3

EXPERIMENTAL PROCEDURE

The present research focused on **"Antioxidant and Antitumorigenic efficacy of methanolic extracts of *Gloriosa superba* and Silver Nanoparticles of methanolic extracts of *Gloriosa superba* to DLA tumor cells"** was conducted in three different phases.

Phase I

3.1 Preparation of methanolic extracts of *Gloriosa superba* seed, tuber and leaves (MGsSTL)

3.1.1 Synthesis of AgNPs of *Gloriosa superba* seed, tuber and leaves (AgMGsSTL)

3.2 Characterization of MGsSTL and AgMGsSTL

3.2.1 Preliminary phytochemical screening of MGsSTL

3.2.2 High Performance Thin Layer liquid Chromatography (HPTLC) analysis of MGsSTL

3.2.3 UV-Visible Spectroscopy

3.2.4 Fourier Transform InfraRed (FTIR) Analysis

3.2.5 Scanning electron microscope (SEM) with Energy Dispersive Spectroscopy (EDX)

3.2.6 Transmission electron microscope (TEM)

3.2.7 Dynamic light Scattering (DLS)

3.2.8 Zeta-Potential

3.2.9 X-RAY Diffraction (XRD) Analysis

Phase II

3.3 Assessment of *in vitro* antioxidative role of MGsSTL and AgMGsSTL

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3.3.2 Hydroxyl radical scavenging assay ($OH^{\cdot}$)

3.3.3 Superoxide radical scavenging assay ($^{\cdot}O_2^-$)

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3.4 Assessment of *in vitro* antitumorigenic activity of MGsSTL and AgMGsSTL to Dalton lymphoma ascites tumor cells

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3.4.2 Trypan blue exclusion assay

3.4.3 Apoptotic activity

Phase III

3.5 Assessment of *in vivo* antioxidative and antitumorigenic activity of MGsSTL and AgMGsSTL in DLA induced Swiss albino mice.

3.5.1 Assessment of activities of enzymic antioxidants

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3.5.1.2 Estimation of Superoxide dismutase activity

3.5.1.3 Estimation of Glutathione S-Transferase activity

3.5.1.4 Estimation of Glutathione Peroxidase activity

3.5.1.5 Estimation of Glutathione Reductase activity

3.5.1.6 Estimation of Protein

3.5.2 Assessment of levels of non enzymic antioxidants

3.5.2.1 Estimation of Vitamin A

3.5.2.2 Estimation of Vitamin E

3.5.2.3 Estimation of Vitamin C

3.5.2.4 Estimation of Reduced glutathione (GSH)

3.5.3 Assessment of levels of lipid peroxides (LPXs)

3.5.4 Assessment of antitumorigenic effect of MGsSTL and AgMGsSTL by percentage of mortality rate in *in vivo* cytotoxic studies

3.5.5 Histological appearance of the liver of control and experimental Swiss albino mice

The protocols adapted for the present study are presented in this chapter:

Phase I

This phase involved the preparation of MGsSTL, synthesis of AgMGsSTL and the characterization of MGsSTL and AgMGsSTL.

Collection of plant material

Fresh seed, tuber and leaves of *Gloriosa superba* were collected from the outskirts of Thanjavur district, Tamilnadu. The collected samples were washed thoroughly in tap water, shade dried and finely powdered.

3.1 Preparation of methanolic extracts of *Gloriosa superba* seed, tuber and leaves (MGsSTL)

Ten gram of each seed, tuber and leaf powders of *Gloriosa superba* was filled individually in separate thimbles and extracted with 150 ml of methanol using a soxhlet extractor for 24 hours. The methanol extracts of seed, tuber and leaf were distilled and evaporated to dryness. The concentrated extract of seed, tuber and leaf were then accurately weighed and stored in small vials at -20^0 C for further use.

