain (0.15 g / 100 ml methanol) for 2 to 3 min. For diluting the stain, an equal amount of distilled water was added over slide and kept for another 3 min. The excess stain was removed by washing with tap water and air dried. In order to count WBCs, the slide was observed by moving in a zig-zag manner under light microscope (40 X magnification). The percentage of various counted WBCs was calculated by counting up to 100 cells.

3.10.2.2.5 Determination of Platelet Count

The platelets in the blood sample were counted manually according to the method of Cheesbrough and McArthur (Cheesbrough M; McArthur J., 1976).

Principle:

While all erythrocytes are lysed, the isotonic balance of the diluent (1 % ammonium oxalate solution) retains platelets intact. Then the cells were counted in a specific area of the Neubauer counting chamber under high power of light microscope.

Procedure:

The method of platelet counting was same as that of RBC count. As in RBC, 1:200 dilution of blood was done using diluting fluid (1 % ammonium oxalate). Gently mixed for 2 min and counted using Neubauer counting chamber by placing under the high power of light microscope (40 X). The platelet count was calculated using the following formula;

Platelet count = (Number of cells counted (N) x dilution factor) / (Depth factor x Area counted)

Here, dilution factor = 200; the area counted = 5 / 25 (platelets of 5 squares are counted, out of the 25 squares (1 mm^2) of the larger central square) and depth of counting chamber is 0.1mm. So, the total number of platelets per mm^2 = N x 10,000

3.10.2.3 Determination of Hepatic function

To separate the serum, blood collected in non-EDTA tubes were placed for centrifugation at 5000 rpm for 10 min. This serum was used to analyze the activities of hepatic function marker enzymes including SGPT, SGOT and ALP. Moreover, the amount of bilirubin and total protein was also determined.

3.10.2.3.1 Estimation of serum glutamate pyruvate transaminase (SGPT)

The activity of SGPT or alanine aminotransferase (ALT) was determined according to International Federation of Clinical Chemistry (IFCC) kinetic method (W. Thefeld, H. Hoffmeister, E.-W. Busch, 1974) using the diagnostic kit of Agappe Pvt. Ltd., Ernakulum, India.

Principle:

The transfer of amino group from alanine to α-ketoglutarate by ALT generates pyruvate and L-Glutamate. In this assay system, in the presence of lactate dehydrogenase enzyme, pyruvate reacts with NADH + H^+ and gives L-lactate and NAD^+. NAD does not absorb light at 340 nm, but NADH can. So the activity of ALT in the sample is directly proportional to the rate of decrease in absorbance at 340 nm.

$$\text{L-alanine + 2-oxoglutarate} \xrightarrow{\text{ALT}} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + } H^+ \xrightarrow{\text{LDH}} \text{D-lactate + } NAD^+$$

Procedure:

The diagnostic kit of SGPT includes two reagents; R1 and R2. Reagent 1 is a combination of tris buffer (110 mM), L-alanine (600 mM) and LDH (> 1500 U / l). Reagent 2 consists of α-ketoglutarate (16 mM) and NADH (0.24 mM). The working reagent was prepared by mixing R1 and R2 in the ratio 4:1 by volume. The working reagent is stable for 30 days when stored at 2 – 8 °C. About 100 µl of serum and 1 ml of working reagent were mixed well and kept at 37 °C for 1 min. Then, the change in absorbance was recorded at 340 nm for 3 min with an interval of 60s. The activity of SGPT was calculated using the following formula;

$$\text{The activity of SGPT (U / l)} = \Delta A / \text{min} \times 1746$$

3.10.2.3.2 Estimation of serum glutamate oxaloacetic transaminase (SGOT)

The activity of SGOT or aspartate aminotransferase (AST) was determined by IFCC kinetic method (H.U Bergmeyer, 1974; W. Thefeld, H. Hoffmeister, E.-W. Busch, 1974) using diagnostic kit of Agappe Pvt. Ltd., Ernakulum, Kerala, India.

