3

Expreimental Procedure

Green synthesis of nanoparticles employing plant materials drawn an unequivocal attention. The bioactive constituents of the plant act as reducing and capping agent which increases the rate of reduction and stability of nanoparticles. Biologically synthesized nanoparticles have upsurge applications in various sectors as it is free from toxic chemicals (Mohammadlou *et al.*, 2016). Hence the present research focused on **"Antioxidative and Antitumorigenic Potential of PEG Functionalized Silver Nanoparticles from Ethanolic Extract of *Volkameria inermis* Leaves to EAC Cells by *in vitro* and *in vivo* Studies"** was conducted in five different phases.

***In vitro* Studies**

PHASE I

3.1 ASSESSMENT OF PHYTOCHEMICAL CONSTITUENTS, EVALUATION OF ANTIOXIDATIVE POTENTIAL OF DIFFERENT EXTRACTS AND CHARACTERIZATION OF ETHANOLIC EXTRACT OF *Volkameria inermis* LEAVES BY HPLC AND HPTLC TECHNIQUES

3.1.1. Collection of Plant Sample

3.1.2. Preparation of Different Extracts of *Volkameria inermis* Leaves

3.1.3. Preliminary Phytochemical Analysis

3.1.4. Evaluation of Antioxidative Potential of *Volkameria inermis* leaves

3.1.4.1 The DPPH radical scavenging assay

3.1.4.2 Superoxide radical scavenging assay

3.1.4.3 Hydroxyl radical scavenging assay

3.1.4.4 Hydrogen peroxide scavenging assay

3.1.4.5 Nitric oxide scavenging assay

3.1.5 Ferric Reducing Antioxidant Power Assay (FRAP)

3.1.6 Characterization of Ethanolic Extract of *Volkameria inermis* Leaves by HPLC and HPTLC Techniques

3.1.6.1 Analysis of ethanolic extract of *Volkameria inermis* by HPLC

3.1.6.2 Analysis of ethanolic extract of *Volkameria inermis* by HPTLC

PHASE II

3.2 SYNTHESIS OF BIOLOGICALLY ACTIVE SILVER NANOPARTICLES (AgNPs), IT'S FUNCTIONALIZATION (PEGylated AgNPs) AND THEIR CHARACTERIZATION

3.2.1 Synthesis of Biologically Active AgNPs from Ethanolic Extract of *Volkameria inermis* Leaves

3.2.1.1 Heating in water bath

3.2.1.2 Heating by microwave oven

3.2.1.3 Exposure to sunlight

3.2.2 Separation of Silver nanoparticles

3.2.3 Synthesis of Functionalized AgNPs

3.2.4 Characterization of AgNPs and PEGylated AgNPs

3.2.4.1 Spectral analysis of biologically active AgNPs and PEGylated AgNPs

3.2.4.2 Transmission Electron Microscopy (TEM) with Energy Dispersive X-ray Spectroscopy (EDX)

3.2.4.3 X- ray Diffraction (XRD)

3.2.4.4 Fourier Transform Infrared spectroscopy (FTIR)

3.2.4.5 Zeta potential

PHASE III

3.3 EVALUATION OF BIOCOMPATIBILITY ROLE AND DRUG RELEASING CAPACITY OF PEGylated AgNPs

3.3.1 Biocompatibility of PEGylated AgNPs

3.3.1.1 Extent of hemolysis

3.3.1.2 Morphological changes of red blood cells

3.3.2 Drug Releasing Profiles at Different pH Conditions

PHASE IV

3.4 DETECTION OF APOPTOTIC EFFECT OF PEGylated AgNPs ON EAC CELL LINES

3.4.1 Cell Culture

3.4.2 Assessment of the Viability of EAC Cells by MTT assay

3.4.3 Microscopic Examination of EAC Cells by AO/EtBr Dual Staining Method

3.4.4 Evaluation of DNA Fragmentation in EAC cells by Agarose Gel Electrophoresis

3.4.5 Analysis of Cell Cycle by Flow Cytometry

***In vivo* Studies**

PHASE V

3.5 EVALUATION OF *In vivo* ANTIOXIDATIVE AND ANTITUMORIGENIC ACTIVITY OF PEGylated AgNPs IN EAC INDUCED SWISS ALBINO MICE

