3

METHODOLOGY

3.1 Research Design

The current study aims to synthesize AgNPs and AgNP loaded Liposomes and to analyze their drug release profile, antioxidant and anticancer potential using acute lymphoblastic leukaemia cells (MOLT-3) and peripheral blood lymphocytes (Normal cells). The study was designed into four different phases. In phase I, an attempt was made to synthesize and characterize Silver nanoparticles and Silver nanoparticles loaded Liposomes from *Tabebuia pallida* leaf extract. In Phase II, evaluation of Drug Release profile of Silver Nanoparticles Loaded Liposomes and their validation using various mathematical models was done. In Phase III, The Free radical scavenging Potential of Silver nanoparticles and Silver nanoparticles loaded Liposomes of *Tabebuia pallida* was evaluated. Fourth phase was designed to analyse Anticancer potential of Silver nanoparticles and Silver nanoparticles loaded Liposomes of *Tabebuia pallida* against Molt-3 cells.

Phase I

3.2 Synthesis and Characterisation of Silver nanoparticles and Silver nanoparticles loaded Liposomes from *Tabebuia pallida* leaf extract

3.2.1 Collection of plant species

The Leaf sample of *Tabeubia pallida* was collected from a place near Avinashi of Tirupur District, Tamil Nadu. The leaves were washed 2-3 times with tap water. Then the leaves were dried under shade and roughly powdered and stored in well sealed for to carry out further processing in laboratory.

3.2.2 Authentication of Plant species

The plant was authenticated by the Botanical Survey of India [BSI], Southern Circle, Coimbatore, India. Authentication no: BSI/SRC/5/23/2019/Tech./193.

3.2.3 Extraction of the Plant Materials

Using Soxhlet apparatus, the various solvent extracts of rougly grounded powder (10g) of the *T. pallida* leaves were prepared. The solvents used for the extraction of phytoconstituents are ethanol and water in different proportions like ethanol 20: water 80, ethanol 80: water 20, ethanol 40: water 60, ethanol 60: water 40, and ethanol 50: water 50 for synthesizing the silver nanoparticles.

3.2.4 Sunlight mediated green synthesis of silver nanoparticles

Sunlight meadiated green synthesis of *T. pallida* silver nanoparticles was carried out as reported by Mounil *et al.,* (2020) and Rautela *et al.,* (2019) as given in Appendix I.

3.2.5 Characterisation of green synthesized *Tabebuia pallida* silver nanoparticles

3.2.5.1 UV-Visible spectroscopy

UV-Visible spectroscopy is mainly used to record the optical absorption spectra of the synthesized silver nanoparticles by dissolving it in DMSO by sonication. DMSO alone serves as a blank. The particle solution was measured in UV-Visible nanospectrophotometer (Shimadzu Biospec nano) with an absorption range of 200-800nm.

3.2.5.2 Energy dispersive X-ray analysis

The synthesized silver nanoparticles were analyzed for its elemental composition using EDAX APEX which leads to the formation of peaks that corresponds to the elemental composition of the sample.

3.2.5.3 X-Ray Diffraction

The crystalline nature of the synthesized silver nanoparticles was examined using X-ray Diffraction. X'pert Pro X-ray diffractometer is the instrument used for X-ray diffraction study. **3.2.5.4 Preparation of silver nanoparticles loaded liposomes**

The silver nanoparticles encapsulated liposomes were developed by thin-film hydration method as described by Fathy *et al.,* (2019) as given in appendix II.

3.2.5.5 Encapsulation efficiency of silver nanoparticles loaded liposomes

The silver nanoparticle encapsulation efficiency was determined using the indirect spectrophotometric method described by Nayyer *et al.,* (2019) as given in appendix III

3.2.5.6 FTIR Spectroscopy

The functional group present in the synthesized silver nanoparticles and silver nanoparticles loaded liposomes were analysed using SHIMADZU, FTIR spectroscopy- miracle 10, and was scanned within a range of 500-4000cm^{-1}.

3.2.5.7 Field Emission Scanning Electron Microscope

FESEM is a main tool for the visualization of topographic detail of Nanoparticles. The surface morphology of the silver nanoparticles and silver nanoparticles loaded liposomes were analyzed using FESEM (MIRA 3 TESCAN). The samples were sputtered using gold, dried and analysed.

3.2.5.8 Dynamic Light Scattering

To determine the Polydispersity index and hydrodynamic size the Dynamic Light Scattering analysis was done. The instrument used for the analysis was Malvern Panalytical, Zetasizer Ver. 7.13.

3.2.5.9 Zeta potential

To ascertain the stability of the synthesized silver nanoparticles and silver nanoparticles loaded liposomes of *Tabebuia pallida* Zeta potential analysis was carried out. The analysis was performed using Malvern Panalytical, Zetasizer Ver. 7.13.

Phase II

In phase II, the *in vitro* drug release analysis of silver nanoparticles loaded liposomes was done which was validated using various mathematical models of drug release.

3.3.1 *in vitro* drug release study

The *in vitro* release of silver nanoparticles from the liposomes was analyzed using three different pH; pH 5.5, pH 6.8 and pH 7.4, and decribed by Kavithaa *et al.*, (2016) as given in the appendix IV.

