An *In-Vitro* Study to Investigate Anti-inflammatory and Thrombolytic Activity of Methanolic Extract of *Blumea lacera* Leaves

By
Sumaiya Ahmed
ID: 15346018

A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

Department of Pharmacy
Brac University
September, 2019

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Sumaiya Ahmed
15346018

Approval

The thesis titled "An *In-vitro* study to investigate anti-inflammatory and thrombolytic activity of methanolic extract of *Blumea lacera* leaves" submitted by Sumaiya Ahmed (15346018) of Summer, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on 03-10-2019.

Examining Committee:

Supervisor:
(Member)

Faria Tahsin
Lecturer, Department of Pharmacy
Brac University

Program Coordinator:
(Member)

Dr. Hasina Yasmin
Associate Professor, Department of Pharmacy
Brac University

Departmental Head:
(Chair)

Dr. Eva Rahman Kabir
Chairperson, Department of Pharmacy
Brac University

Ethics Statement

This study does not involve animal trial or human trial.

I have donated my own blood for the experiments done using blood in the current project.

This work will only be published after ethical permission has been taken.

Abstract

Blumea lacera is a genus flowering plant which is a valuable ayurvedic medicine for centuries. Phytochemical contents of *Blumea lacera* extract figured the presence of different biochemical components like alkaloids, steroids, terpenoids, flavonoids, glycosides, tannins and phenolic compounds. The aim of the current study was to investigate the anti-inflammatory and thrombolytic property of *Blumea lacera* by observing inhibition of cell lysis and percent clot lysis. Five different concentrations of *Blumea lacera* extract were used as a test drug and aspirin, streptokinase were used as a standard drug for two experiments. The study showed dose dependent manner of haemolysis inhibition and good clot lysis activity as well. The maximum membrane stabilization of extract was found 72.80% at 500 µg/mL conc. in heat induced process and 74.08% at 500 µg/mL conc. in hypotonicity induced process and 61.79% clot lysis by extracts were found. *Blume lacera* possess significant anti-inflammatory as well as thrombolytic activity. However, further investigation can be carried out on isolation of individual phytocomponents and haemolysis inhibition by *in-vivo* procedure.

Keywords: Anti-inflammation, Thrombolytic, Heat induced, Hypotonicity, Haemolysis, *Blumea lacera*, Aspirin, Streptokinase, Blood Clot.

Dedication

Dedicated to my parents and supervisor for their great support, encouragement, love and care.

Acknowledgement

All the blessings and mercy of the Almighty Allah who is the greatest of all and the source of strength, knowledge and patience, has blessed me a sound health with a sound mind to continue my project work with full diligence throughout the whole journey. A successful outcome of a successful work needs a lot of assistance and guidance from many esteemed persons and I find myself utmost a fortunate one to have all of them till the very end of my thesis work.

First of all, I would like to express my earnest pleasure and deepest gratitude to my one of the most obliging and dedicated supervisor Faria Tahsin (Lecturer, Department of Pharmacy, Brac University) for her enormous amount of support, guidance and motivation. Whatever I have done, it's only due to the constant assistance of her with her enriched knowledge. I am also immensely thankful to Dr. Eva Rahman Kabir (Chairperson, Department of Pharmacy, Brac University) for providing me the chance to carry out the project at an individual level to flourish my knowledge. Besides I would also like to thank all the lab who cooperated me a lot throughout the lab work.

Last but not the least, I would plead my uttermost appreciation to my parents who have always remained as a protective umbrella over my head from the very first day of my life and appeared as a source of strength when it was needed.

Table of Contents

List of Tables

List of Figures

List of Acronyms

- mg = Microgram
- mL = Millilitre
- µg = Microgram
- µl = Microlitre
- kg = Kilogram
- WHO = World Health Organization
- ST = Streptokinase
- BL = *Blumea lacera*
- DW = Distilled water
- UV = Ultraviolet
- Conc. = Concentration
- HRBCs = Human red blood cells

Chapter 1

1.1 Introduction

Inflammation is a complex process associated with pain, increased vascular permeability, protein denaturation and decreased stabilization of membrane. Despite of being a sign of primary defense mechanism, uncontrolled and persistent inflammation may cause various chronic illness such as heart attacks, septic shocks, various rheumatoid arthritis, systemic lupus, osteoarthritis etc (Rietsch, 1882). Lysosomal enzymes release during inflammation leading to tissue damage through damage to macromolecules and lipid peroxidation membrane mainly responsible for pathological problems. It is a part of a body's immune responses. Thus it is most important to stabilize the membrane to limit inflammatory response by inhibiting the release of lysosomal constituents. Several anti-inflammatory mediators and the recruitment of monocytes to remove cell debris influence inflammation resolution. There are various non-steroidal anti-inflammatory drugs (NSAIDs) that can inhibits different lysosomal enzymes or stabilize the lysosomal membrane.

Blood clot formation recently became a severe blood circulation problem. Therefore, thrombus or embolus blocks blood flow by blocking the blood vessel and reducing the amount of oxygen. These results in tissue necrosis in that area. Thrombin is a blood clot formed by fibrinogen and is lysed by plasmin, which is activated by tissue plasminogen activator (tPA) from plasminogen. A fibrinolytic drug has the purpose of dissolving thrombin in acutely occluded coronary arteries, thus restoring blood supply to ischemic myocardium, limiting necrosis and improving prognosis. It is observed that many thrombolytic agents still have important deficits, including the need for high-dose efficacy, restricted fibrin specificity and a related major tendency to bleed. Steps must therefore be taken to create enhanced versions of these medicines to minimize the shortcomings of the thrombolytic medications

available (Ramjan, Hossain, Runa, Md, & Mahmodul, 2014). Together with active plant extracts, the trend of using natural products has increased normally for few drug discoveries. Plants have been used for thousands of years as traditional medicine systems around the world. *Blumea lacera* is widely distributed in Bangladesh and it has remarkable medicinal importance. Borneol, germacrene D, β-bisabolene, β-caryophyllene, γ-terpinene, sabinene, alkaloids, steroids, terpenoids, flavonoids, glycosides, tannins and phenolic compounds. These constituents showed more therapeutic activities such as anti-inflammatory, anti-arthritis, thrombolytic, analgesic, anxiolytic activities etc. So, therefore the study was designed to analyze anti-inflammatory and thrombolytic activity of methanolic extract of *Blumea lacera.*

1.2 Other species of Blumea:

1.2.1. *Blumea densiflora* DC

Blumea densiflora DC have a significant meidicinal use and it is known as a traditional plant, also familiar mostly to the traditional practitioner “kabiraj”. It belongs to the family of Asteraceae which is mainly found in herb topical region. One of the chemical constituents of the plant is essential oil containing camphor; the areal part it composed with sequiterpene, lactones, tagitinin A, tiroludin ethyl ether and iso-alanolactone derivatives. The anthelmintic property is laid in leaf extract and can be used for rheumatism and analgesia (M. Hossain, Islam, Azad, & Faruq, 2016).