Principle:

Oxaloacetate and L-glutamate is formed by the transamination reaction between L-aspartate and 2-oxoglutarate. The reaction is catalyzed by AST. In the presence of malate dehydrogenase (MDH), the oxalate formed in this reaction is then reacted with NADH + H^+ and form NAD^+. The AST activity in the sample is proportional to the rate of decrease in absorbance at 340 nm.

L-aspartate + 2-oxoglutarate → oxaloacetate + L-glutamate

NAD Oxaloacetate + NADH + H^+ → L-malate + NAD^+

Procedure:

The diagnostic kit of SGOT included two reagents; R1 and R2. Reagent 1 contained tris HCl (pH 7.8 and 88 mM), L-aspartate (260 mM), LDH (> 1500 U / l) and MDH (> 900 U / l) whereas reagent 2 is a combination of α ketoglutarate (12 mM) and NADPH (0.24 mM). The working reagent was prepared by mixing 4 volumes of R1 and 1 volume of R2, which is stable for a month when stored at 2 – 8 °C. The working reagent (1000 µl) and 100 µl of serum were kept at 37 °C for 1 min. Further, the change in absorbance was recorded at 340 nm for 3 min with 60s interval. The activity of SGOT was calculated from the following formula;

SGOT activity (U / l) = ΔA / min x 1745

3.10.2.3.3 Estimation of alkaline phosphatase (ALP) activity

The activity of ALP was determined using kinetic reaction (Schlebusch, Rick, Lang, & Knedel, 1974). The ALP diagnostic kit supplied by Agappe Diagnostics Ltd., Ernakulum, Kerala, India was used for estimation of ALP activity.

Principle:

The diagnostic kit of ALP includes two reagents; Rl and R2. Rl contains diethanolamine buffer (pH 10.2, 125 mM) and magnesium chloride (0.625 mM) and R2 contains p-nitrophenyl phosphate (50 mM). The working reagent was prepared by mixing RI and R2 in a ratio of 4:1 by volume. The working reagent (1 ml) and serum (20 µl) was mixed well, incubated for 37 °C. The absorbance was taken at 405 nm for 3 min with 60s intervals. The activity of ALP was calculated using the following formula:

$$\text{The activity of ALP (U / l)} = \Delta A / \text{min} \times 2750$$

3.10.2.3.4 Estimation of Total Bilirubin

The level of bilirubin was determined spectrophotometrically based on the method of Walters and Gerard (Walter M, 1970) using Bilirubin kit (Agappe Pvt. Ltd., Ernakulum, Kerala, India).

Principle:

The reaction between sulfanilic acid and sodium nitrite yields diazotized sulfanilic acid. In the presence of diazo reagent (TAB), bilirubin reacts with diazotized sulfanilic acid forms pink coloured azobilirubin complex which is measured at 546 nm using spectrophotometer.

Procedure:

A volume of 1 ml of total bilirubin reagent (constituents: 28.9 mM of sulfanilic acid and 9 mM of TAB) was pipetted out into test tubes. To this, 20 µl of activator total and 50 µl of serum was added and incubated for 5 min at room temperature. Then the absorbance of this reaction mixture was read at 546 nm. The reagent blank without serum was also prepared simultaneously. The concentration of bilirubin present in sample was calculated using the formula:

Total bilirubin concentration (mg / dl) = (OD of the test - OD of blank) x 25

3.10.2.4 Determination of Renal Function

The renal function tests namely urea and creatinine were analyzed with the help of commercially available diagnostic kits using serum separated from blood collected in tubes without EDTA.

3.10.2.4.1 Estimation of Serum Urea

The amount of urea present in serum was analyzed according to Urease-GLDH (enzymatic UV method) (Young, Fajardo, Murray, Rand, & Scrimshaw, 1975) using diagnostic kit provided by Euro Diagnostic Systems Pvt. Ltd., Chennai, India.

Principle:

Urea present in the sample is converted into ammonia and carbon dioxide in the presence of the enzyme urease. In this modified method, glutamate and NAD is formed as a result of reaction between ammonium ions, α-ketoglutarate and NADH. The rate of oxidation of NADH to NAD is proportional to the urea concentration. The concentration of urea is measured at 340 nm as decrease in absorbance in fixed time intervals.