3.1.1 Synthesis of AgNPs of *Gloriosa superba* seed, tuber and leaves (AgMGsSTL)

The 1mM silver nitrate ($AgNO_3$) was prepared using deionized water and used for the synthesis of silver NPs (AgNPs) by the method *of* Harborne, 1998. To 90 ml of aqueous solution of 1 mM silver nitrate, 10 ml of each MGsS, MGsT and MGsL in separate test tubes was added for the reduction of Ag^+ to Ag^o and incubated for a period of 15 h at room temperature. Here the extract act as the stabilizing agent for the reduction of Ag^+ to Ag^o. AgNPs exhibited yellowish brown to dark brown color in aqueous solution as shown in Plate I due to the excitation of surface plasmon resonance (Ahmad and Sharma, 2012).

Plate I

Synthesis of AgMGsSTL from MGsSTL

Overall, lower extract concentrations yield smaller and more monodisperse AgNP populations; tailoring the reducing agent and/or stabilizing ligand concentrations afford control over nanoparticle size and size-distribution (Masurkar *et al*., 2011).

3.2 Characterization of MGsSTL and AgMGsSTL

This phase involved the characterization of MGsSTL and AgMGsSTL by phytochemical screening, High Performance Thin Layer liquid Chromatography (HPTLC), UV-VisibleSpectroscopy, Fourier Transform Infrared Spectroscopy (FT-IR), Scanning Electron Microscope (SEM) with Energy Dispersive Spectroscopy (EDX), Transmission Electron Microscope (TEM), Dynamic Light Scattering (DLS), Zeta-Potential and X-RAY Diffraction analysis (XRD).

3.2.1 Preliminary phytochemical screening of MGsSTL

Phytochemical screening was performed using standard procedures. The procedures for detection of alkaloids, flavonoids, saponins, phenols, glycosides (Raaman, 2006), tannins, carbohydrates (Iyengar, 1995), steroids and terpenoids (Siddiqui and Ali, 1997) are given in Appendix I.

3.2.2 High Performance Thin Layer liquid Chromatography (HPTLC) analysis of MGsSTL

A densitometry-C of HPTLC analysis was performed for the development of characteristic finger printing profile. The MGsS, MGsT and MGsL was dissolved with HPLC grade methanol 100 mg/0.5ml.The solution was centrifuged at 3000 rpm for 5 min and used for HPTLC analysis. Then, 2 µl of the samples were loaded as 5 mm band length in the 5 x 10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase (alkaloid compound) and the plate was developed in the respective mobile phase (Ethyl acetate-Methanol-Water 10:1.35:1) up to 90 mm. The developed plate was dried using hot air to evaporate solvents from the plate and sprayed with drangendroff reagent followed by 10% ethanolic sulphuric acid. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at Visible light, UV 254nm and UV366 nm. Finally, the plate was fixed in scanner stage and scanned at 254nm. The Peak table, Peak display and Peak densitogram was identified (Puri *et al*., 2010).

3.2.3 UV-Visible Spectroscopy

The AgNPs were characterized in a Perkin-Elmer UV-Visible spectrophotometer, Lambda-19 to know the kinetic behavior of AgNPs. The scanning range for the samples was 200-800 nm at a scan speed of 480 nm/min. The spectrophotometer was equipped with “UV Winlab” software to record and analyze data. Base line correction of the spectrophotometer was carried out by using a blank reference. The UV-Visible absorption spectra of all the samples were recorded and numerical data were plotted in the “Origin 6.5” (Sooväli *et al*., 2006).

3.2.4 Fourier Transform InfraRed (FTIR) Analysis

Perkin-Elmer spectrometer FTIR Spectrum ONE in the range 4000–400 cm−1 at a resolution of 4 cm−1.was used. The sample was mixed with KCl procured from Sigma. Thin sample disc was prepared by pressing with the

disc preparing machine and placed in Fourier Transform InfraRed (FTIR) for the analysis of the NPs (Prati *et al*., 2010).