1.2.2. *Blumea mollis:*

Blumea mollis is a medicinal plant belongs to the family Asteraceae which is small in size and grows up to 30-60 cm. in height. It is known as annual herb and the leaves are curved irregularly with shaped ovate. The plant consists of soft, erect stem glandular hair. It appears

as pink purple in color when the flower blooms. South India, Myanmar, China, South Africa in Malaysia are known for *Blumea mollis* habituations. Due to the presence of ethereal fatty oils, resins and bitter principles, the plant *Blumea mollis* imparts great medicinal and commercial value. *Blumea mollis* possesses several medicinal properties and prevention against skin diseases, asthma, dropsy, wound healing, diarrhea are well known properties among them. Antioxidant, anti-cancer, anti-bacterial, larvicidal, anti-inflammatory and hepatoprotective properties are also evident in the plant's leaves due to the presence of 39 phytochemicals in total where linalol, gamma-elemene, copaene, estragole, allo-ocemene, gamma-terpinene and allo-aromadendrene are considered to be major components (Sreelekha, K. P., Krishna T. P. A., Krishna, T. P. A., Deepa, P. E., Darsana, U., Juliet, S., Nair, S. N., Ravindran, 2017).

1.2.3. *Blumea eriantha*:

Blumea eriantha is one of the Asteraceae family's annual herbs, because of the presence of high camphor quantities, it imparts a camphorous odor. It is basically a small herb, growing at a height of up to 1 m. The leaves are with soft hair like fibers and commonly found in topical countries. Due to the presence of essential oils, the plant shows potential anti-bacterial, anti-fungal, insecticidal properties. Traditionally, *Blumea eriantha* is used as a carminative and diuretic drug as well as preventive disease measurement such as diarrhea and cholera (Gore & Desai, 2014).

1.2.4. *Blumea megacephala*:

Blumea megacephala is an herbal plant. It is contained with most effective component essential oil, which is obtained through steam distillation of samples. The plant found in Shiwang Mountains in Guangxi Province, China, which was analyzed using GC-FID and GC-MS. Among the 65 compounds identified in the oil, the main chemical constituents with

the compounds named borneol (13.6 %), β-caryophyllene (9.56 %), germacrene D (9.09 %), sabinene (6.37 %), and α-humulene (4.78 *%). B. megacephala* has antimicrobial activity revealed that the essential oil (1000 µg/disc) has promising antimicrobial effects against several pathogens, giving satisfactory inhibition zone diameter values (21.5, 21.6, 23.4, 23.8, 21.9) and MIC values (125, 125, 62.5, 125, 125 µg/mL) against some Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), some gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), and yeast (*Hansenula anomala*). Antioxidant and antimicrobial activities were correlated with chemical composition.

1.3 Plant Description (*Blumea lacera*)

Blumea Lacera Burm.f, synonyms: Conyza lacera Burm. f. (Local name, Jangli muli, Kukurmuti, Shialmutra, English name, Asteraceae) with a strong pungent smell specially when squeezed. *Blumea lacera* came from the Plantae kingdom and the Mangoliophyta class. It can be found in the region of Bangladesh, India and Africa. There are at least 80 species of *Blumea lacera* (Jahan, Kundu, & Bake, 2014). *Blumea lacera* is considered as annual herb and it grows up to 45 to 60 cm in height. It has flowering time from January to April and the flowers appears as yellowish color. After blooming the flower prominently, the yellow spike like portion turns into grey/white color to provide the flower a ball shape (Salisu, Veronica, Ogechi, Uwem, & Olakunle, 2015). *Blumea lacera* is commonly found in moist and shady place near old buildings and pond sides. It contains chemical like camphor, cineol 66, d-fenchone10 and citral, acetylene compounds, thiophene derivatives etc. which has been using for various kind of disease management. In case of disease management the whole plant is used. It has a vast number of medicinal properties like, astringent, acrid, antipyretic, anti-inflammatory, digestant, anthelmintic, diuretic etc. The alcoholic extraction of the herb possesses anti-inflammatory activity against carrageenan and bradykinin induced inflammation. Also evident from the essential oils derived from the leaves are analgesic,

hypothermic and tranquilizing properties. The plant also provides anti-breast cancer cells with antiviral, cytotoxic and anti-leukemic activity (Khair et al., 2014).

Taxonomic Classification:

Kingdom - Plantae

Sub kingdom - Tracheobionta – Vascular plants

Super division - Spermatophyte – Seed plants

Division - Magnoliophyta – Flowering plants

Class - Magnoliopsida – Dicotyledons

Subclass - Asteridae

Order - Asterales

Family – Asteraceae – Aster family

Genus - Blumea

Species – lacera

1.4 Chemical Composition (*Blumea lacera*)

By gas chromatography and mass spectroscopy, 46 compounds have found in total from *Blumea lacera* upon which the major chemical compounds identified were-

Table 1: Chemical constituents of Blumea lacera (Haque, Chowdhury, & Harun, 2018).

Chemical compound	Percentage
Borneol	11.43%
Germacrene D	8.66%

β-bisabolene	4.24%
β-caryophyllene	6.68%
γ-terpinene	4.35%
Sabinene	4.34%

Furthermore, *Blumea lacera* is considered as a great source of multiple essential phytocomponents like alkaloids, steroids, terpenoids, flavonoids, glycosides, tannins and phenolic compounds which shows great deal in disease management (Phyu Myint, Nyein Sann, Mar Soe, & Phyu Phyu Myint, 2017).

1.5 Background studies of *Blumea lacera*:

1.5.1 Anxiolytic property:

From the literature it has been found that *Blumea lacera* has the significant anxiolytic activity examined by using the hole board test and open field test (OFT) in case of diazepam treated animal as compared to control animal. For both of these two test, animals were divided into four groups with each consisting seven mice. First group received normal saline; second group received diazepam (1 mg/kg); third and fourth groups were received plant extract at 200 mg/kg and 400 mg/kg body weight respectively. In case of hole board assay, test groups orally received crude methanolic extract of *Blumea lacera* and were examined by counting of passing one chamber to another through hole for a period of 5 min at 30 min after oral administration. The result showed significant number of line crossing as compared to control animals. On the other hand, in case of open field test, general motor activity, explanatory behaviors and measures of anxiety of animals were observed in a plain wooden box having sixteen small squares. It is similar to hole cross test. After drug administration both treated and control groups were placed in the open field and recorded their behavior for 5 min.

During this time, the number of entries to the open center, peripheral corners, number of crossings (measure the distance traveled), rearing and assisted rearing were carefully observed. The results significantly increase the number of squares crossed at intervals of 5 min compared to the control group (Khair, 2014).

1.5.2 Analgesic property:

In the investigation of analgesic property of *Blumea lacera* by hot plate method showed a significant analgesic activity which reveals that the analgesic activity may be produced analgesic property. *Blumea lacera* have essential oil in its chemical constituents which have an effective activity as an analgesic. For this experiment animals were divided into 3 groups having 3 in each. Two groups orally having test substance and standard (diclofenac). Placed animals to the hot plate and the temperature was maintained at 45 ± 1°C. The reaction time was considered between the placement of mice on the hot place and the flicking moment after having the heat. On the other hand, group 1 was received a comparable volume of vehicle. The observation was counted from the 0 min and 30 min later the latent reaction time.

1.5.3 Antipyretic activity:

From the study regarding antipyretic property it has been found that *Blumea lacera* has significant property of reducing temperature depend on dose level. Methanolic extract of *Blumea lacera* have effective activity on the highest dose of 400 µg/mL; ethanolic extract of *Blumea lacera* showed effectiveness at dose of 200 and 400 µg/mL; chloroform extract of *Blumea lacera* have the highest effective activity on the lowest dose of 200 µg/mL. It means it varies depending on the dose and the medium of the extract. The experiment was done by brewer's yeast induced pyrexia model where pyrexia/fever is injected inside the body of experimental animals (Bhuiya et al., 2017). Swiss albino mice were divided into 8 groups with 5 in each group and must be fasted overnight before the experiment. Temperature were

measured after inducing pyrexia and then administer the test drug and standard (diclofenac sodium) drug and also with the control (0.9% NaCl). After 3 hours, again temperature measured and then calculate the percentage of reduction of temperature and this is how the antipyretic property can be identified by *in-vivo* method.