Procedure:

The working reagent was prepared by mixing R1 (BUN reagent) and R2 (Urease enzyme) in the ratio 4:1 by volume. The working reagent (1 ml) and serum (10 µl) was mixed well at 37 °C. The absorbance was taken after 30 s and 60 s as Al and A2 respectively at 340 nm. The change in absorbance (AA) was calculated by subtracting A2 - Al. The same experiment was done with standard provided along with the kit. Using the following formula, concentration of urea present in the serum was calculated;

$$\text{Urea (mg / dl)} = (\Delta A_T / \Delta A_S) \times 50$$

Here, ΔAT= change in absorbance of sample; ΔAS = change in absorbance of standard and 50 = concentration of standard.

3.10.2.4.2 Estimation of Serum Creatinine

Modified Jaffe's two point kinetic method (Bonsnes, and Taussky, 1945) was used for the estimation of creatinine present in the serum with the help of creatinine kit supplied by Euro Diagnostic Systems Pvt. Ltd., Chennai, India.

Principle:

In alkaline condition, sodium picrate (originates from picric acid present in reagent) reacts with creatinine present in sample and generates creatinine picrate complex. This orange coloured complex shows maximum absorbance at 520 nm.

Sodium picrate + creatinine → creatinine picrate complex

Procedure:

The kit for creatinine estimation consists of two reagents; R1 - picric acid reagent, (comprises >8 mM of picric acid, >475 mM of NaOH and >2 mM of EDTA) and R2 - alkaline buffer. R1 and R2 were mixed in equal volume for preparing working reagent. About 1 ml of working reagent and 50 ul of serum was mixed well and absorbance of this mixture was measured at 520 nm after 30s (A0) and 90s (A1). Also conducted the similar experiments with creatinine aqueous standard (2 mg / dl) provided along with kit and the amount of creatinine in the serum was calculated according to the formula:

$$\text{Serum creatinine (mg / dl)} = (\Delta A_T / \Delta A_S) \times 2$$

Here, ΔAT = change in absorbance of sample; ΔAs = change in absorbance of standard and 2 = concentration of standard

3.10.2.5 Analysis of Lipid Profile

3.10.2.5.1 Estimation of Total Cholesterol

The amount of total cholesterol in serum was estimated by CHOD-POD method.

Principle:

Cholesterol esters in serum are hydrolysed by the enzyme cholesterol esterase. The resulted free cholesterol is oxidized by cholesterol oxidase into 4-cholesterol-3-one and H_2O_2. In the presence of peroxidase 4-cholesterol-3-one couples with 4-aminoantipyridine and phenol to yield a red quinone. The product which is red quinone is measured at 505 nm.

Procedure:

The reagents in total cholesterol kit include pipes (90 mM, pH 6.9), phenol (26 mM), cholesterol esterase (100 U / l), cholesterol oxidase (300 U / l), peroxidase (650 U / l) and 4-aminophenazone (0.4 mM). A volume of 10 µl serum and 1 ml of reagent was mixed well. The reaction mixture was incubated for 5 min at 37 °C and thereafter, the absorbance of this reaction mixture was recorded at 505 nm. A standard solution was prepared in the similar manner by using 10 µl of standard instead of serum.

Total cholesterol (mg / dl) = (Absorbance of sample / Absorbance of standard) x 200.

3.10.2.5.2 Estimation of Triglycerides

The amount of triglycerides present in the sample was evaluated according to glycerol phosphate dehydrogenase – peroxidase (GPO – POD) method using kit provided by Euro diagnostic systems, Chennai, India.

Principle:

The lipoprotein lipase acts on serum triglycerides to liberate glycerol and free fatty acids. Glycerol kinase phosphorylates glycerol into glycerol-3 phosphate which is eventually converted to dihydroxyacetone phosphate and H_2O_2 by glycerol phosphate dehydrogenase (GPO). H_2O_2 reacts with 4-aminophenazone (4-AP) and p-chlorophenol in the presence of peroxidase (POD) and forms red coloured quinone. The intensity of the formed quinone is proportional to the concentration of triglycerides in serum.