3.5.1 Maintenance of Experimental Animals

3.5.2 Assessment of EC_{50} of PEGylated AgNPs by Trypan Blue Exclusion Assay

3.5.3 Grouping of Animals

3.5.4 Evaluation of PEGylated AgNPs on the Mortality Rate of EAC Cells Induced Swiss albino Mice

3.5.5 Evaluation of PEGylated AgNPs on the Activities of Liver Marker Enzymes in Serum of EAC Challenged Swiss albino Mice

3.5.5.1 Aspartate transaminase (AST, EC. 2.6.1.1)

3.5.5.2 Alanine transaminase (ALT, EC. 2.6.1.2)

3.5.5.3 Alkaline phosphatase (ALP, EC. 3.1.31)

3.5.6 Evaluation of PEGylated AgNPs on the Activities of Enzymic Antioxidants

3.5.6.1 Estimation of Catalase (CAT, EC.1.11.1.6)

3.5.6.2 Estimation of Superoxide dismutase (SOD, EC.1.15.1.1)

3.5.6.3 Estimation of Glutathione peroxidase (GPx, EC.1.11.1.9)

3.5.7 Evaluation of PEGylated AgNPs on the Levels of Non Enzymic Antioxidants

3.5.7.1 Estimation of Vitamin A

3.5.7.2 Estimation of Vitamin E

3.5.7.3 Estimation of Reduced glutathione (GSH)

3.5.8 Evaluation of PEGylated AgNPs on the Rate of Lipid Peroxidation

3.5.9 Evaluation of PEGylated AgNPs on the Histological Status of the Liver of Control and EAC Cells Challenged Swiss albino Mice

PHASE I

3.1 ASSESSMENT OF PHYTOCHEMICAL CONSTITUENTS, EVALUATION OF ANTIOXIDATIVE POTENTIAL OF DIFFERENT EXTRACTS AND CHARACTERIZATION OF ETHANOLIC EXTRACT OF *Volkameria inermis* LEAVES BY HPLC AND HPTLC TECHNIQUES

The phytochemical constituents and antioxidative activity of *Volkameria inermis* leaves was assessed in the different extracts namely petroleum ether, chloroform, ethylacetate, ethanol and water in the increasing order of polarity.

3.1.1. Collection of Plant Sample

Fresh leaves of *Volkameria inermis* (Plate 1) were collected in an area free of pesticides and other contaminants from Tamil Nadu Agricultural University (TNAU). The collected leaves were washed thoroughly in tap water, shade dried and powdered. Authentication was done at Botanial Survey of India at TNAU Coimbatore district, India **(BSI/SRC/5/23/2015/Tech/2082)**.

Plate 1

Volkameria inermis

3.1.2. Preparation of Different Extracts of *Volkameria inermis* Leaves

Five different solvents namely petroleum ether, chloroform, ethylacetate, ethanol and water were taken, in which 10g of powdered plant sample was added individually in all solvents (10g/100ml). Plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 hours. After 24 hours the extract was filtered and the filtrate was concentrated using flash evaporator and stored in air tight containers at 4°C and used for the experiments (Santhi *et al.,* 2011).

3.1.3. Preliminary Phytochemical Analysis

Standard procedures were used for performing phytochemical screening. The different leaf extracts of *Volkameria inermis* were analysed for the presence of alkaloids, flavonoids, phenols, steroids, saponins, terpenoids, tannins, glycosides, amino acids and proteins (Appendix I).

3.1.4. Evaluation of Antioxidative Potential of *Volkameria inermis* Leaves

The antioxidative potential was evaluated by assessing the scavenging activity of DPPH, superoxide, hydroxyl radical, hydrogen peroxide and nitric oxide scavenging assays and FRAP assay using the different extracts namely petroleum ether, chloroform, ethylacetate, ethanol and water extracts of *Volkameria inermis* leaves.