1.6 The aim of the project

The aim of the project is to-

- Find out the anti-inflammatory property of *Blumea lacera* extract by investigating the heat induced haemolysis and hypotonicity induced haemolysis by *in-vitro* method.
- Find out the thrombolytic property by clot lysis method by *in-vitro.*

1.7 Objective of the project

The objective of the study are to-

- Estimate the percentage of cell lysis inhibition in case of both test drug and standard drug.
- Estimate the percentage of clot lysis in case of both test and standard drug.
- Determine the concentration at which both the drugs are providing highest range of inhibition of erythrocyte lysis and highest clot lysis.
- Find out the potency and effectiveness of the drug in case of inflammation and thrombosis treatment.

Chapter 2

Methodology

2.1 Chemicals used in experiment

Here is the following list of chemicals used in the conduction of full investigation:

Table 2: Name of the chemicals used in the experiment.

Name of the chemicals	Source / Manufacturer company
Sodium chloride (NaCl)	Merck Specialties Private Limited, India
Potassium chloride (KCl)	Merck, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Active Fine Chemicals Limited, Bangladesh
Potassium dihydrogen phosphate (KH_2PO_4)	Active Fine Chemicals Limited, Bangladesh
Normal saline (0.9%)	Solo (Square Pharmaceuticals Limited, Bangladesh)
Aspirin - 75 mg	Ecospirin tablet (ACME Laboratories Limited, Bangladesh)
Sodium hydroxide (NaOH)	Brac University (Pharmaceutical Technology lab)
Streptokinase	STK (Incepta Pharmaceuticals Limited, Bangladesh)
Distilled water	Brac University (Physiology lab)
Potassium oxalate ($C_2K_2O_4$)	Brac University (Physiology lab)

2.2 Membrane stabilization

The erythrocyte membrane resembles to the lysosomal membrane and as such the effect of drug on the membrane stabilization of the erythrocyte could be extrapolated to the stabilization of the lysosomal membrane. The membrane stabilizing activity of the extractives as assayed by using heat induced haemolysis and hypotonicity induced haemolysis method. These methods has been used to study the *in-vitro* anti-inflammatory activity because the stabilization implies that the extract may well stabilize lysosomal membrane. Stabilization of lysosomal is important for limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bacterial enzymes and proteases which occurs tissue inflammation and damage upon extra cellular release.

2.3 Preparation of red blood cells (HRBCs) suspension

The blood was collected from the healthy human volunteer with the condition of not taking any NSAIDs (Non-steroidal anti-inflammatory Drug) for last 2 weeks prior to the experiment. 5 ml blood was collected with sterile injection from the volunteer. Placed the blood to the centrifuge tube with potassium oxalate ($C_2K_2O_4$) (anti-coagulant) and immediately mixed the blood with it and transferred to the centrifuge tube. Then centrifuged for 10 minutes at 3000 rpm and the serum was removed carefully without disturbing the HRBCs with a dropper and the cells were washed 3 times with normal saline (0.9%).Three times wash were done with equal volume of saline. The volume of blood was reconstituted as 10% v/v suspension with normal saline.

2.4 Heat induced haemolysis

2.4.1 Preparation of standard drug of different concentrations

Aspirin was used as standard drug and different concentration (100-500 µg/mL) was used. 5 tablets of aspirin were taken and the average weight of the tablet was 148.5 mg and the strength of the each tablet was 75 mg. By calculation, it was found that 99 mg in weight of crushed tablet consists of 50 mg of aspirin. Therefore, 99 mg crushed tablet was taken and dissolved in 100 ml of normal saline to make the concentration of the solution 500 µg/mL. After that 8 ml was taken from the stock solution (standard drug) and made the volume up to 10 ml by adding normal saline to dilute the concentration to 400 µg/mL. Furthermore, 6 ml, 4ml and 2 ml of solution was taken from 500 µg/mL concentrated stock solution (standard drug) and made it up to 10 ml by adding normal saline to finalize the concentrations respectively 300 µg/mL, 200 µg/mL and 100 µg/mL.

2.4.2 Flow diagram of preparation of standard drug with different concentrations

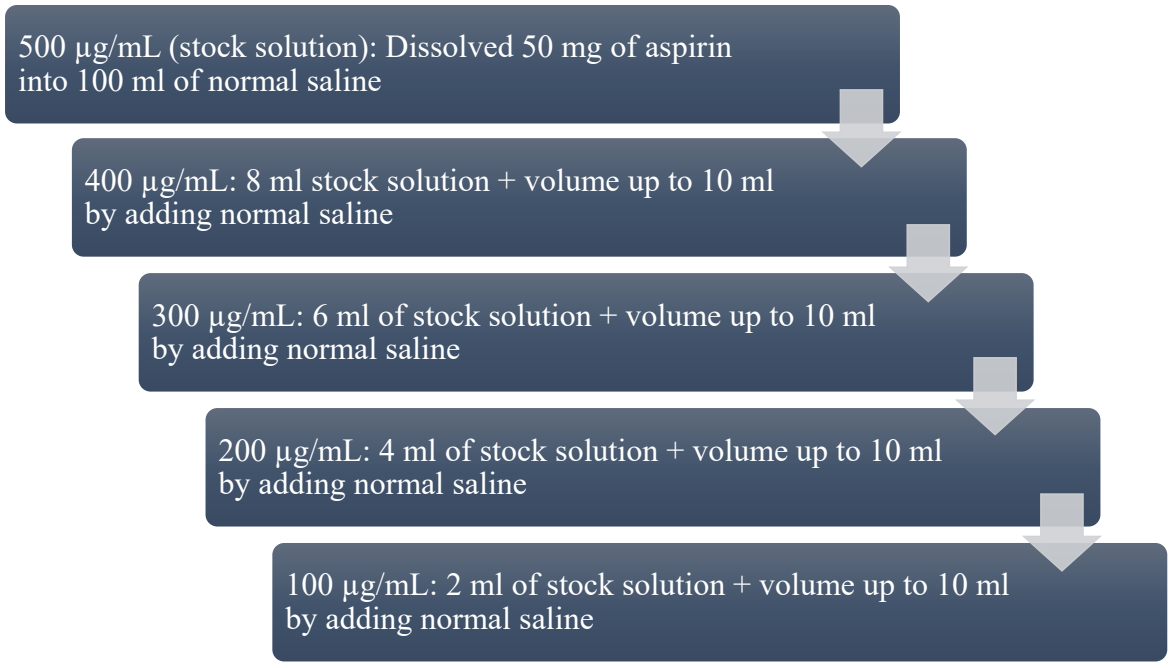

Figure 1: Flow diagram of preparation of standard drugs with different concentrations

2.4.3 Preparation of test drug (*Blumea lacera*) with different concentrations:

50 mg of extract was taken to dissolve in 100 ml of normal saline to make the concentration of 500 µg/mL. After that 8 ml was taken from the 500 µg/mL concentrated stock solution and made the volume up to 10 ml by adding normal saline to dilute the concentration to 400 µg/mL. Furthermore, 6 ml, 4ml and 2 ml of solution was taken from 500 µg/mL concentrated stock solution (standard drug) and made it up to 10 ml by adding normal saline to finalize the concentrations respectively 300 µg/mL, 200 µg/mL and 100 µg/mL.