Procedure:

About 1 ml of reagent (constitutes 50 mM (pH 6.3), 2 mM p - chlorophenol, 500 U / l glycerol kinase, 150000 U / l lipoprotein lipase, 0.1 mM 4-aminophenazone, 3500 U / l glycerol 3 – kinase and 0.1 mM ATP) and 10 µl of serum were incubated for 5 min at 15 - 20°C. The absorbance was measured at 505 nm. A standard solution was prepared in the similar manner by substituting standard (200 mg / dl provided along with kit) instead of serum. Total triglyceride (TG) was calculated using the following formula:

Triglycerides (mg / dl) = (Absorbance of sample / Absorbance of standard) x 200

3.10.2.5.3 Estimation of HDL

The concentration of high density lipoprotein cholesterol (HDLc) in serum was done by the enzymatic photometric method with a commercial HDL kit (Euro Diagnostic Systems Pvt. Ltd., Chennai, India).

Principle:

The principle involved is the direct enzymatic colorimetric assay. It is based on the solubilization of HDL cholesterol by detergent (< 2 %) present in the reagents. The released HDL cholesterol reacts with cholesterol oxidase, cholesterol esterase and chromogens to produce a colour. The intensity of the colour formed is directly proportional to the concentration of HDLc in the serum. The non - HDL lipoproteins including LDL, VLDL and chylomicrons will be absorbed on the surface by the detergents which inhibits their reaction with reagents.

Procedure:

A volume of 450 µl of R1 (contains < 1000 U / l cholesterol oxidase and < 1 mM N,N-bis (4-sulphobutyl)-m-toluidine-disodium) was added to test tubes labeled as blank, calibrator and sample. Then 10 µl of serum and calibrator (lyophilized human serum, con 39.2 mg / dl) were added to respective tubes and incubated for 5 min at 37 °C. After that, 150 µl of R2 (contains < 3000 U/l ascorbic oxidase < 1500 U / l cholesterol esterase, < 1300 U / l peroxidase < 1 mM, 4-aminoantipyrine, and < 2% detergent) were added to all tubes. The reaction mixture was again incubated for 5 min at 37 °C. The absorbance of calibrator and sample was taken at 600 nm against reagent blank. HDL in the serum was calculated using the following formula:

$$\text{HDLc (mg / dl)} = (\Delta A \text{ sample} / \Delta A \text{ calibrator}) \times \text{concentration of calibrator}$$

3.10.2.5.4 Estimation of LDL and VLDL

The concentration of low density lipoprotein (LDL) present in the serum was calculated using Friedewald's formula (William T Friedewald, Robert I Levy, 1972). The formula is as follows:

$$\text{LDLc (mg / dl)} = \text{TC} - \text{HDLc} - (\text{TG} / 5)$$

Here, TC = Total Cholesterol, HDLc = High Density Lipoprotein Cholesterol, TG = Triglycerides.

$$\text{VLDL (mg / dl)} = \text{Triglycerides (mg / dl)} / 5$$

Here, VLDL = Very Low Density Lipoprotein.

3.10.2.6 Histological Analysis

A small portion of organs (brain, heart, lungs, liver, stomach, kidney and testis / ovary from each group was fixed in 10 % neutral buffered formalin solution. Thin sections of thickness 4 μm of paraffin embedded tissues were taken, deparaffinized, hydrated and stained using haematoxylin- eosin stain. The sections were examined using light microscope (200 X magnification) and photographs were taken using Magnus inverted laboratory microscope.