3.2.1 Synthesis of Biologically Active AgNPs from Ethanolic Extract of *Volkameria inermis* Leaves

Silver nanoparticles were prepared from ethanolic extract of *Volkameria inermis* leaves. To 10 ml of the ethanolic leaves extract 90 ml of 1mM silver nitrate solution was added (Donda *et al.,* 2013). The extent of nanoparticles synthesis was monitored by measuring the absorbance at 400-600nm.

3.2.1.1 Heating in water bath

The ethanolic extract of *Volkameria inermis* leaves in the presence of silver nitrate was heated for various durations (5, 10, 15 and 20 minutes) in a water bath at a temperature of 60 °C (Gulcin *et al.,* 2011).

3.2.1.2 Heating by microwave oven

The ethanolic extract of *Volkameria inermis* leaves with silver nitrate solution was heated in microwave for various durations namely 10, 20, 30 and 40 seconds (Nooroozi *et al.,* 2012).

3.2.1.3 Exposure to sun light

The ethanolic extract of *Volkameria inermis* leaves with silver nitrate solution was exposed to sunlight for various durations (5, 10, 15 and 20 minutes) (Sulaiman *et al.,* 2013).

3.2.2 Separation of Silver Nanoparticles

To separate the synthesized silver nanoparticles, samples were centrifuged at 13,000 rpm for 20 minutes under refrigeration and washed 3 times with deionized water. A dried powder of the silver nanoparticles was obtained by freeze drying.

3.2.3 Synthesis of Functionalized AgNPs

The PEGylated AgNPs were synthesized by means of direct PEGylation. PEG – 4000 (80 mM) was added to 50ml of freshly synthesized silver nanoparticles. The prepared solution was cooled down for 8hours and stored at 5°C for future use (Kumar *et al.*, 2016).

3.2.4 Characterization of AgNPs and PEGylated AgNPs

The synthesized AgNPs and PEGylated AgNPs were characterized by means of the following techniques:

3.2.4.1 Spectral analysis of biologically active AgNPs and PEGylated AgNPs

The optical property of the AgNPs was analysed by UV – visible absorption spectroscopy (Shimadzu – BioSpec – nano, Japan). A volume of 100µl of synthesized AgNPs were diluted with 900µl of distilled water and subjected to spectral analysis in the wavelength range from 220-800 nm.

3.2.4.2 Transmission Electron Microscope (TEM) with Energy Dispersive X-ray Spectroscopy (EDX)

The morphology and size of the silver nanoparticles and functionalized silver nanoparticles (PEGylated AgNPs) were examined by Hitachi 7000H, Tokyo, Japan Transmisssion electron microscopy operated at an accelerating voltage of 120 KV. The colloidal dispersion of the samples were prepared and placed on a carbon coated copper grid and evaporated under vacuum conditions. The TEM images were obtained and the energy dispersive X-ray analysis (TECNAI F30, Genesis Rev.3.0 software) was performed to determine the composition of the synthesized AgNPs and PEGylated AgNPs.

3.2.4.3 X-ray Diffraction (XRD)

The XRD is an analytical technique used to determine phase crystallinity of the material. The XRD pattern was obtained by placing the prepared samples on a glass slide and dried under hot air oven at 50°C. The samples were dried and analyzed under the XRD instrument (PAN analytical, XPERT- PRO diffractometer) with a Cu source at 1.5406 A^{o} wavelength as X - ray source in thin film mode.

3.2.4.4 Fourier Transform Infrared spectroscopy (FTIR)

The FTIR spectrum was used to identify the functional groups in the plant extract responsible for the reduction of silver ions for the synthesis of silver nanoparticles. The FTIR spectrum was recorded for the plant extract, silver nanoparticles (AgNPs) and

3.4.1 Cell Culture

EAC (Ehrlich Ascites Carcinoma) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10 per cent FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5 per cent CO_2 incubator. The viability of cells were evaluated by MTT assay method.

3.4.2 Assessment of the Viability of EAC Cells by MTT assay

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml of PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period 30µl of reconstituted MTT solution was added to EAC cell lines and samples treated in test wells. The plate was gently shaken and incubated at 37ºC in a humidified 5 per cent CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and solubilized with 100µl of MTT solution. DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm Igarashi and Miyazawa (2001).