2.4.4 Flow diagram of preparation of test drug (*Blumea lacera*)

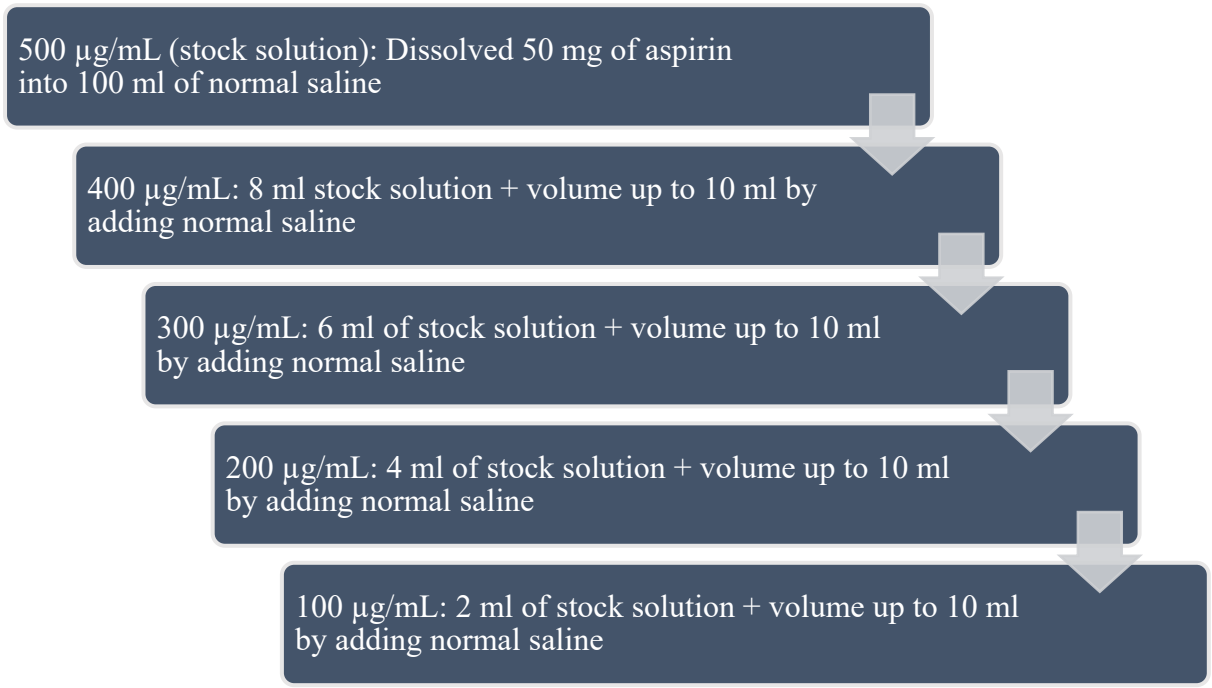

Figure 2: Flow diagram of preparation of test drugs with different concentrations

2.4.5 Preparation of reaction mixtures

• Test control solution:

Total volume was 2 ml where 1 ml of normal saline was added to 1 ml of 10% HRBCs suspension.

• Test solution:

Total volume was 2 ml where 1 ml of test solution (solution with *Blumea lacera* extract) of various concentrations (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL) was mixed with 1 ml 10% HRBCs suspension.

• Standard solution:

Total volume was 2 ml where 1 ml of standard solution (solution with aspirin with the strength of 75 mg) of different concentration (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL) was added to1 ml 10% HRBCs suspension.

2.4.6 Procedure

- Different concentrations of *Blumea lacera* extract (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL) and various concentrations of standard drug (aspirin) (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL) were prepared properly.
- Reaction mixtures containing 2 ml of test solution (1 ml test drug and 1 ml blood suspension), standard solution (1 ml standard drug and 1 ml blood suspension) and test control solution (1 ml blood suspension and 1 ml saline) were prepared appropriately.
- All the reaction mixtures were incubated in water bath for 30 minutes at 56°C.

- After completion of incubation, all the samples were kept undisturbed for few minutes to make them cool or kept under running water tap for cooling.
- Then centrifuged all the reaction mixtures tubes at 2500 rpm for 5 minutes.
- Lastly the absorbance of the supernatant was measured using UV-visible spectrophotometer at 560 nm (G & S, 2011).
- The experiments were done in triplicates manner for all the test samples.
- The percentage of inhibition of protein denaturation was calculated by the following formula**:**

  Percentage of inhibition = (Abs control – Abs sample) × 100 / Abs control

  [Here, Abs = Absorbance].