3.11 Protective effect of CZAgNPs on Sodium Fluoride Induced Oxidative Stress in Animal model

In the antioxidant study, the protective effect of the plant based silver nanoparticles (CZAgNPs) against sodium fluoride induced oxidative stress was evaluated. Thirty Swiss albino male mice (25 - 30 g b. wt) were divided into 6 groups of 5 animals each. Group 1 was maintained as untreated while the remaining five groups were given NaF (600 ppm) in drinking water for 7 days to induce oxidative stress. To study the antioxidant efficacy of CZAgNPs , groups III, IV and V animals were orally administered with three different safe doses of nanoparticles (25, 50, 75 mg / kg b. wt respectively) while group II served as positive control (Ascorbic acid, 10 mg / kg b. wt) and group VI as negative control (NaF 600 ppm). The animals in group II, III, IV, V were pre-treated for seven days with respective doses of drugs and were continued simultaneously with NaF administration started; on the 8 th day onwards for another 7 days. All animals were sacrificed on the 15 th day following overnight fasting. At the end of experiment, the antioxidant profile was analyzed in the liver tissue of the mice.

The blood was collected directly by heart puncture and stored in vials coated with anticoagulant EDTA. Serum was separated for biochemical analysis and kept at -20 °C. Liver was carefully excised, weighed, immediately cleaned with normal saline solution (0.9 % NaCl) and the tissue sample was kept at − 80 °C for further analysis. Liver tissue was homogenized and the following antioxidant assays were carried out. The assays included Catalase, SOD, GSH, GPx, GST, GR and TBARs.

3.11.1 Preparation of hemolysate from blood

The blood collected in tubes with anticoagulant EDTA was centrifuged for 10 min at 1500 rpm. After the removal of the upper layer, the packed RBC was washed repeatedly using phosphate buffer saline. Drabkin's method (Drabkin & Austin, 1935) was used for the determination of concentration of haemoglobin in packed RBC. Haemolysis of packed RBC was obtained by mixing 0.1 ml of packed RBC with 0.9 ml of hypotonic phosphate buffer. To this mixture 0.25 ml of chloroform and 0.5 ml of ethanol was added with vigorous shaking followed by cold centrifugation at 8600 rpm for 45 min. The supernatant thus obtained was used for the estimation of blood catalase and blood SOD.

3.11.2 Preparation of tissue homogenate

The liver tissue dissected out from the animals immediately after collection of blood was washed with ice cold 0.9 % saline to remove the contaminants. The liver was wiped with filter paper and the weight of liver was taken using electronic weighing balance. The 10 % liver homogenate was prepared using 0.1 M Tris-HCl buffer having pH 7.4 using homogenizer in ice cold condition. One portion of the homogenate was used for the estimation of lipid peroxidation. The remaining portion of the homogenate was centrifuged at 8600 rpm for 30 min. Thus obtained

supernatant was used in the estimation catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione – s-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), lipid peroxidation (TBARs) and total protein.

3.11.3 In Vivo Antioxidant Enzyme Analysis

3.11.3.1 Estimation of superoxide dismutase (SOD)

The activity of SOD in tissue and blood was estimated according to the method of McCord and Fridovich (McCord, 1969).

Principle:

This assay is based on the inhibitory effect of SOD present in the sample on reduction of nitroblue tetrazolium (NBT) by superoxide radicals. The superoxide radicals are generated by photo reduction of riboflavin in the presence of EDTA. The superoxide radical acts on NBT. Upon reduction NBT results in the formation of blue coloured formazan complex. The blue coloured complex has absorption maxima at 560 nm.

Procedure:

The reaction mixture was prepared by mixing 100 µl of tissue supernatant or haemolysate, 200 µl of KCN / EDTA (0.0015 % KCN in 0.1 M EDTA) and 2550 µl of phosphate buffer (67 mM, PH 7.8). After the addition of 100 µl of NBT (1.5 mM) and 50 µl of riboflavin (0.12 mM), absorbance of this reaction mixture was measured at 560 nm (initial OD). These tubes were illuminated under incandescent light for 20 min and absorbance was taken at 560 nm immediately after illumination (final OD). Control tube was kept without tissue supernatant. The percentages of inhibition were

calculated from absorbance of the test sample (At) and control (Ac). The activity of SOD was calculated using the following formula;

The activity of SOD (U / Hb) in blood = (% inhibition x 1750) / 50 x 100 x Hb

The activity of SOD (U / mg protein) in tissue = % inhibition / 50 x mg protein

Here; % inhibition = (Ac –At / Ac) x 100

3.11.3.2 Estimation of catalase (CAT)

The activity of blood catalase was determined as per the method of Aebi (Aebi, 1984) and of tissue catalase according to the method of Beers and Sizer (Beers Jr. & Sizer, 1952).