3.3.2 Application of *in vitro* drug release data on mathematical models:

The *in vitro* drug release results of silver nanoparticles encapsulated liposomes are correlated with various drug release kinetic models like Zero order model (Dash *et al.,* 2010), First order model (Dash *et al.*, 2010), Higuchi model (Subal, 2006), Korsmeyer and peppas model (Singhvi and Singh, 2011) and Hixson and crowell model (Singhvi and Singh, 2011) as given in appendix V.

Phase-III

3.4 Free radical scavenging assays

In phase III, the free radical scavenging potential of Hydroethanolic extract, silver nanoparticles and silver nanoparticles loaded liposomes of *Tabebuia pallida* was done.

3.4.1 DPPH Radical Scavenging Assay

DPPH radical scavenging ability of the synthesized silver nanoparticles and silver nanoparticles loaded liposomes was assessed according to the method described by Brand-Williams *et al.,* (1995) as given in Appendix VI.

3.4.2 ABTS radical scavenging assay

The free radical scavenging activity of the samples was determined using ABTS radical scavenging assay as reported by Shirwaikar *et al.,* (2006) as given in Appendix VII.

3.4.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging potential of AgNPs and AgNP loaded liposomes was analyzed according to Klein *et al.,* (1991) as given in Appendix VIII.

3.4.4 Hydrogen peroxide scavenging activity

The ability of the AgNPs and AgNP loaded liposomes to scavenge hydrogen peroxide was tested as stated by Ruch *et al.,* (1989) as given in Appendix IX.

3.4.5 Reducing Power assay

The reducing abilty of the synthesized AgNPs and AgNP loaded liposomes was analyzed according to the method of Yıldırım *et al.,* (2001) as given in Appendix X.

3.4.6 Nitric oxide radical scavenging assay

Nitric oxide scavenging potential of AgNPs and AgNP loaded liposomeswas carried out as described by Shirwaikar and Somashekar (2003) and Sreejayan and Rao (1997) as given in Appendix XI.

Phase-IV

In phase IV, the Anticancer potential of AgNPs of *T. pallida* leaves and their Nanoliposomes were evaluated using Molt-3 leukaemic cells and Peripheral Blood Lymphocytes.

3.5.1 Culturing of Molt-3 cells

The Molt-3 cells were purchased from National Centre for Cell Science (NCCS), Pune, India. RPMI-1640 was the medium used for the culturing of Molt-3 cells with 10% Fetal Bovine Serum and 0.5% penicillin streptomycin. The flasks were incubated at 37ºC with 5% CO_2 in a CO_2 incubator.

3.5.2 Separation and Culturing of Peripheral Blood Lymphocytes (PBL)

Fresh blood was collected from a healthy individual under sterile condition by vein puncture (Ethical clearance no: AUW/IHEC/BC-16- 17/XMT-01). The blood was then heparinized to prevent it from clotting. The heparinized blood was then subjected to dilution using PBS in the ratio of 1:1. This diluted blood was added to a centrifuge tube containing lymphosep in the ratio 1:2. The heparinized blood along with lymphosep was subjected to centrifugation at 400 g for 30 mins at a temperature of 18-20ºC. After 30 mins of centrifugation the lymphocytes were separated as a grey coloured layer at the top of the centrifuge tube. This lymphocytes layer was carefully separated without disturbing the remaining contents. Thus the obtained lymphocytes were washed using phosphate buffered saline thrice. Then the cells were subjected to centrifugation to obtain pellet form. These pellets were then resuspended in RPMI 1640 medium with 10% Fetal Bovine Serum and 0.5% penicillin streptomycin and phytohemagglutinin. The flasks were incubated at 37ºC with 5% CO_2 in a CO_2 incubator.

3.5.3 Treatment groups

1. Cells alone
2. Cells + hydroethanolic extract of *Tabebuia pallida*
3. Cells + silver nanoparticles of *Tabebuia pallida*
4. Cells + silver nanoparticles loaded liposomes of *Tabebuia pallida*

3.5.4 MTT Dye Reduction Assay

Cell viability was evaluated by the reduction of MTT dye was analyzed as described by Igarashi and Miyazawa (2001) as given in the Appendix XII.

3.5.5 Sulphorhodamine B assay

The cytotoxic effect exerted towards the Molt-3 cell line and PBL by the hydroethanolic extract, silver nanoparticles and silver nanoparticles loaded liposomes was studied by the SRB assay as elucidated by Skehan *et al.,* (1990) as given in Appendix XIII.

3.5.6 Measurement of Apoptosis- Annexin V/FITC staining

The Apototic and necrotic cell death caused by the AgNPs and AgNP loaded liposomes was detected using Annexin V/FITC-PI staining using flow cytometry as presented in Appendix XIV.

3.5.7 Measurement of Mitochondrial Membrane Potential (ΔΨ) by JC-1 staining

The mitochondrial membrane potential was detected using flow cytometry as outlined in Appendix XV.

3.5.8 Cell Cycle analysis

Cell cycle analysis was performed as per the protocol explained by Krishan (1975) using flow cytometry as given in Appendix XVI

3.6 Statistical analysis:

In vitro drug release analysis, Free radical scavenging and cytotoxic assays were performed in triplicates and the values are expressed as mean ± SD. Statistical significance of the data was analyzed using one-way ANOVA by using Microsoft Excel, 2007 and P value < 0.05 are considered significant.