2.4.7 Flow diagram of heat induced haemolysis method

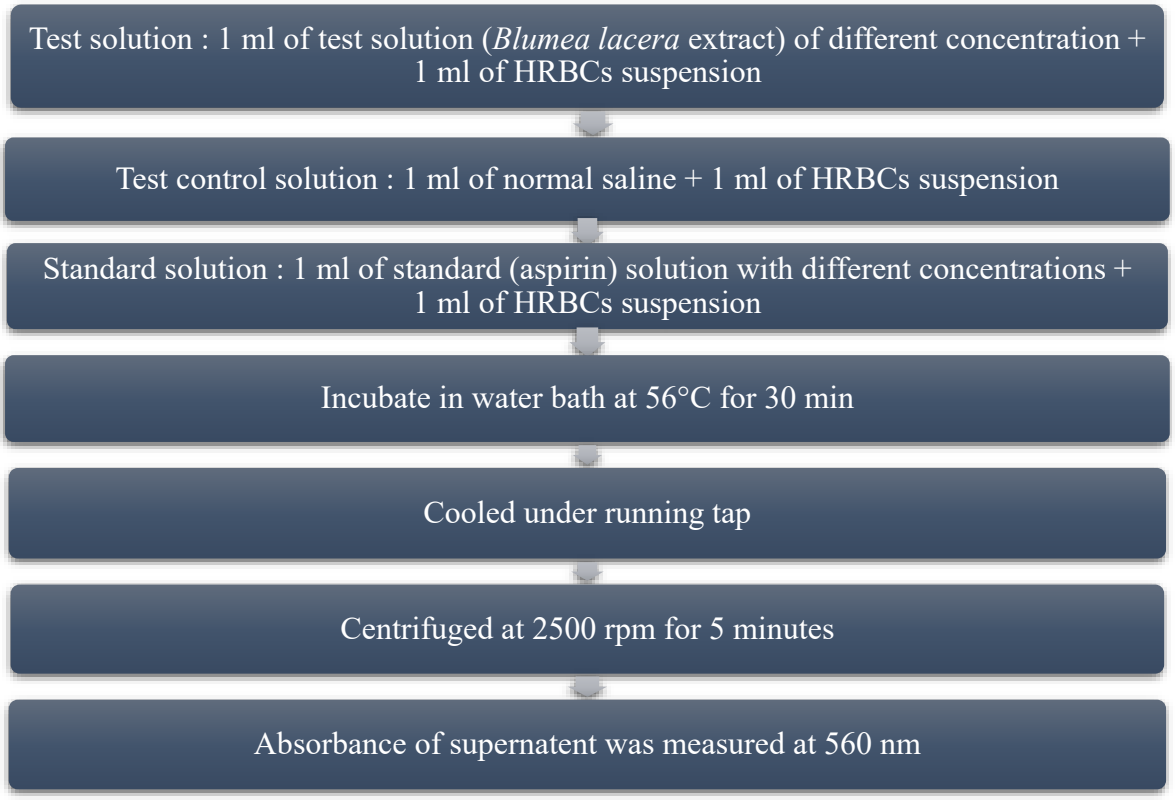

Figure 3: Flow diagram of heat induced haemolysis method procedure

2.5 Hypotonicity induced haemolysis

2.5.1 Preparation of standard drug with different concentrations

Aspirin was used as standard drug and different concentrations (100-500 µg/mL) were used. 5 tablets of aspirin were taken and the average weight of the tablet was 143.4 mg and the strength of the each tablet was 75 mg. By calculation, it was found that 95.6 mg in weight of crushed tablet is consist of 50 mg of aspirin. Therefore, calculated weight of crushed tablet was taken and dissolved in 100 ml of hyposaline (0.36%) to make the concentration of the solution 500 µg/mL. After that 8 ml was taken from the concentrated stock solution (standard drug) and made the volume up to 10 ml by adding hyposaline to dilute the concentration to 400 µg/mL. Furthermore, 6 ml, 4 ml and 2 ml of solution was taken from 500 µg/mL concentrated stock solution (standard drug) and made it up to 10 ml by adding normal saline to finalize the concentrations respectively 300 µg/mL, 200 µg/mL and 100 µg/mL.

2.5.2 Flow diagram of preparation of standard drug with different concentrations

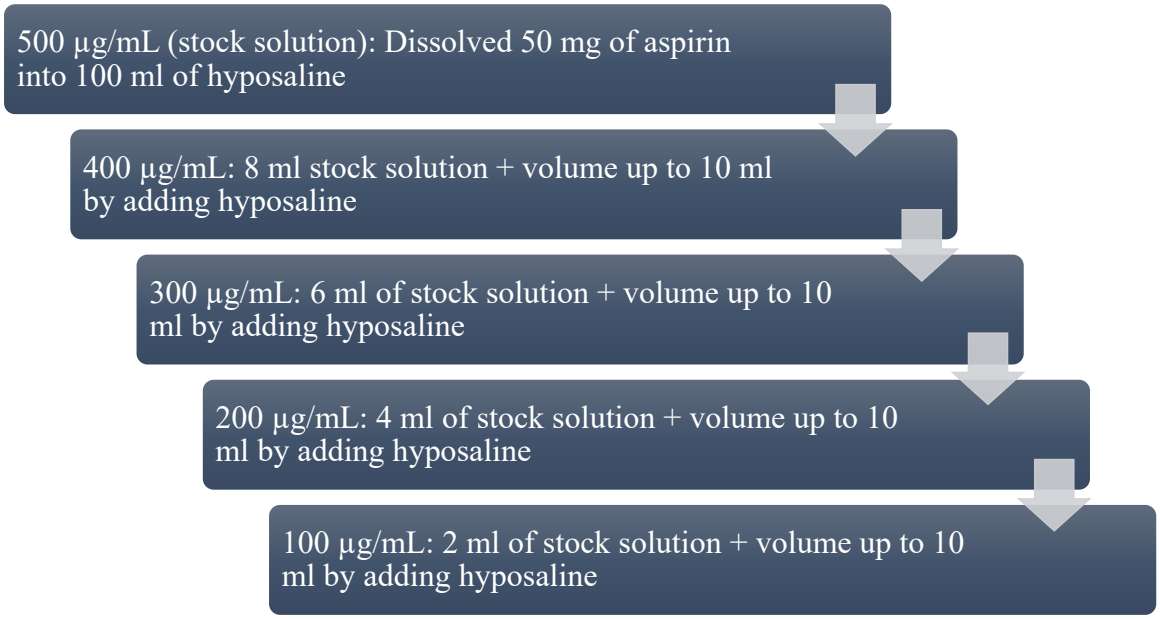

Figure 4: Flow diagram of preparation of standard drug (aspirin) with different concentrations

2.5.3 Preparation of test drug with different concentrations

50 mg of extract was taken to dissolve in 100 ml of hyposaline to make the concentration at 500 µg/mL. After that 8 ml was taken from the 500 µg/mL solution and made the volume up to 10 ml by adding hyposaline to dilute the concentration to 400 µg/mL. Furthermore, 6 ml, 4ml and 2 ml of solution was taken from 500 µg/mL stock solution (standard drug) and made it up to 10 ml by adding normal saline to finalize the concentrations respectively 300 µg/mL, 200 µg/mL and 100 µg/mL.

2.5.4 Flow diagram of preparation of test drug with different concentrations

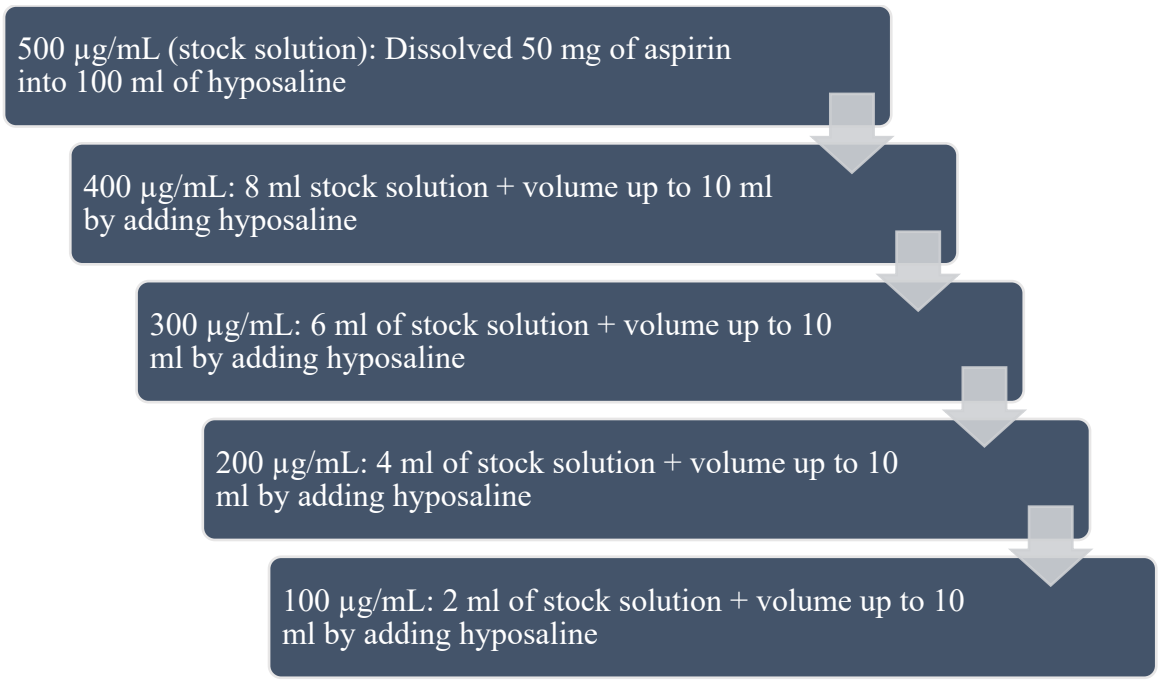

Figure 5: Preparation of test drugs (Blumea lacera extract) with different concentrations

2.5.5 Preparation of reagents and reaction mixtures

Phosphate Buffer Saline (PBS) [pH 7.4] :

Sodium chloride (NaCl) – 2.25 g, disodium hydrogen phosphate (Na_2HPO_4) – 0.0287 g and potassium dihydrogen phosphate (KH_2PO_4) – 0.065 g were taken accurately and dissolved in 800 ml of distilled water. The pH of the solution was then adjusted to 7.4 by using sodium hydroxide (NaOH) and made the volume up to 1000 ml by adding distilled water.

