3.0 MATERIALS AND METHODS

The experimental procedure for the present topic entitled **"Evaluation of Thrombolytic and Antioxidant Potential of *Moringa pterygosperma* Gaertn."** is discussed under the following headings:

3.1. COLLECTION AND IDENTIFICATION OF THE PLANT SAMPLE

The leaves and flowers of *Moringa pterygosperma* Gaertn. were collected from Coimbatore city, Tamilnadu. Taxonomical identification was made by the taxonomist of Botanical Survey of India, Southern circle, Tamilnadu Agricultural University, Coimbatore. No. BSI/SRC/5/23/2015-16/Tech./1585.

3.2. PREPARATION OF THE PLANT EXTRACTS

3.2.1. Preparation of the extract for phytochemical analysis and Thrombolysis

The aqueous, ethanol and petroleum ether extracts of leaves and flowers of *Moringa pterygosperma* Gaertn. were prepared at a concentration of 500 mg/ml and analyzed for phytochemicals and thrombolytic activity.

3.2.3. Preparation of alkaloid extract

Fresh leaves and flowers of *Moringa pterygosperma* Gaertn. (5g each) were crushed in a mortar and pestle with 10% acetic acid in ethanol (200ml) and incubated for 4 hours in the dark. After incubation, the extract was filtered and the solution was concentrated to ¼ th volume in a boiling water bath. To the extract, 25% ammonium hydroxide or 25% ammonia was added until a precipitate was formed and then centrifuged at 2500 rpm for 5 minutes. The residue obtained was washed with 1% NH_4OH and filtered. The residue that contained alkaloids was then weighed, dissolved in ethanol and stored at 4°C.

3.2.4. Preparation of phenolic extract

Leaf and flower samples (1g) were taken and crushed using a mortar and pestle sparately. To the crushed sample, 20ml of 80% ethanol was added and transferred to the conical flask. The conical flask was plugged and placed in a boiling water bath for 15 minutes with occasional shaking. The content was then centrifuged and the supernatant thus collected was the phenolic extract.

3.2.5. Preparation of flavonoid extract

Approximately half the volume of the phenolic fraction was transferred to a 50ml separating funnel. The sample was then extracted with petroleum ether (40-60°C). The aqueous layer thus obtained was the flavonoid extract.

3.3 Qualitative analysis of phytoconstitutents

The aqueous, ethanolic and petroleum ether extracts of leaves and flowers of *Moringa pterygosperma* Gaertn. were screened for the presence of phytochemicals, according to the method proposed by Raaman (2006) and the details are given appendix I.

3.4. QUANTITATIVE ANALYSIS OF PHYTOCONSTITUENTS

3.4.1 Estimation of carbohydrate

The method of Anthrone *et al.,* 1999 was followed for determination of carbohydrate and the procedure is stated in appendix II.

3.4.2 Estimation of protein

The method of Lowry *et al.,* 1951 was used to determine the total protein and the procedure is given in appendix III.

3.4.3 Estimation of flavonoids

The flavonoid content of the sample was estimated by the method of Zhishen *et al.,* 1999 and it is described in appendix IV.

3.4.4 Estimation of total phenols

The method of Malick and Singh, 1980 was followed for determination of total phenol and the procedure is stated in appendix V.

3.4.5 Estimation of alkaloids

The method of Muthumani *et al.,* 2010 was followed for determination of alkaloids and the procedure is explained in appendix VI.

3.5 HPTLC PROFILING

The methanolic extract of leaves and flowers of *Moringa pterygosperma* Gaertn. were subjected to HPTLC for the screening of phytochemicals by the method of Wagner *et al.,* 1996. The procedure is given in appendix VII.

3.6 DETERMINATION OF ANTIOXIDANTS:

3.6.1 ASSESSMENT OF TOTAL ANTIOXIDANT CAPACITY

Moringa pterygosperma Gaertn. leaves and flowers were analysed for the total antioxidant capacity by the method of Prieto *et al.*, 1999 as explained in appendix VIII.

3.6.2 ENZYMIC ANTIOXIDANTS

The samples were analysed for the presence of enzymic antioxidants such as catalase, superoxide dismutase, polyphenol oxidase and peroxidase.

Catalase:

The activity of catalase was estimated by the method of Luck *et al.*, 1974. The procedure is given in appendix IX.

Superoxide dismutase:

Assay of superoxide dismutase was carried out by the method of Misra and Fridovich, 1972 as explained in appendix X.

Polyphenol oxidase:

The method of Esterbauer *et al.*, 1977 was followed for the determination of polyphenol oxidase as stated in appendix XI.

Peroxidase:

The peroxidase activity of the samples was estimated by the method of Reddy *et al.*, 1995 as described in appendix XII.

3.6.3 NON-ENZYMIC ANTIOXIDANTS:

The analysis of non-enzymic antioxidants included ascorbic acid, α-tocopherol and reduced glutathione was carried out as described below.

