3

METHODOLOGY

Natural sources have provided an abundance of therapeutic plants, and a remarkable number of new drugs have been identified from them. Medicinal plants have been proven to be an important source of therapeutic medications and the therapeutic potential of these plants is owing to a variety of chemical substances, primarily secondary metabolites (Varkey and Kasthuri, 2016). The popularity of herbal drugs in global health care has increased due to its noteworthy efficacy in the treatment of various ailments (Dharmapal *et al.,* 2018).

The present study intends to identify the bioactive components present in the ethanolic extract of *T. roseo-alba* and to assess the antioxidant capacity with the focus on the anticancer activity on A549 lung cancer cell lines. The designed study was planned into four phases. In phase I, bio active phyto constituents present in the ethanolic extract of *T. roseo-alba* were screened and *in vitro* antioxidative potential of *T. roseo-alba* was assessed. In the second phase, an attempt was made to synthesis and characterize the silver nanoparticles of *T. roseo-alba.* Third phase was designed to analyze antiproliferative and apoptotic inducing potential of the ethanolic extract of *T. roseo-alba* and their AgNps in A549 lung cancer cell lines. In phase IV, molecular docking studies were performed to find the interactions between the bioactive compounds present in *T. roseo-alba* and apoptotic and lung cancer targets using Autodock.

Phase I

3.1 Phytochemical characterization and Assessment of Free radical scavenging potential of ethanolic extract of *T. roseo-alba*

Collection of Plant Sample

Fresh leaves of *T. roseo-alba* were collected in the campus of P.S.G College of Arts and Science, Coimbatore district and authenticated at Botanical Survey, Tamil Nadu Agriculture University (TNAU) Coimbatore, India (BSI/SRC/5/23/2019/Tech/3236).

3.1.1 Phytochemical screening of *Tabebuia roseo- alba*

Different solvent extracts (chloroform, petroleum ether, ethanol and water) were employed to analyse the presence of bioactive metabolites such as phenols reducing sugars, alkaloids, saponins, tannins, flavonoids, terpenoids and steroids. The phytochemical analysis of the various solvent leaf extracts of *T. roseo-alba* was performed by following the method of Raaman (2006) as given in Appendix I.

3.1.2 Quantification of Phytochemicals

The amount of protein present in the plant material was estimated by the method of Lowry *et al.* (1951), total carbohydrate content was estimated by the method of Hedge and Hefreiter, (1962), total phenol content was estimated by the method of Malik and Singh, (1980), flavonoids were analysed by the method of Zhishen *et al.* (1999), and tannins were estimated according to the method given by (Van Buren and Robinson, 1969) and are given in Appendices II-VI.

3.1.3 HPTLC fingerprinting analysis of ethanolic extract of *T. roseo- alba* leaves

HPTLC fingerprinting profiling of ethanolic extract of *T. roseo- alba* leaves was performed by following the procedure as described in Appendix VII.

3.1.4 GC-MS Profiling

GC MS analysis of ethanolic extract of *T. roseo- alba* leaves was performed by following the procedure as described in Appendix VIII.

3.1.5 Assessment of *in vitro* antioxidative role of *T. roseo-alba* leaves

3.1.5.1 DPPH radical scavenging assay

DPPH radical scavenging activity was assessed based on the method of Blois, (1958) as given in Appendix IX

3.1.5.2 $ABTS^+$ radical scavenging activity

$ABTS^+$ radical scavenging activity was assessed by the method of Re *et al.* (1999) as described in Appendix X

3.1.5.3 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured based on the method of Klein *et al.* (1991) as in Appendix XI

3.1.5.4 Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging activity was determined according to the method described by Ruch *et al*. (1989) as in Appendix XII.

3.1.5.5 Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined according to the method described by Green *et al.* (1982) as given in Appendix XIII.

3.1.5.6 Superoxide radical scavenging assay

Superoxide radical scavenging activity was determined by the method of Liu *et al.* (1997) as elaborated in Appendix XIV.