Test solution:

Total volume was 4.5 ml where 1 ml PBS, 2 ml hyposaline, 1 ml of test solution (solution with *Blumea lacera* extract) of various concentration (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL) was mixed with 0.5 ml of 10% HRBCs suspension (G & S, 2011).

Test control solution:

Control was separately mixed with 1 ml PBS, 3 ml of hyposaline, 0.5 ml of HRBC suspension (G & S, 2011).

Standard solution:

Total volume was 4.5 ml where 1 ml PBS, 2 ml hyposaline, 1 ml of standard solution (solution with aspirin with the strength of 75mg) of different concentration (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL) and 0.5 ml of 10% HRBC suspension (G & S, 2011).

2.5.6 Procedure

- Different concentrations of (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL) test drug (*Blumea lacera* extract) and various concentrations of (100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL and 500 µg/mL) standard drug (aspirin) were prepared properly.
- Test solutions, standard solutions, test control solutions were prepared appropriately.
- All the reaction mixtures were incubated for 30 minutes at 37°C.
- After incubation all of them were centrifuged at 3000 rpm for 3 minutes.
- The supernatant liquid was decanted.
- The hemoglobin content was estimated by UV-visible spectrophotometer at 560 nm and the percentage haemolysis was estimated by assuming the haemolysis produced in the control as 100%.

  Percentage of Protection = 100 – (OD Sample / OD Control) × 100

  [Here, OD = Optical Density].

2.5.7 Flow diagram of hypotonicity induced haemolysis method

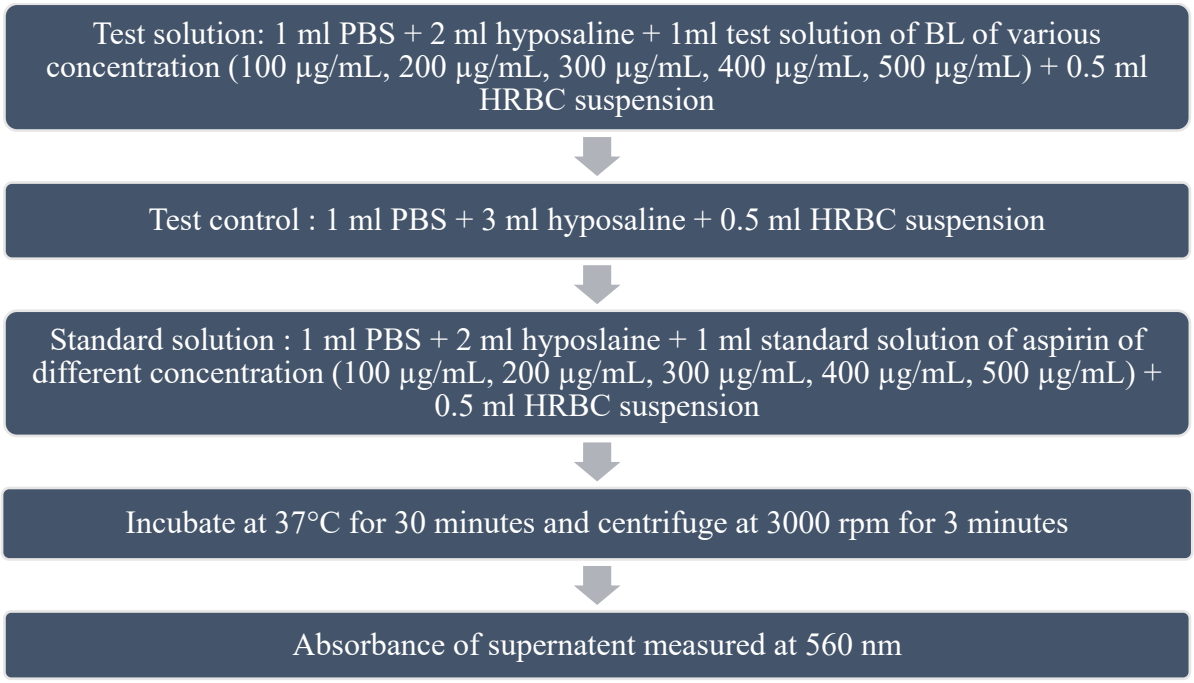

Figure 6: Flow diagram of hypotonicity induced haemolysis process

2.6 Thrombolytic activity test

Thrombolysis could be the breakdown (lysis) of blood clots simply by pharmacological means. It is colloquially termed as clot busting that is why it works by stimulating fibrinolysis by plasmin through infusion of analogs regarding tissue plasminogen activator (tPA), the protein that normally activates plasmin. The aim of the present study was to investigate the thrombolytic activity of methanolic extract and its different fraction of leaves of *Blumea lacera.*

2.6.1 Preparation of Extract solution for thrombolytic test

The thrombolytic activity of all extractives were evaluated by a method developed by using streptokinase (SK) as standard substance. The crude drug was taken 100 mg and suspended in 10 ml of distilled water. To mix it up properly it was shaken vigorously with a vortex mixture until it dissolved properly. Then the suspension was decanted to eliminate the soluble

supernatant which was filtered with the filter paper. The solution was ready for *in-vitro* evaluation of clot lysis activity (M. I. Hossain & Mahmood, 2015).

2.6.2 Preparation of streptokinase (SK) solution for thrombolytic test

Commercially available streptokinase (STK) vial (Incepta pharmaceutical Ltd) of 15,00,000 I.U.) was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *in-vitro* thrombolysis.

Figure 7: Streptokinase (STK)

2.6.3 Preparation of specimen for thrombolytic test

1.5 ml blood was drawn from each healthy human volunteers (here blood was taken from 2 volunteers) without a history of oral contraceptive or anticoagulant therapy and 500µl of blood was transferred to the previously weighed eppendorf tubes and was allowed to form clots (M. I. Hossain & Mahmood, 2015).

2.6.4 Procedure for thrombolytic test

- Blank eppendorf tube were weighted before going to the main experiment for calculating the clot.
- 1.5 ml of blood were taken from both healthy human volunteers and 500 µl were distributed in 6 eppendorf tubes.
- All the tubes were kept in incubator at 37°C for 45 minutes to form clots.
- After the clots were formed serum was removed without disturbing the clot.
- Weighed again to determine the clot weight **(Clot weight = Weight of clot containing tube – Weight of blank tube).**
- To each eppendorf tube containing pre-weighed clot, 100 µl of solutions of different partitionates along with the crude extract was added separately. As a positive control streptokinase (SK) and as a negative distilled water (non thrombolytic control), were added separately.
- Again incubated for 90 minutes at 37°C.
- After incubation removed excess serum from the clots and again weighed to observe the difference in weight after clot disruption.
- Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis (M. I. Hossain & Mahmood, 2015).