3.2.5 Scanning Electron Microscope (SEM) with Energy Dispersive Spectroscopy (EDX)

Jeol JSM-6480 LV SEM machine were used to characterize mean particle size, morphology of NPs. The freeze dried sample of AgNP solution was sonicated with distilled water, small drop of this sample was placed on glass slide allowed to dry. A thin layer of platinum was coated to make the samples conductive Jeol JSM-6480 LV SEM machine was operated at a vacuum of the order of 10-5 torr. The accelerating voltage of the microscope was kept in the range 10-20 kV. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM. The EDX analysis of Ag sample was done by the SEM (JEOLJSM 5800) machine. The EDX normally reveals the presence of phases (McMullan, 2006).

The EDAX is an analytical technique which utilizes x-rays that are emitted from the specimen when bombarded by the electron beam to identify the elemental composition of the specimen. When the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the surface of specimen. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The EDS X-ray detector measures the number of emitted X-rays versus their energy. The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum of the energy versus relative counts of the detected X-rays is obtained and evaluated for qualitative and quantitative determinations of the elements (Dhanalakshmia and Rajendran, 2012).

3.2.6 Transmission Electron Microscope (TEM)

Sample is dispersed in double distilled water. A drop of thin dispersion is placed on a “staining mat”. Carbon coated copper grid is inserted into the drop with the coated side upwards. After about ten minutes,the grid is removed and air dried.

Then screened in JEOL JEM 100SX Transmission Electron Microscope at an accelerating voltage of 80kv (Fultz, and Howe 2007).

3.2.7 Dynamic Light Scattering (DLS)

This is one of the most popular technique which is used to determine the size of particles. Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler Shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. From DLS (Zetasizer, Malvern) it is possible to compute the sphere size distribution and give a description of the particle's motion in the medium, measuring the diffusion coefficient of the particle and using the autocorrelation function (Block and Scheffold 2010). For the green synthesized AgNPs the nanostructure diameter measurements were performed using the Zetasizer Nano-S system (Malvern Instruments Ltd., Malvern, UK). A Sample was dispersed in water at a concentration of 50 g/ml (Saxena *et al*., 2010).

3.2.8 Zeta-Potential

Zeta-potential measurements were performed on a Malvern Zetasizer Ver. 6.32 at 25◦C with an incident wavelength of 633nm and a 173◦ back scattering angle. Clear disposable zeta-potential cells (1 cm path length) were rinsed with ethanol, followed by deionized water prior to sample loading. The viscosity, refractive index, and absorption values were provided in the Malvern software for water (μ = 0.8872 cP, RI = 0.1330) and crystalline silver (RI = 1.3330, absorption = 3.987). Twenty runs were averaged for each liquid sample for accurate determination of zeta-potential measurements (Dougherty and Rose 2008).

3.2.9 X-RAY Diffraction Analysis (XRD)

The formation and quality of compounds were checked by XRD spectrum. The mixture was centrifuged at 10000 rpm for 10 minutes in a refrigerated centrifuge, followed by redispersion of the pellet in acetone. The dispersed pellets were dried in an incubator at 37 °C for 1 week. The size of the purified AgNPs was

analyzed by X-ray powder diffraction crystallography SEIFERT JSO-DEBYEREX-2002 (Germany) diffractometer with Cu-Kα radiation (λ=1.540 nm) with a scan rate of 0.04^{o} per second and a scan range between 10 -70^{o}, 2 Θ in flat plate geometry with Cu radiation. The average crystalline particle size of AgNps was calculated by the use of full width at half maximum (FWHM) of *face-centered cubic* (111) using the Debye–Scherrer equation, Kλ/ β CosΘ, where K is the Scherrer constant with value from 0.9 to 1, λ is the wavelength of the X-ray, β is the full width at half maximum and θ is the Bragg angle in radians (Geerlof, *et al*., 2006).

Phase II

3.3 Assessment of *in vitro* antioxidative role of MGsSTL and AgMGsSTL

Antioxidative role of MGsSTL and AgMGsSTL was evaluated by assessing their scavenging of DPPH, $OH^{\cdot}$, $^{\cdot}O_2^{-}$. radicals and non radicals such as H_2O_2 and NO against the standard Vitamin C.

3.3.1 DPPH radical scavenging assay (DPPH)

DPPH radical scavenging activity was measured according to the method of Mensor *et al*. (2001) as given in Appendix II.