3.1.5.7 Reducing power assay

Reducing power assay capacity was assessed according to the method described by Oyaizu, (1986) as in Appendix XV

Phase II

3.2 Synthesis and Characterization of the AgNPs of *T. roseo-alba*

This phase involved the Soxhlet extraction of ethanolic extract of *T. roseo-alba,* synthesis of AgNPs and the characterization of AgNPs of *T. roseo-alba*.

Preparation of Ethanolic leaf extract

The fresh *T. roseo-alba* leaves were air dried for the period of 2 to 3 weeks at room temperature, powdered and extracted with ethanol using Soxhlet apparatus. The collected extract was then filtered using muslin cloth and evaporated under reduced pressure. The collected residue was stored in air tight containers at 4°C and used for further experiments.

3.2.1 Synthesis of AgNPs of *T. roseo-alba*

3.2.1.1 Preparation of silver nitrate solution

For the preparation of the 1mM $AgNO_3$ solution, accurately weighed 16.987 mg of $AgNO_3$ was dissolved in 100 ml of double distilled water and mixed thoroughly. It was **p**reserved in the brown bottle till further use.

3.2.1.2 Synthesis of silver nanoparticles

In a sterile conical flask, 90 ml of 1mM silver nitrate solution was added to 10 ml of ethanolic plant extract. The solution was mixed thoroughly and continuously stirred with a magnetic stirrer at 800rpm for 30 minutes (Rautela *et al.,* 2019). The silver nanoparticles formation was indicated by the change in colour of the solution from light yellow to dark brown which may be because of the reduction of silver ions by the ethanolic plant extract.

3.2.1.3 Lyophilization of sample

The synthesized silver nanoparticle solution was then centrifuged at 15,000 rpm for 15 mins and the pellet was collected. It was washed twice with distilled water and lyophilized. The lyophilized sample was used for XRD and FTIR analysis.

3.2.2 Characterization of crude extract and AgNPs of *T. roseo-alba*

The AgNPs of *T. roseo-alba* were characterized by adopting various parameters as given below.

3.2.2.1 UV-Visible Spectroscopy

Using UV-vis spectroscopy, preliminary characterization was carried out. The bio reduction of Ag+ ions was examined by the measurement of UV–Vis spectra using 0.5 ml of the diluted aliquot with deionized water as a reference. UV–Vis spectroscopic analysis of the biosynthesized silver nanoparticles were carried out using UV–Vis spectrometer (Shimadzu Biospec Nano) in the range of wavelength from 300 to 800 nm. The presence and reduction of silver ions was indicated by a peak of absorption in the 300 to 500 nm range (Yasmin *et al.,* 2020).

3.2.2.2 Fourier Transform Infrared Analysis

The functional groups of the phytochemicals involved in the bioreduction of Ag ions and subsequent capping and stabilisation of biosynthesized silver nanoparticles were determined using FTIR analysis (Huq *et al.,* 2020). It was carried out by using Shimadzu 8400S Fourier-transform infrared spectrometer operated at a resolution of 4 cm−1 in the range of 400–4000 cm−1 (Femi-Adepoju *et al.,* 2019).

3.2.2.3 Dynamic light scattering

Using DLS (Zetasizer Nano ZS, ZEN 3600, Malvern, UK), size distribution and Zeta potential of bio-reduced AgNPs were assessed. This equipment can measure particle size distributions in the range of 2 nm to 3 nm at 25˚C.

3.2.2.4 X-RAY Diffraction analysis

X-ray diffraction spectroscopy, reveals the crystalline structure, size, and shape of the unit cell and lattice parameters. The characterization of purified $AgNO_3$ was carried out with X-Pert Pro PANalytical diffractometer working at 40 kV voltage and 30 mA current with cu kα radiation in 2θ configurations. The average crystallite domain size D was calculated from the diffraction pattern by using the Debye Scherer's formula, which can be given as

$$D = \frac{0.94}{\beta \cos \theta}$$

Where the λ is the X-ray wavelength used for diffraction, β is the full width at of maximum (FWHM) and θ is the diffraction angle (Metha *et al.,* 2017).