Percentage of clot lysis = (Weight of released clot / Clot weight) × 100

2.6.5 Flow diagram of Thrombolytic test method

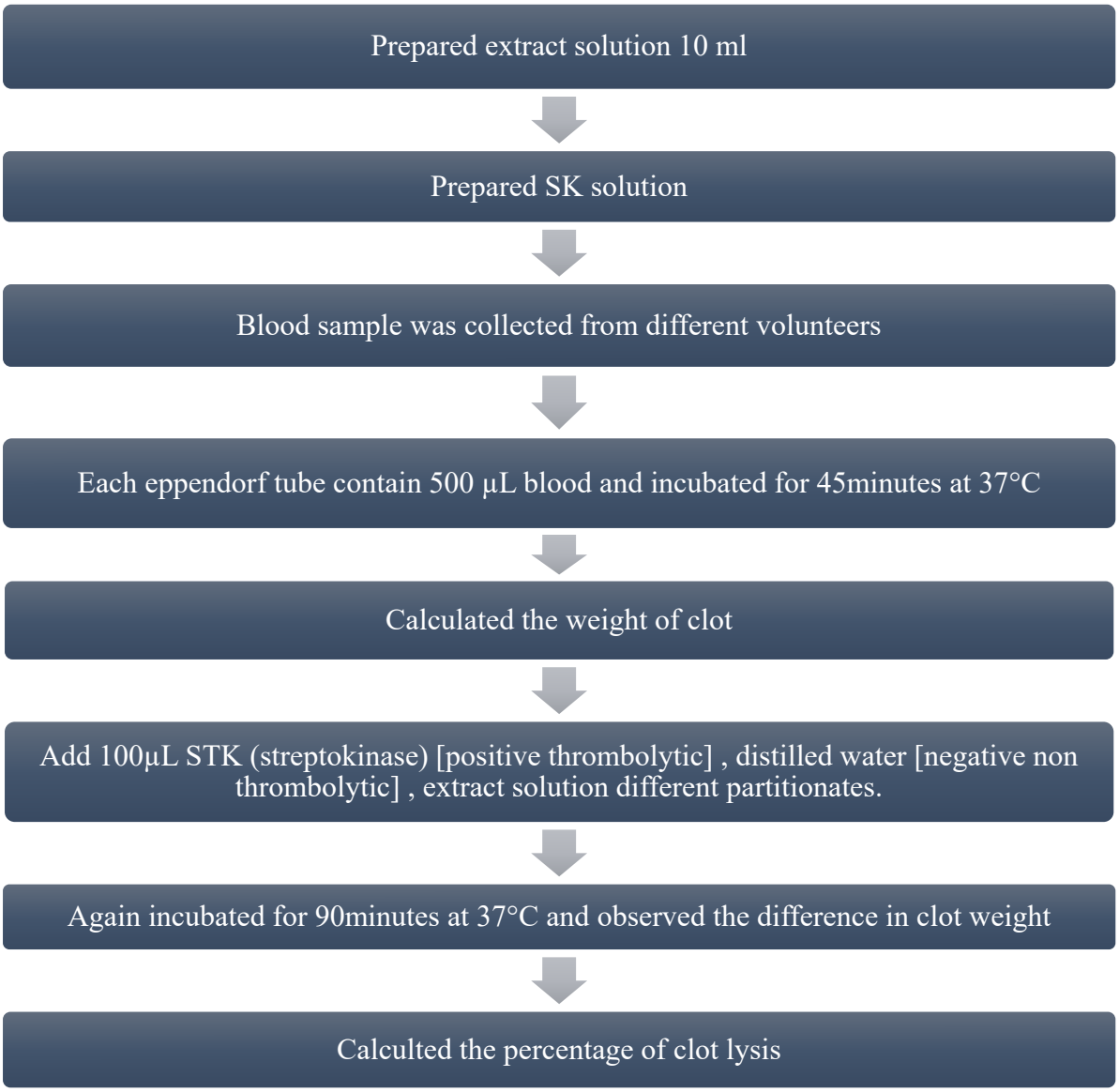

Figure 8: Thrombolytic activity testing procedure

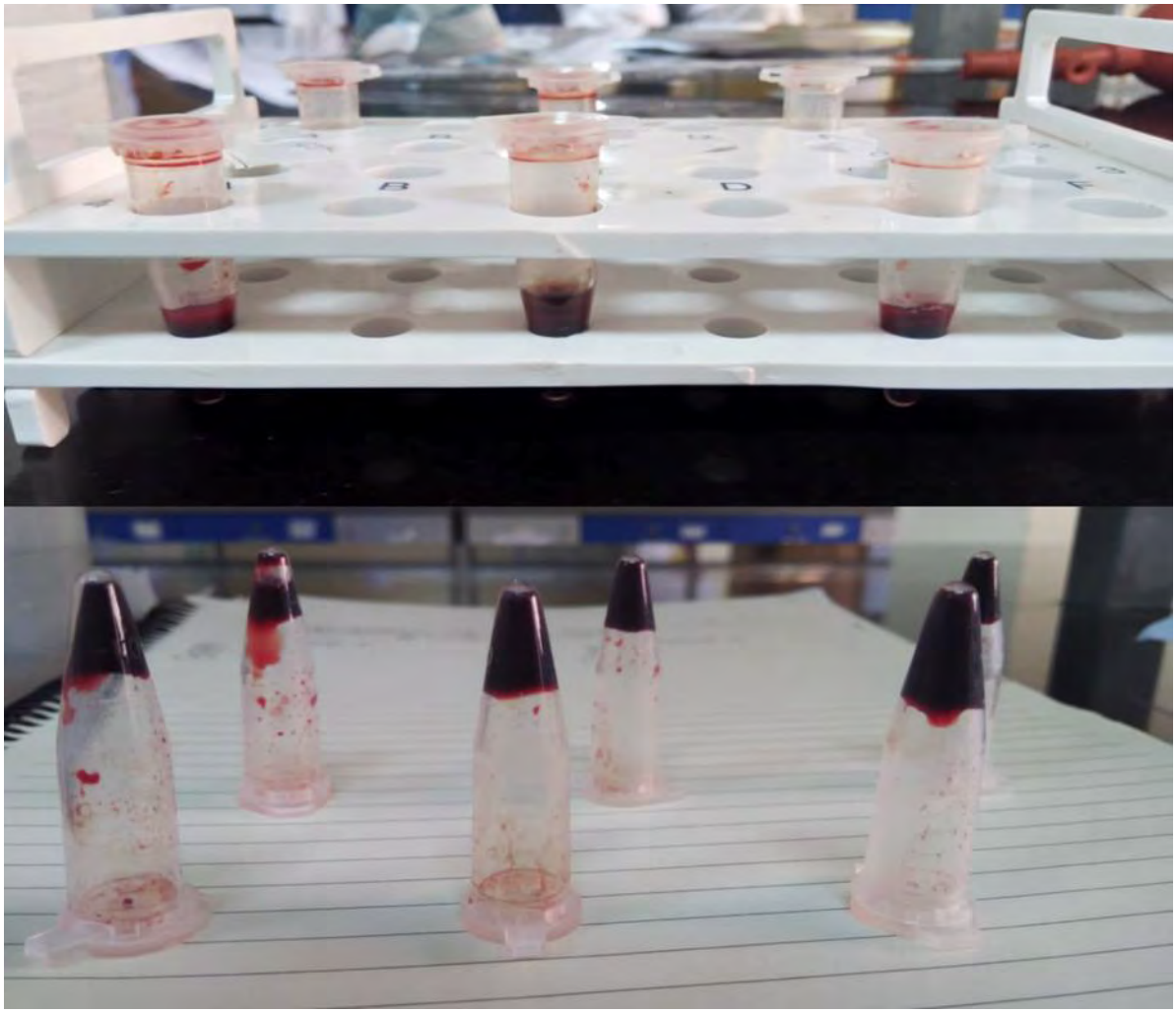

Figure 9: Eppendorf tubes after lysis

Chapter-3

Results

3.1 Heat induced haemolysis

3.1.1 Table of haemolysis inhibitory activity of aspirin:

The concentration and percentage of inhibition of haemolysis done by standard drug (aspirin) is shown below in the table-

Table 3: The lysis inhibition percentage of aspirin

Concentration (µg/mL)	Absorbance ± SEM	Inhibition of lysis (%)
100 (µg/mL)	0.360 ± 0.007	12.83%
200 (µg/mL)	0.331 ± 0.012	19.85%
300 (µg/mL)	0.232 ± 0.013	43.83%
400 (µg/mL)	0.212 ± 0.005	48.66%
500 (µg/mL)	0.224 ± 0.010	45.76%

Interpretation:

From the above set of information mentioned on the table indicated that with the any increment of concentration, the haemolysis inhibitory activity of aspirin was also increased which proves the dose dependent haemolysis inhibitory activity of aspirin.