3.3.2 Hydroxyl radical scavenging assay ($OH^{\cdot}$)

The hydroxyl radical scavenging activity was analyzed according to the method of Elizabeth and Rao, 1990 as in Appendix III.

3.3.3 Superoxide radical scavenging assay ($^{\cdot}O_2^{-}$)

Superoxide radical scavenging activity of MGSSTL and AgMGSSTL was determined by the method of McCord and Fridovich, 1968 as elaborated in Appendix IV.

3.3.4 Hydrogen peroxide scavenging assay (H_2O_2)

Hydrogen peroxide scavenging activity of MGSSTL and AgMGSSTL was determined according to the method described by Ruch *et al*.(1989) as in Appendix V.

3.3.5 Nitric oxide scavenging assay (NO)

Nitric oxide scavenging activity of MGSSTL and AgMGSSTL was determined by the method described by Green *et al*.(1982) (Appendix VI).

3.4 Assessment of *in vitro* antitumorigenic activity of MGsSTL and AgMGsSTL to Dalton lymphoma ascites tumor cells

In vitro antitumorigenic activity of MGsSTL and AgMGsSTL was evaluated by assessing their cytotoxic effect to Dalton lymphoma ascites (DLA) tumor cells by MTT and tryphan blue assay. Apoptotic activity of AgMGsSTL was also carriedout by flow cytometry

Propagation of tumor cells

The DLA tumor cell lines were procured from Amala Cancer Research Centre, Thrissur, Kerala and were propagated in Swiss albino mice by intraperitoneal transplantation of 1×10^6 cells in 100 µl of PBS. After 14 days, tumor cells were drawn from the intraperitoneal cavity and were used for the *in vitro* cytotoxic study.

3.4.1 MTT assay

The proliferation of tumor cells by MGsSTL and AgMGsSTL in Swiss albino mice against DLA cells was judged by MTT proliferation assay (Loosdrecht *et al*., 1994) as in Appendix VII. The concentration of extracts which killed 50 % of DLA tumor cells was designated as the 50 percent inhibitory concentration (IC_{50}) of the MGsSTL and AgMGsSTL and was used in Phase II *in vivo* studies.

3.4.2 Trypan blue exclusion assay

In vitro cytotoxic activity of MGsSTL and AgMGsSTL in Swiss albino mice against Dalton lymphoma ascites tumor cells was studied by Trypan blue exclusion method of Salomi and Panikker (1989) as in Appendix VIII.

3.4.3 Apoptotic activity

The apoptotic measurement activity of AgMGsSTL to DLA tumor cells of was determined using flow cytometry. The DNA of the DLA cancer cells was

stained by 5 µl of annexin V-Biotin and 5 µl propidium iodide (PI) staining solution by incubating 1.0 × 10^6 cells in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and Hoechst 33258 (1.0 µg/ml) for 15 min in the dark. The DNA histograms were recorded on a Skatron Argus 100 flow cytometer (Skatron, Tranby, Norway) and analyzed using the Multiplus Program (Phoenix Flow systems, San Diego, CA, USA). Cell cycle phases and apoptotic cells/bodies were distinguished by their DNA content (Hoechst fluorescence) and cell size (forward light scatter). Apoptosis was defined as the registered counts/signals left to the G1 peak, with background subtracted. This background was set to approximately 20% of the G1 channel number. Observed the G1 peak at channel 100, and the background at channel 20 or below. The apoptotic index was determined as percentage of counts/signals in the area between the G_1 peak and the background, relative to the total area excluding background and aggregates. The relative abilities of all the tested particles to induce apoptosis were quantified by estimating the area under the curve (AUC) of the concentration-effect curves (0–600 µg/ml) by use of GraphPad Prism software (Refsnes *et al*., 2006).

Reagents required

Annexin V- Biotin (50 µg/mL) 100 µL

10X Binding Buffer 5 mL

Propidium Iodide (50 µg/mL) 1 mL

Store components at 4°C in the dark

Phase III

This phase involved the *in vivo* assessment of antioxidative and antitumorigenic efficacy of MGsSTL and AgMGsSTL during 20 days and 60 days period of tumorigenesis in DLA tumor cells induced Swiss albino mice.