3.2.2.5 Scanning Electron Microscope with Energy Dispersive Spectroscopy

TESCAN-MIRA3 XMU coupled with energy dispersive X-ray spectrometer was used to study the morphology and elemental analysis of biosynthesized silver nanoparticles. The lyophilized silver nanoparticles were sonicated using distilled water and drop coating of the nanoparticle suspension on glass slides was made and it was dried under ambient temperature. The energy dispersive X-ray spectroscopy attached with the SEM was used to perform compositional analysis on the sample and the existence of elemental silver was confirmed through EDS (Umoren *et al.,* 2014).

3.2.2.6 Transmission Electron Microscopy

TEM was used to measure the size and shape of the silver nanoparticles and to perform morphological examination of the nanoparticles**.** The TEM micrograph images were captured on the JEOL 1200 EX instrument, operated at 100 to 200 kV voltage. The high resolution images were captured and SAED pattern was also observed (Elamawi *et al.,* 2018).

Phase III

3.3 Antiproliferative and apoptotic activity of crude extract and AgNPs of *T. roseo-alba*

Cancer has been a constant battle around the world, with many advances in treatment and prevention. It is characterized by continually proliferating cells in the human body that are unable to be controlled, resulting in malignant cell tumours with the potential to spread to other parts of the body. Current therapies, including chemotherapy, radiation therapy, and chemically derived medications, may impose a lot of pressure on patients and further harm their wellbeing. Hence, a greater emphasis on alternative cancer treatments and therapies is required (Padmaharish and Lakshmi, 2017).

In the current study, the antiproliferative activity and apoptotic activity of ethanolic extract of *T. roseo-alba* and their AgNPs in Lung cancer cell line was elucidated.

Culturing of lung cancer cell line

Lung cancer cell line A549, was purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured by using DMEM medium which is supplemented with 10% FBS, 0.5% penicillin- streptomycin, and 1mM sodium pyruvate and incubated at 37˚C.

Treatment groups

1. Cells alone
2. Cells + Ethanolic extract of *T. roseo-alba*
3. Cells + AgNPs of *T. roseo-alba*

Cell viability assays

In order to ascertain the optimal dose, antiproliferative and cytotoxic effect of the ethanolic extract of *T. roseo-alba* and their AgNPs, MTT assay was performed.

3.3.1 MTT dye reduction assay

Viability of the cells were examined by the reduction of 3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) as given by Igarashi and Miyazawa, (2001) Appendix XVI.

3.3.2 Measurement of Apoptosis

3.3.2.1 Analysis of Apoptosis by Annexin V/FITC staining

The magnitude of apoptosis elucidated by ethanolic extract of *T. roseo-alba* and their AgNPs was determined using Annexin V/FITC Apoptosis detection kit (BD Biosciences) as given in the Appendix XVII.

3.3.2.2 Analysis of Mitochondrial Membrane Potential by JC-1 staining

The Apoptotic potential of ethanolic extract of *T. roseo-alba* and their AgNPs was also determined by JC-1 staining kit procedure as given in the Appendix XVIII.

3.3.2.3 Analysis of DNA Fragmentation

The DNA fragmentation analysis was carried out by following the procedure as described in Appendix XIX.

3.3.2.4 Western blotting analysis

The regulation of apoptotic protein (caspase-3) in treated cells was evaluated using Western blotting as given in Appendix XX.

3.3.3 Analysis of cell cycle

The effect of ethanolic extract of *T. roseo-alba* and their AgNPs on the cell cycle distribution of A549 cells were examined using flow cytometer kit method as given in Appendix XXI.

