Materials and Methods

3.0 MATERIALS AND METHODS

Thrombolysis is a complex mechanism which interacts with clot components and surrounding plasma. In this interaction, plasmin, plasminogen, plasminogen activator and fibrin are involved. Aspirin and Heparin are significantly effective for activation of lysis and prevention of reocculusion. The new studies and investigations in this area will give new imminent that encourage the advancement of the ideal thrombolytic therapy. Herbal medicines are considered safer due to their natural activity (Sherwani *et al.*, 2013).

The experimental procedure for the present topic entitled **"Evaluation of thrombolytic and antioxidant potential of *Piper betle L.*"** is discussed under the following headings:

3.1. COLLECTION AND IDENTIFICATION OF THE PLANT SAMPLE

The leaves of *Piper betle L.* were collected from Coimbatore district, Tamilnadu. Taxonomical identification was made by the taxonomist of Botanical Survey of India, Southern circle, Tamilnadu Agricultural University, Coimbatore.

3.2. PREPARATION OF THE PLANT EXTRACTS

3.2.1. Preparation of the extract for phytochemical analysis

Fresh leaves of the plant were collected, washed and homogenized using water and ethanol for the preparation of aqueous and organic extracts respectively. These extracts were then filtered using Whatmann no.1 filter paper and used further for the study.

3.2.2. Preparation of the extract for thrombolysis and Cytotoxicity screening

The aqueous extracts of *Piper betle L.* were prepared and analyzed for thrombolytic and cytotoxic effect.

3.2.3. Analysis of antioxidant potential

The plant samples were extracted with various buffers and used for the analysis of enzymic and non-enzymic antioxidants.

3.3. QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTO-CONSTITUENTS

Phytochemical analysis was carried out to detect the phytocomponents of the plant extracts. The procedure is given in appendix I.

Estimation of flavonoids

Flavonoids with some specific chemical structure can react with Al^{3+} and form a red complex which gives a maximum absorption at 510nm (He, 2009). The method of Zhishen *et al.,* 1999 was followed for determination of flavonoids and the procedure is stated in appendix II.

Estimation of tannins

Quantification of tannins by the method of Robert (1971) is explained in Appendix III.

Estimation of protein

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

3.4. DETERMINATION OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS

3.4.1 Enzymic antioxidants

The samples were analyzed for the presence of enzymic antioxidants such as Catalase, Peroxidase, Superoxide dismutase, Polyphenol oxidase, Glutathione s-transferase and Glutathione peroxidase.

Catalase

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

Peroxidase

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

Superoxide dismutase

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

Polyphenol oxidase

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

Glutathione s-transferase

Glutathione s-transferase was estimated by the method of Habig *et al.*, 1974 and the procedure is explained in appendix IX.

Glutathione peroxidase

The method of Rotruck *et al.*, 1973 was used to assess the activity of glutathione peroxidase as explained in appendix X.

3.4.2 Non-enzymic antioxidants

The analysis of non-enzymic antioxidants includes ascorbic acid, α-tocopherol, polyphenols and reduced glutathione.

Ascorbic acid

Ascorbic acid was estimated by the method of Roe and Keuther, 1953 and the procedure is explained in appendix XI.

α-Tocopherol

Tocopherol was estimated by the method of Rosenberg *et al.*, 1992. The procedure is stated in appendix XII.

Polyphenols

Polyphenols are potent inhibitor of lipid peroxidation. It was determined by the method of Malick and Singh, 1980 as described in appendix XIII.

Reduced glutathione

Reduced glutathione was assessed by the method of Moron *et al.*, 1979. The procedure is explained in appendix XIV.

3.5. DETERMINATION OF TOTAL ANTIOXIDANT ACTIVITY

The method of Prieto *et al.*, 1999 was followed to determine the total antioxidant activity of the selected plant samples. The procedure is described in appendix XV.

3.6. 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.

3.7. 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. CYTOTOXICITY SCREENING

Brine shrimp lethality bioassay was carried out to investigate the cytotoxic effect of the plant extracts by the method of Oladimeji *et al.*, 2006. The detailed procedure is stated in appendix XVIII.

3.9. HPTLC PROFILING

The methanolic extracts of *Piper betle L.* was subjected to HPTLC for the quantification of phytochemicals by the method of Wagner *et al.*, 1996. The procedure is given in appendix XIX.

3.10. STATISTICAL ANALYSIS

The significance between clot lysis and plant extracts by means of weight difference was tested by two-way ANOVA. Serum cholesterol level and clot lysis

were compared by correlation analysis. LC_{50} values for brine shrimp lethality bioassay were calculated by probit analysis. Experimental results of both enzymic and non-enzymic antioxidant activities were expressed by one-way ANOVA.

3.11. IN SILICO STUDIES

The methodology on molecular docking studies of hydroxychavicol is 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. Hydroxychavicol 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 VIIa, VIII and X was retrieved from Protein Data Bank.

3. Selection of target proteins

Thrombotic myocardial infarction may be secondary to complicated or ruptured atherosclerotic plaques with further exposure of procoagulant proteins that initiate blood coagulation or due to contact between blood and damaged endothelium. Tissue factor (TF) is a key enzyme in extrinsic coagulation pathway, present in the adventitia of normal blood vessels, atherosclerotic plaques, in addition to a circulating pool in the blood. The activation of extrinsic coagulation pathway is mediated via the binding of FVII to TF to form a TF-FVIIa complex, further activation of factor X, IX, XI, the formation of prothrombinase complex and thrombin generation (Rehab *et al*, 2010).

Elevated levels of factors VIII, IX, and XI can prove to be equally problematic. Studies have shown that individuals with VIII above the upper limit of normal have roughly 5 times the possibility of venous thromboembolism. Similarly, levels of IX and XI in the top 10% have twice the risk of thromboembolism. High levels of factor XI have also been associated with an increased risk of heart attack, stroke, and ischemia (Gailani and Renne, 2007). Tissue factors namely VIIa, VIII and X are some of the target proteins were used as the target proteins. The PDB ID for TF VIIa, VIII and X are 4JYU, 1WBG and 2Y7X respectively.

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 hydroxychavicol 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 hydroxychavicol 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 Schrödinger 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 TF VIIa, VIII and X 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 Standard Precision Mode (SP). 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.