The percentage of growth inhibition was calculated using the formula:

$$\text{Per centage viability} = \frac{\text{Mean OD of samples}}{\text{Mean OD of control}} \times 100$$

3.4.3 Microscopic Examination of EAC Cells by AO/EtBr Dual Staining Method

Approximately 1 ml of a dye mixture (100 mg/ ml acridine orange (AO) and 100 mg/ mL of ethidium bromide (EtBr) in distilled water was mixed with 9 ml of cell suspension (1×10^6 cells/ ml) on clean microscope cover slips. The EAC cells were collected, washed with phosphate buffered saline (pH 7.2) and stained with 1 ml of AO/EtBr. The cells were incubated for 2 minutes and washed twice with PBS (5 minutes each) and visualized under a fluorescence microscope (Nikon Eclipse, Inc., Japan) at 40 X magnification with an excitation filter at 480 nm (Parks *et al.*, 1979).

3.4.4 Evaluation of DNA Fragmentation in EAC cells by Agarose Gel Electrophoresis

The EAC cells were treated with ethanolic leaves extract (25 µg/ml) and PEGylated AgNPs (10 µg/ml). Treated and untreated cells were collected by centrifugation at 3000 rpm for 15 min at 4°C. The cell pellet was suspended in cell lysis buffer (Tris HCl 10 mmol/L pH 7.4, triton x-100, 0.5 per cent) and kept at 4 °C for 10 minutes. The lysate was centrifuged at 25000 rpm for 20 minutes. The supernatant was incubated with RNAase of 40 µg at 37°C for 1 hour then incubated with proteinase K 40 µg at 37 °C for 1 hour. To the final aqueous phase 40µl of 3.5 M ammonium acetate was added, to this ice cold isopropanol was added and centrifuged at 25000 rpm for 15 minutes and dried. After drying, DNA was dissolved in TE buffer and separated by 2 per cent agarose gel electrophoresis at 100 V for 50 minutes and the DNA damage was analyzed by (alpha innotech image analyzer) gel documentation (Rosarin *et al.*, 2012).

3.4.5 Analysis of Cell Cycle by Flow Cytometry

The EAC cells (2×10^5 cells/10 cm dish) were treated with the ethanolic leaves extract and PEGylated AgNPs for 24 hours. The cells were harvested by centrifugation, washed with ice-cold PBS, and then resuspended with ice-cold 70 per cent ethanol overnight. The cells were treated with 10µg/ml of RNase at 37° C, then spun down and stained with 40µg/ml of propidium iodide (PI) for 30 minutes. The DNA content was then measured by (BD facs) flow Cytometry (Krishan 1975).

In vivo Studies

PHASE V

3.5 EVALUATION OF *In vivo* ANTIOXIDATIVE AND ANTITUMORIGENIC ACTIVITY OF PEGylated AgNPs IN EAC INDUCED SWISS ALBINO MICE

In vivo antioxidative and antitumorigenic potential of PEGylated AgNPs was evaluated by the assessment of serum liver marker enzymes, enzymic, non enzymic antioxidants and lipid peroxidation in EAC induced Swiss albino mice.

3.5.1 Maintenance of Experimental Animals

Male Swiss albino mice of 5-7 weeks old (25-30g) were bought from a small animal breeding station, Thrissur, Kerala. The mice were maintained in a controlled hygienic environment at temperature 25 ± 2°C and 12hours dark/light cycle. The mice were acclimatized for 15 days before the commencement of the experiments. They were fed with standard healthy diet and water *ad libitum*. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee **(AUW: IAEC.2015.BC:03)**.

PLATE 2

Swiss albino mice

EAC cells induced mice and normal mice

3.5.2 Assessment of EC_{50} of PEGylated AgNPs by Trypan Blue Exclusion Assay

The antitumorigenic activity of PEGylated AgNPs was assessed by trypan blue dye exclusion method. The EC_{50} of PEGylated AgNPs was calculated using the intraperitoneally propagated EAC cells by the method of Salomi and Panikkar (1989) as in Appendix VIII.

3.5.3 Grouping of Animals

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

Group II (DMSO) mice received 35µl of DMSO and served as vehicle control for group V and VI.

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 in 100µl of paraffin oil.