Plate II

Medicinal plant selected for the study

Gloriosa superba

Gloriosa superba seed

Gloriosa superba tuber

Gloriosa superba leaves

Plate III

Swiss albino mice (*Mus musculus)*

Normal mouse and Dalton's lymphoma ascites tumor induced mouse

3.5 Assessment of *in vivo* antioxidative and antitumorigenic activity of MGsSTL and AgMGsSTL in DLA induced Swiss albino mice.

In vivo antioxidative and antitumorigenic potential of MGsSTL and AgMGsSTL was evaluated by the assessment of enzymic antioxidants, levels of non enzymic antioxidants and lipid peroxide levels in DLA induced Swiss albino mice.

Maintenance of animals

Swiss albino mice of 5-7 weeks old (20-25g) were bought from a small animal breeding station, Kerala Agricultural University, Thrissur. The mice were maintained under dark/light cycle (14/10h). The animals were kept in neat cages, bottomed with husk and fed with standard pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 15 days before the commencement of the experiments. All procedures described were reviewed and approved by the University Animals Ethical Committee (Reg no: 623/02/b/CPCSEA).

Grouping of animals

The Swiss albino mice were divided into 17 groups with six animals in each treatment groups as follows:

Group I (**PBS**) mice received 100µl of PBS and served as vehicle control for experimental group XVII.

Group II (**DMSO**) mice received 100µl of DMSO and served as vehicle control for group V, VI, VII, VIII, IX, X.

Group III (**Paraffin oil)** mice received 100µl of paraffin oil and served as vehicle control for Silymarin group IV.

Group IV **(Silymarin)** mice received the standard antioxidant silymarin (25mg/kg body weight) in 100µl of paraffin oil.

Group V (**MGsS)** and VI **(AgMGsS)** mice received MGsS (33 µg in 100 µl (ED_{50}) of DMSO) and AgMGsS (25µg in 100 µl of DMSO).

Group VII **(MGsT)** and VIII **(AgMGsT)** mice received MGsT (38 µg in 100 µl (ED_{50}) of DMSO) and AgMGsT (30 µg in 100 µl of DMSO).

Group IX **(MGsL)** and X **(AgMGsL)** mice received MGsL (82 µg in 100 µl of (ED_{50}) of DMSO) and AgMGsL (79 µg in 100 µl of DMSO).

Group XI **(DLA+ MGsS)**, XII **(DLA + AgMGsS)**, XIII **(DLA +MGsT)**, XIV **(DLA+ AgMGsT)**, XV **(DLA +MGsL)** and XVI **(DLA + AgMGsL)** received one acute dose of $1x10^6$ DLA tumor cells in 100 µl of PBS on the first day of the experimental period and also ED_{50} dose of MGsS, AgMGsS, MGsT, AgMGsT, MGsL and AgMGsL throughout the experimental tenure.

In Group XVII **(DLA)** mice, tumor was induced by the administration of one acute dose of 1×10^6 DLA tumor cells in 100 µl of PBS on the first day of the experimental period.

The experiments were carried out for 20 days and 60 days. At the end of the study the mice were sacrificed after an overnight fasting. The liver was dissected, blotted of blood and washed with PBS of pH 7.2. A part of the liver homogenate was prepared using PBS and used for the determination of enzymic and nonenzymic antioxidants. A part of the liver homogenate was prepared using Tris HCl for the assessment of the rate of lipid peroxidation. The histological examination of the liver of all the experimental mice was also carried out.

3.5.1 Assessment of activities of enzymic antioxidants

The activities of enzymic antioxidants like Catalase (CAT), Superoxide dismutase (SOD), Glutathione S-tranferase (GST), Glutathione Peroxidase (GPx), and Glutathione reductase (GR) were analyzed in the liver homogenate.

3.5.1.1 Estimation of Catalase (CAT, EC.1.11.1.6) activity

CAT activity was assessed by the method of Luck (1974) as given in Appendix IX.