Principle:

The decomposition of H_2O_2 with maximum absorbance in UV region (240 nm) is catalyzed by the activity of catalase enzyme. With breakdown of H_2O_2, the absorbance decreases and change in absorbance per unit time determines the measure of catalase activity.

Procedure:

Catalase activity in blood: In order to adjust the Hb count to 4 - 5 g / dl, 10 µl of haemolysate was diluted with phosphate buffer to 5 ml (0.5 M, pH 7). Then, 2 ml of the diluted solution was mixed with 1 ml of phosphate buffer in a quartz cuvette as reference. The absorbance was measured at 240 nm. A volume of 2 ml haemolysate and 1 ml of H_2O_2 (30 mM) was mixed instead of phosphate buffer. The decrease in absorption was measured (at 25 °C) at 240 nm for 1 min with 15s intervals. The

change in absorbance is termed as ΔA. The activity of catalase in haemolysate was calculated using the following formula;

Catalase activity (U / Hb) in blood = (ΔA/min x 3000 x 0.153) / Hb

Here, ΔA /min = A2 - Al; Al = A240 at t = 0 sec and A2 = A240 at t = 15 sec; 3000 = dilution factor and 0.153 = 2.303 / 15

Catalase activity in tissue: Taken 2.7 ml of phosphate buffer (pH 7, 0.5 M) and 0.3 ml H_2O_2 (30 mM) in a quartz cuvette and measured the absorbance at 240 nm. Removed 50 µl from the reaction mixture and added 50 µl of tissue (10%). The absorbance was read at 240 nm for 1 min with 15 second intervals.

Catalase (U / mg protein) activity in tissue = (ΔA /3 x total volume of reaction) / (43.6 x 1cm x volume of enzyme sample x mg / ml protein.

Here, (ΔA / min = A2 - A1), A1 = A240 at t = 0 sec and A2 = A240 at t 15 sec; 43.6 = molar extinction coefficient of H_2O_2.

3.11.3.3 Estimation of reduced glutathione (GSH)

Principle:

The reduced glutathione react with 5-5'Dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) to yield 5 - thionitrobenzoic acid (a yellow coloured chromophore). 5 - thionitrobenzoic acid has maximum absorption at 412 nm.

Procedure:

A volume of 500 µl of tissue supernatant (10 %) along with 125 µl TCA (25 %) was placed on ice for 6 min. This reaction mixture was spun at 1500 rpm for 10

min followed by the addition of 0.6 ml of TCA (5 %). Further, 0.3 ml of supernatant was mixed with 0.7 ml of phosphate buffer (0.2 M, pH 8) and absorbance was taken immediately at 420 nm after the addition of 0.7 ml of DTNB (0.6 mM). The level of GSH (nmol / mg tissue protein) in tissue was calculated from standard graph (y = 0.0053x) plotted using different concentrations of GSH.

The level of GSH (n mol / mg protein) in tissue = Value from graph (X) / mg protein

3.11.3.4 Estimation of glutathione – s - transferase (GST)

The activity of GST in tissue was estimated according to the method of Habig *et al*. (Habig, Pabst, & Jakoby, 1974).

Principle:

The determination of GST is based on the rate of increase in the conjugate formation between GSH and 1-chloro-2, 4-dinitrobenzene (CDNB), with absorbance maxima at 340 nm.