PHASE IV

3.4 In silico docking of protein targets with active components of *T. roseo-alba* leaves

Phytochemicals derived from plants appear to be effective agents in the development of new drugs. The analysis of a variety of bioactive phytochemicals as powerful carcinogenic blockers was possible with *in silico* investigations using online and offline drug design programmes such as Auto dock and Schrodinger. In addition, phytochemical components can serve as drugs of the new generation as well as a mixture of inhibitors along with existing drugs to manage cancer and other infectious diseases with minimum risk factors (Umesh *et al.,* 2020).

Molecular docking has been shown to be a very effective approach for discovering new drugs that target proteins (Pinzi *et al.,* 2019). Therefore, the current study is aimed at *in silico* docking analysis on selected phytochemicals from *T. roseo-alba* as lead compounds in order to explore their binding efficiencies to the modelled apoptotic and anticancer proteins, thereby providing leads for next-generation cancer therapies.

3.4.1 Retrieval of Ligand

The phytochemicals present in the leaves of *T. roseo-alba* were identified by the chromatographic techniques, which were considered as the ligand molecules for the *in silico* studies against the anti-apoptotic and onco-protein targets. The 3D structures were retrieved in SDF format and were changed to PDB format using OpenBabel-2.4.1. The pre-clinical inhibitors were used as the controls which were also retrieved from the PubChem database. Gasteiger charges were assigned to the ligands, for merging the nonpolar hydrogens using Autodock Tools 4.2.6 and the equipped ligands were saved in PDBQT format (Shaji *et al.,* 2018).

3.4.2 Retrieval of Target

The 3-D X-ray crystal protein structures of Apoptotic and lung cancer targets were retrieved in PDB format from the RCSB PDB. To carry out docking analysis, all water molecules were removed to facilitate the interaction of only inhibitors or ligands with the selected receptor and polar hydrogen atoms were included in the refined model using AutoDock Tools (ADT). Moreover, based on the computation of Kollman charges, protein was prepared with the addition of polar hydrogens using Autodock Tools 4.2.6. The equipped protein was saved in PDBQT format (Shaji *et al.,* 2018).

3.4.3 Active sites Prediction in the modelled protein

In order to predict the active sites in the modelled protein, Computed Atlas of Surface Topography of Proteins (CASTp) 3.049 programme was employed. It is a web-based tool for detecting and measuring voids in three-dimensional protein structures. Following the submission of the modelled 3D protein to the server, the required amino acids for binding interactions were determined.

3.4.4 Predictions of *in silico* drug-likeness and toxicity

Drug-likeness specifies whether there are properties associated with the chance for a specific pharmacological agent to develop as an orally dynamic drug. This analysis

is based on the proven principle called the Lipinski rule of five by Lipinski *et al*. (2012). When a chemical has more than 5 H-bond donors, a molecular weight larger than 500 *daltan,* 10H-bond acceptors and a measured LogP (CLogP) greater than5, the rule predicts poor absorption or penetration. The criterion known as drug score is also used to select molecules as drug candidates. The *in silico* drug-likeness and toxicity predictions of the proposed ligands were evaluated using the Swiss ADME predictor. The compounds' analyses were compared to those of a reference drug, and only compounds that satisfied all of the screenings were employed in the molecular docking analysis (Oduselu *et al.,* 2019).

3.4.5 Molecular Docking Analysis

AutoDock Tools, a free Graphic User Interface (GUI) for the AutoDock 4.2.6 software, was used to perform the molecular docking research. The amino acids were chosen based on the CASTp results and the resemblance of the simulated 3D structure to the template structure. Furthermore, the docking study was performed using Lamarckian Genetic Algorithm 4.2. Grid maps have been developed and spacing has been modified to 0.8 Å to allow binding of ligands. The number of runs of the genetic algorithm was fixed at 10, and the further docking factors were left at their default values. For every ligand scored using AutoDock 4.2.6, ten different conformations were produced and scoring functions were graded based on their binding energies. For the post-docking analysis AutoDock Tools 4.2.6 and PyMOL were used (Oduselu *et al.,* 2019).

Statistical Analysis

All the radical scavenging and cytotoxic assays were performed in triplicates (n = 3) and values are expressed as mean ± SD.