Group V (Ethanolic extract) mice received ethanolic leaves extract of *Volkameria inermis* (EC_{50} - 38 µg in 35 µl of DMSO).

Group VI (PEGylated AgNPs) mice received PEGylated AgNPs (EC_{50} - 24 µg in 35 µl of DMSO).

Group VII (EAC) received one acute dose of 1×10^6 EAC cells (i.p) in 100 µl of PBS that served as EAC control.

Group VIII (EAC+ Ethanolic extract) and **group IX (EAC+ PEGylated AgNPs)**, received one acute dose of $1x10^6$ EAC cells in 100 µl of PBS on the first day of the experimental period and also EC_{50} dose of ethanolic leaves extract and PEGylated AgNPs throughout the experimental tenure.

The experiments were carried out for 15 and 60 days. At the end of the study the mice were sacrificed and the liver and blood was taken. In the serum the activity of liver marker enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were assessed. In the liver homogenate the enzymic and non enzymic antioxidants were assessed. The histological examination of the liver of all the experimental mice was also carried out.

3.5.4 Evaluation of PEGylated AgNPs on the Mortality Rate of EAC Cells Induced Swiss albino Mice

The increase in life span of Swiss albino mice transplanted with EAC cells was studied by treating the animal with EC_{50} of ethanolic leaves extract and PEGylated AgNPs. 1×10^6 EAC cells were intraperitoneally administered to the control group. Administration of ethanolic leaves extract and PEGylated AgNPs to the experimental groups was repeated for 60 days. The mortality rate of animals dying with tumor and the average life span was noted (Geran *et al.*, 1972).

3.5.5 Evaluation of PEGylated AgNPs on the Activities of Liver Marker Enzymes in Serum of EAC Challenged Swiss albino Mice

Liver plays a major role in detoxification and excretion of many exogenous and endogenous compounds. To assess the normal functioning of the liver treated with ethanolic extract and PEGylated AgNPs in the presence and absence of EAC cells, selected liver marker enzymes such as AST, ALT and ALP were determined.

3.5.5.1 Aspartate transaminase (AST, EC. 2.6.1.1)

Aspartate transaminase (AST) was assayed by the method of Reitman and Frankel (1957) as in Appendix IX.

3.5.5.2 Alanine transaminase (ALT, EC. 2.6.1.2)

Alanine transaminase (ALT) was assayed by the method of Reitman and Frankel (1957) as in Appendix X.

3.5.5.3 Alkaline phosphatase (ALP, EC. 3.1.31)

Alkaline phosphatase (ALP) was assayed by the method of King (1965) as in Appendix XI.

3.5.6 Evaluation of PEGylated AgNPs on the Activities of Enzymic Antioxidants

The activities of enzymic antioxidants like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were analyzed in the liver homogenate.

3.5.6.1 Estimation of Catalase (CAT, EC.1.11.1.6)

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

3.5.6.2 Estimation of Superoxide dismutase (SOD, EC.1.15.1.1)

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

3.5.6.3 Estimation of Glutathione peroxidase (GPx, EC.1.6.4.2)

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

3.5.7 Evaluation of PEGylated AgNPs on the Levels of Non Enzymic Antioxidants

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

3.5.7.1 Estimation of Vitamin A

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

3.5.7.2 Estimation of Vitamin E

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

3.5.7.3 Estimation of Reduced glutathione (GSH)

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

3.5.8 Evaluation of PEGylated AgNPs on the Rate of Lipid Peroxidation

The levels of MDA in the liver were determined by the method of Nichans and Samuelson (1968) as given in Appendix XVIII.

3.5.9 Evaluation of PEGylated AgNPs on the Histological Status of the Liver of Control and EAC Cells Challenged 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 per cent 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 XIX.

Statistical analysis

The data presented here are mean ± standard deviation of six animals in each group. Significant difference between the means of the six animals was statistically analyzed by one way and two way analysis of variance (ANOVA) and Duncans multiple

range test (DMRT). The significance level was set at $P < 0.05$ for all the tests. Statistical analysis was carried out using WASP 2.0 (www.ccari.res.in / wasp2.0/ index.php).

The results obtained for the various parameters analyzed and the findings were discussed in the light of relevant literature in the next chapter.