Ascorbic acid:

Ascorbic acid was estimated by the method of Roe and Keüther, 1953. The procedure is explained in appendix XIII.

α-Tocopherol:

α-tocopherol was estimated by the method Rosenberg *et al.*, 1992. The procedure is stated in appendix XIV.

Reduced glutathione:

Reduced glutathione was assessed by the method of Moron *et al.*, 1979 which is described in appendix XV.

3.7 THROMBOLYTIC ACTIVITY

Thrombolytic activity of the plant extracts was determined using human blood samples by the method of Prasad *et al.,* 2007. The procedure followed is given in appendix XVI. All the experimental procedures were initiated after reviewed by the Institutional Human Ethics Committee (IHEC/14-15/XPD-07).

3.7.1 ESTIMATION OF TOTAL CHOLESTEROL

Total cholesterol was estimated in serum obtained from human blood used for the determination of thrombolytic activity by kit method (Allian *et al.,* 1974). The details are given in appendix XVII.

3.8 STATISTICAL ANALYSIS

The significance between clot lysis and plant extracts by means of weight difference was tested by ANOVA. Serum cholesterol level and clot lysis were compared by correlation analysis.

3.9 *IN SILICO* STUDIES

The methodology on molecular docking studies of ligand such as IXa, Xa, plasmin and thrombin were discussed under the following headings:

1) PubChem

2) Protein data bank

3) Selection of target proteins

4) Ligand

5) Preparation of target protein structure

6) Docking analysis using Maestro

6.1 Maestro

6.2 QikProp 3.0

6.3 LigPrep 2.1

6.4 GLIDE

6.5 Docking

1. PubChem

The PubChem Compound Database contains validated chemical depiction information provided to describe substance in PubChem substance. Structures stored within pubchem compounds are pre-clustered and cross referenced by identity and similarity groups. Pubchem also provides a fast chemical structure similarity search tool. Ligand structure was retrieved from the PubChem Compound Database.

2. Protein Data Bank

The Protein Data Bank (PDB) is a repository for the 3D structural data of large biological molecules, including proteins and nucleic acids. The structure of Tissue factors IXa, Xa, plasmin, thrombin and plasminogen was retrieved from Protein Data Bank.

3. Selection of target proteins

Blood coagulation is initiated when a vessel wall is damaged, exposing tissue factor (TF) to the circulating factor VII/factor VIIa (FVII/FVIIa) which results in the formation of the TF:FVIIa complex and thereby the initiation of blood coagulation. One of the substrates for the TF:FVIIa complex is factor X (FX), which is activated to factor Xa (FXa), subsequently leading to a series of reactions resulting in clot formation. The fact that TF is the main initiator of the coagulation makes this an interesting protein to study, in the hunt for means to interfere with players involved in the blood clotting process. (Carlsson, 2010). Thrombin formed blood clot from fibrinogen and is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator (tPA) (Ali *et al.*, 2013).

4. Ligand

LigPrep is a robust collection of tools designed to prepare high quality, all atom 3D structures for large numbers of drug-like molecules, starting with 2D or 3D structures in SD or Maestro format. The resulting structure of ligand can be saved in either SD or Maestro format.

5. Preparation of target protein structure

The protein preparation wizard panel allows to take a protein from its raw state to a state in which it is properly prepared for use by Schrodinger.

6. Docking analysis using Maestro

6.1 Mastero

Mastero is the graphical user interface for all of Schrodinger products as Combi Glide, Epik, Glide, Impact, Liasion, LigPrep, Phase, Maestro model, Prime, QikProp, Qsite. Maestro is a powerful and versatile molecular modeling environment and the portal to the most advanced science in computational chemistry.

6.2 QikProp 3.0

Qikprop 3.0 module of Schrodinger is a quick, accurate, easy to use absorption, distribution, metabolism and excretion (ADME) prediction program. It predicts physically significant descriptor and pharmaceutically relevant properties of organic molecules. The ADME properties of selected ligands was detected by Qikprop.

6.3 LigPrep 2.1

The preparation of the ligand was done using LigPrep 2.1, a module on the Maestro window of Schrodinger is a robust quality, all atom 3D structures for large number of drug like molecules, starting with 2D or 3D structure in Maestro format. The target proteins namely IXa, Xa, plasmin and thrombin were subjected to ligPrep. The output file is produced with extension "_-out.mae.

6.4 GLIDE (Grid based ligand docking with energetics)

Glide searches for favorable interactions between one or more ligand molecules and a receptor molecule usually a protein. The ligand poses that are generated by GLIDE pass through a series of hierarchical filters that evaluate the ligand interaction with the receptor.

6.5 Docking

The ligand was then docked to the target proteins using Glide 4.5 module of Schrodinger. The docking was done in Extra Precision Mode (XP). The docked protein and the ligand were viewed with Glide Pose Viewer. The images of the best docked poses of the ligand and the protein were saved as .jpg files. The above methodology was carried out and the results obtained are represented in the following chapter.