Procedure:

The reaction mixture consisted of phosphate buffered saline at pH 6.5 (2.7 ml), 100 µl cell lysate, 100 mM CDNB in ethanol and 100 mM GSH in a total volume of 3 ml. The activity of GST was calculated from the change in absorbance at 340 nm for 3 min. The GST activity was expressed as nmoles of CDNB conjugates formed/min/mg protein.

$$\text{GST activity} = \Delta\text{OD per min} \times 1000 \times 39.6 \times \text{mg}$$

3.11.3.5 Estimation of glutathione reductase (GR)

The activity of GR in tissue was determined as per the protocol of Mavis and Stellwagen, (Mavis & Stellwagen, 1968).

Principle:

The conversion of glutathione disulfide to reduced glutathione at the expense of NADPH is catalyzed by glutathione reductase. The reaction can be followed by spectroscopy at 340 nm, using the molar extinction coefficient of β- nicotinamide dinucleotide phosphate (reduced).

Procedure:

The reaction mixture consisted of 100 mM potassium phosphate buffer (2.15 ml), 30 mM glutathione disulfide (100 µl), 0.8 mM NADPH (350 µl) and 1 % bovine serum albumin (300 µl). The decrease in absorbance at 340 nm was measured for 3 minutes at 30 sec intervals after the addition of 100 µl of tissue homogenate (10 %). GR activity was calculated using the formula;

Glutathione reductase (U / mg protein) = (Δ Absorbance / 2 x Total volume of reaction) / (6.22 x 1cm x volume of enzyme sample x mg / ml protein)

Here, Total reaction volume = 3 ml, 6.22 = molar extinction coefficient of β-NADPH at 340 nm, DF = Dilution factor, volume of enzyme solution = 0.1 ml

One unit of enzyme activity is defined as the reduction in 1.0 μ mol of oxidized glutathione per minute (at pH 7.6 at 25 ^{0}C).

3.11.3.6 Estimation of glutathione peroxidase (GPx)

The activity of GPx in tissue was determined as per the protocol of Paglia & Valentine (Paglia, D. E., & Valentine, 1967).

Principle:

The enzyme GPx degrades H_2O_2 in the presence of GSH, The remaining amount of GSH was measured by its reaction with DTNB.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Procedure:

The enzyme activity was measured at zero and 10 min intervals. For determining the enzyme activity at zero min, to 50 µl of tissue homogenate, 1 ml of 1.67 % metaphosphoric acid, 50 µl of 5 mM glutathione, 50 µl of 25 mM sodium azide, 50 µl of 25 mM hydrogen peroxide and 300 µl of 0.1 M, pH 7 phosphate buffer was added. The reaction mixture was centrifuged at 3000 rpm for 15 min. To the supernatant 1ml of 0.4 M di sodium hydrogen phosphate and 0.5 ml of 1mM DTNB was added, incubated for 10 min and absorbance was read at 412 nm. In order to determine the enzyme activity after 10 min, after initial incubation the metaphosphoric acid was added to the reaction mixture, centrifuged and collected the supernatant. A volume of 1 ml disodium hydrogen phosphate and 0.5 ml of DTNB was added to the reaction mixture. After incubation the absorbance was measured. The reaction mixture without tissue homogenate served as blank. The unit activity of the enzyme was calculated using the formula,

Unit activity = [(ΔA at 0’ - ΔA at 10’) x 2 x total reaction volume] / [slope x10’ x enzyme volume x mol. wt of GSH]

3.11.3.7 Determination of lipid peroxidation

The level of lipid peroxidation in tissue was estimated in terms of thiobarbituric acid reacting substances (TBARs) according to the method of Ohkawa *et al*.(Ohkawa, Ohishi, & Yagi, 1979).

Principle:

This assay is based on the reaction of malondialdehyde (MDA), end product of lipid peroxidation with TBA to produce a pinkish coloured adduct in acidic medium called TBARs. Thus formed adduct is measured spectrophotometrically.