3.5.1.2 Estimation of Superoxide dismutase (SOD, EC.1.15.1.1) activity

The activity of SOD was estimated by the method of Misra and Fridovich (1972) as given in Appendix X.

3.5.1.3 Estimation of Glutathione S-Transferase (GST, EC.2.5.1.13) activity

The activity of GST was estimated by the method of Habig *et al*. (1974) as given in Appendix XI.

3.5.1.4 Estimation of Glutathione Peroxidase (GPx, EC.1.11.1.9) activity

The activity of GPx in the liver was assessed by the method of Rotruck *et al*.(1973) as expressed in Appendix XII.

3.5.1.5 Estimation of Glutathione Reductase (GR, EC.1.6.4.2) activity

The activity of GR in the liver was assessed by the method of David and Richard (1983) as expressed in Appendix XIII.

3.5.1.6 Estimation of Protein

Protein concentration in the liver homogenate was determined by the modified method of Shakir *et al*., 1994 using BSA as standard to express the enzymic activities in units per mg protein (Appendix XIV).

3.5.2 Assessment of levels of non enzymic antioxidants

The levels of the non enzymic antioxidants such as fat soluble (vitamin A and E), water soluble (Vitamin C) and Reduced glutathione (GSH) were assessed in the liver homogenate of experimental mice.

3.5.2.1 Estimation of Vitamin A

Vitamin A was estimated by the method of Bayfield and Cole (1980) as given in Appendix XV.

3.5.2.2 Estimation of Vitamin E

Vitamin E content was determined by the method of Rosenberg (1992) as expressed in Appendix XVI.

3.5.2.3 Estimation of Vitamin C

Vitamin C was estimated by the method of Roe and Keuther (1943) as given in Appendix XVII

3.5.2.4 Estimation of Reduced glutathione (GSH)

The activity of GSH was determined by the method of Moron *et al*. (1979) as in Appendix XVIII.

3.5.3 Assessment of levels of lipid peroxides (LPXs)

The levels of lipid peroxides in the liver were determined by the method of Nichans and Samuelson, 1968 as given in Appendix XIX.

3.5.4 Assessment of antitumorigenic effect of MGsSTL and AgMGsSTL by percentage of mortality rate in *in vivo* cytotoxic studies

In vivo cytotoxic studies were carried out using the ED_{50} of MGsSTL and AgMGsSTL to follow the antitumor activity in terms of Increase in Life Span (ILS) of Swiss albino mice transplanted with DLA tumor cells. Seven groups of (6 mice/group) Swiss albino mice were used for the *in vivo* antitumorigenic studies. To the control group $1x10^6$ DLA tumor cells were administered intraperitoneally on the 1st day of the experiment for the development of tumor. To the experimental groups $1x10^6$ DLA tumor cells were administered intraperitoneally on the 1st day of the experiment for the development of tumor. At the end of 24 hours, ED_{50} (µg) of MGsS, AgMGsS, MGsT, AgMGsT, MGsL and AgMGsL were administered intraperitoneally. This was repeated for 60 days. The mortality of the animals dying off tumor was noted and the increase in life span in days was calculated as described by Geran *et al*. (1972).

3.5.5 Histological appearance of the liver of control and experimental Swiss albino mice

Histological examination was performed after the experimental tenure. Liver of all the control and experimental mice were submitted to a perfusion with saline solution and to the routine histology process. The organs were fixed in formaldehyde (4.0%v/v, prepared in PBS 0.001M, pH 7.2) for 7 days, dehydrated in methanol at different concentrations. After microtomy (4µm) the sections were stained with hematoxylin-eosin (Chaves *et al*., 2004) as in appendix XX.

Statistical analysis

The data presented here are means ± SD of 6 mice in each group. The biochemical results of in vivo studies using mice for 20 days treatment period alone including DLA were subjected to one-way ANOVA and the results of 20 days and 60 days treatment periods excluding DLA treated group were subjected to one way and two way ANOVA using SigmaStat Statistical Package to test the level of statistical significance at $p<0.05$.