Procedure:

The reaction mixture containing 200 µl tissue homogenate, 200 µl SDS (8 %), 1.5 ml acetic acid (20 %, pH 3.5) and 1.5 ml TBA (0.8 %) was incubated at 95ºC for 1 hour. A volume of 0.4 ml of tissue homogenate (10 %) was added to the reaction mixture containing 0.2 ml of SDS (8 %), 1.5 ml of acetic acid (20 %, pH 3.5), 1.5 ml of TBA (0.8 %) and 0.4 ml of distilled water. Then this reaction mixture was incubated for 1 h at 95°C. After incubation, test tubes were cooled under tap water. The absorbance of the supernatant was recorded at 532 nm. The MDA concentration was calculated from the standard graph (y = 0.0294 x) plotted using different concentrations of MDA (1 - 10 nmol) and expressed in terms of TBARS (nmol of MDA / mg protein).

TBARS (nmol of MDA / mg protein) = (Value from graph (X)) / mg protein

3.11.3.8 Histological analysis of liver tissue

The histological analysis of the liver tissue of all the groups (untreated and treated) was done according to the method described in the section 3.9.2.6.

3.12 *In Vivo* Anti-tumor Activity of AgNPs

3.12.1 Evaluation of the effect of CZAgNPs on Ascites Tumor development and life span

Thirty Swiss albino male mice (25-30 g b. wt) were divided into 5 groups of 6 animals each. Group I was control (untreated group); groups II, III, IV were treated with low dose (25 mg / kg b. wt), medium dose (50 mg / kg b. wt) and high dose (75 mg / kg b. wt) of CZAgNPs respectively, group V was administered with standard, cyclophosphamide (10 mg / kg b. wt).

EAC cells aspirated from the peritoneal cavity of tumor bearing mice were washed with PBS. Then 100 µl of cell suspension containing 1 x 10^{6} cells was injected intra- peritonially into all the animals except group I. The drug administration was initiated 24 h after the induction of tumor and was continued for 10 consecutive days. The animals were observed for the development of ascites tumor and the death due to tumor burden was recorded for 50 consecutive days.

The percentage increase in life span (% ILS) of animal was calculated using the formula,

$$\% \text{ ILS} = [(T-C) / C] \times 100$$

Where, T - the mean survival days of treated animals and C – the mean survival days of control animals.

3.12.2 Evaluation of the effect of CZAgNPs on Solid Tumor development

Inorder to evaluate the effect of CZAgNPs on solid tumor 30 healthy male Swiss albino mice (25 – 30 g b. wt) were taken. They were divided into five groups of six animals each. Group I was set as control, untreated group. Group II, III and IV served as treatment groups and they received low dose (25 mg / kg b. wt), medium dose (50 mg / b. wt) and high dose (75 mg / kg b. wt) of CZAgNPs respectively. Fifth group was treated with cyclophosphamide (10 mg / ml) and remained as standard group.

DLA cells were aspirated from the peritoneal cavity of the tumor bearing mice. The procured DLA cells were washed repeatedly with PBS. A volume of 100 µl of cell suspension having approximately 1 x 10^6 cells were injected intramuscularly into the left hind limb of all the animals. About 24 h after the induction of tumor, the drug administration was initiated and was continued for 10 consecutive days. The development of solid tumor on the animal in each group was determined by measuring the diameter of the tumor growth in two perpendicular planes using Vernier caliper. The readings were taken in a basis of 3 days interval up to the 30^{th} day.

The volume of tumor was calculated according to the following formula:

$$\mu\text{Tumor volume} = 4/3\ \pi\ (r_1^2\ r_2)$$

Where, r_1 corresponds to minor radius and r_2 the major radius.

The percentage of inhibition of the tumor growth was calculated according to the formula,

$$\% \text{ inhibition} = [(C-T) / C] \times 100$$

Where, C = volume of tumor of control group on the 30^{th} day and T = volume of tumor of treatment group on the 30^{th} day.

3.13 Statistical analysis

The *in vitro* studies were performed in triplicates and data represented as mean ± SD. For *in vivo* experiments the values are expressed as mean ± SD for six animals. The mean values were statistically analyzed using two – way and one – way analysis of variance using graph pad Insat 3 software (Graph Pad Software, Inc. La Jolla, USA), traced by Dunnett multiple comparison test. $p > 0.05$ was considered as non-significant and $^{*}p < 0.05$ was considered as statistically significant.