3.1.2 Table of haemolysis inhibitory activity of extract

The concentration and percentage of inhibition of haemolysis done by extract (*Blumea lacera*) is shown below in the table-

Table 4: Lysis inhibition percentage of Blumea lacera extract

Concentration (µg/mL)	Absorbance ± SEM	Percentage of inhibition
100 (µg/mL)	0.231 ± 0.001	30.21%
200 (µg/mL)	0.171 ± 0.001	48.33%
300 (µg/mL)	0.146 ± 0.001	55.89%
400 (µg/mL)	0.129 ± 0.002	61.03%
500 (µg/mL)	0.090 ± 0.001	72.80%

Interpretation:

From the above table, it was seen that the percentage of inhibition of haemolysis was increasing with the increasing number of concentration. The initial inhibition was 30.21% at concentration 100 µg/mL where the inhibition had increased to 72.80% at concentration 500 µg/mL. This indicates the dose dependent relationship of concentration and inhibition. The result showed that at 400 µg/mL and 500 µg/mL concentration significantly protect the erythrocyte membrane against lysis induced by heat.

3.1.3 Comparative relationship of aspirin and *Blumea lacera* extract in anti-inflammatory activity:

The graphical representation of comparative relationship in between aspirin and extract in percentage of erythrocyte lysis inhibitory activity is shown in table and figure below:

Table 5: Percentage of inhibition of dried extract of Blumea lacera by heat induced haemolysis with respect to standard aspirin

Concentration (µg/mL)	Percentage of inhibition by aspirin	Percentage of inhibition by extract
100 µg/mL	12.83%	30.21%
200 µg/mL	19.85%	48.33%
300 µg/mL	43.83%	55.89%
400 µg/mL	48.66%	61.03%
500 µg/mL	45.76%	72.80%

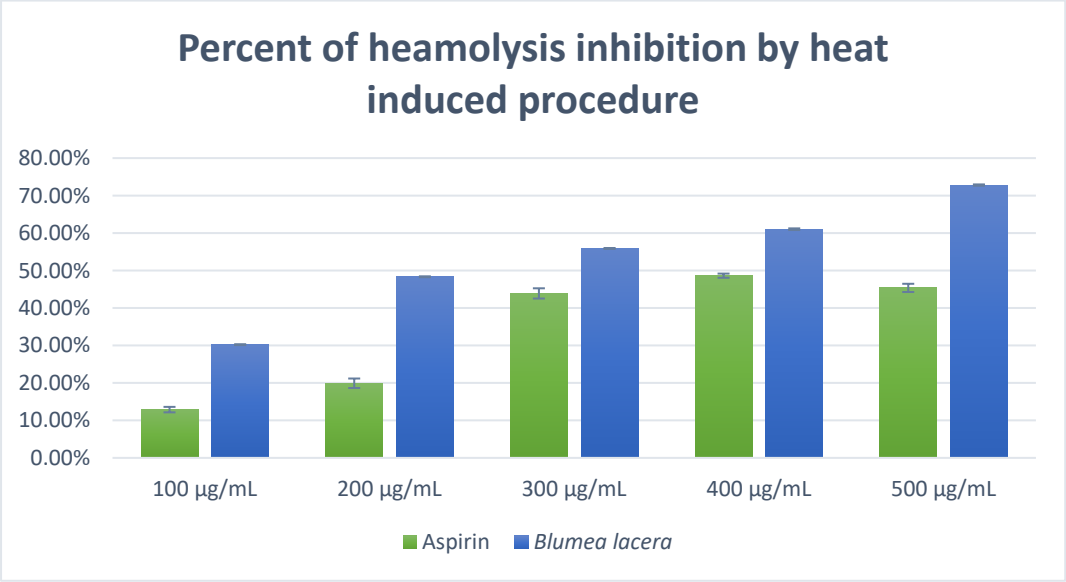

Figure 10: Haemolysis inhibition (%) concentration (µg/mL) Vs. lysis inhibition (%)

3.2 Hypotonicity induced haemolysis

3.2.1 Table of haemolysis inhibitory activity of aspirin:

The concentration and percentage of inhibition of haemolysis done by standard drug (aspirin) is shown below in the table

Table 6: Inhibition percentage of Standard drug (aspirin)

Concentration (µg/mL)	Absorbance ± SEM	Inhibition of lysis (%)
100 µg/mL	0.475 ± 0.001	11.01%
200 µg/mL	0.449 ± 0.001	15.92%
300 µg/mL	0.301 ± 0.003	43.63%
400 µg/mL	0.325 ± 0.006	39.14%
500 µg/mL	0.264 ± 0.010	50.56%

Interpretation:

From the above information set mentioned on the table indicated that the haemolysis inhibitory activity of aspirin, which proves the dose-dependent haemolysis inhibitory activity by hypotonicity-induced procedure, is also increased without any increase in concentration.

3.2.2 Table of haemolysis inhibitory activity of extract:

The concentration and percentage of inhibition of haemolysis done by extract (*Blumea lacera*) is shown below in the Table-

Table 7: Inhibition percentage of test drug (extract of Blumea lacera)

Concentration (µg/mL)	Absorbance ± SEM	Inhibition of lysis (%)
100 µg/mL	0.221 ± 0.006	26.57%
200 µg/mL	0.173 ± 0.001	42.52%
300 µg/mL	0.141 ± 0.003	53.15%
400 µg/mL	0.092 ± 0.001	69.43%
500 µg/mL	0.078 ± 0.014	74.08%

Interpretation:

From the table above, the percentage of haemolysis inhibition is increasing with increasing concentration. At concentration 100 µg/mL, the initial inhibition was 26.57% where the inhibition increased to 74.08% at concentration 500 µg/mL. This indicates the dose dependent relationship of concentration and inhibition. The result showed that at 400 µg/mL and 500 µg/mL concentration protect significantly the erythrocyte membrane against lysis induced by hypotonicity.

3.2.3 Comparative relationship of aspirin and *Blumea lacera* extract in anti-inflammatory activity:

The graphical representation of comparative relationship in between aspirin and extract in percentage of erythrocyte lysis inhibitory activity is shown in table and figure below:

Table 8: Percentage of inhibition of dried extract of Blumea lacera on haemolysis with respect to standard aspirin

Concentration (µg/mL)	Percentage of lysis inhibition by aspirin	Percentage of lysis inhibitionby extract (*Blumea lacera)*
100 µg/mL	11.04%	26.57%
200 µg/mL	15.92%	42.52%
300 µg/mL	43.63%	53.15%
400 µg/mL	39.14%	69.43%
500 µg/mL	50.56%	74.08%

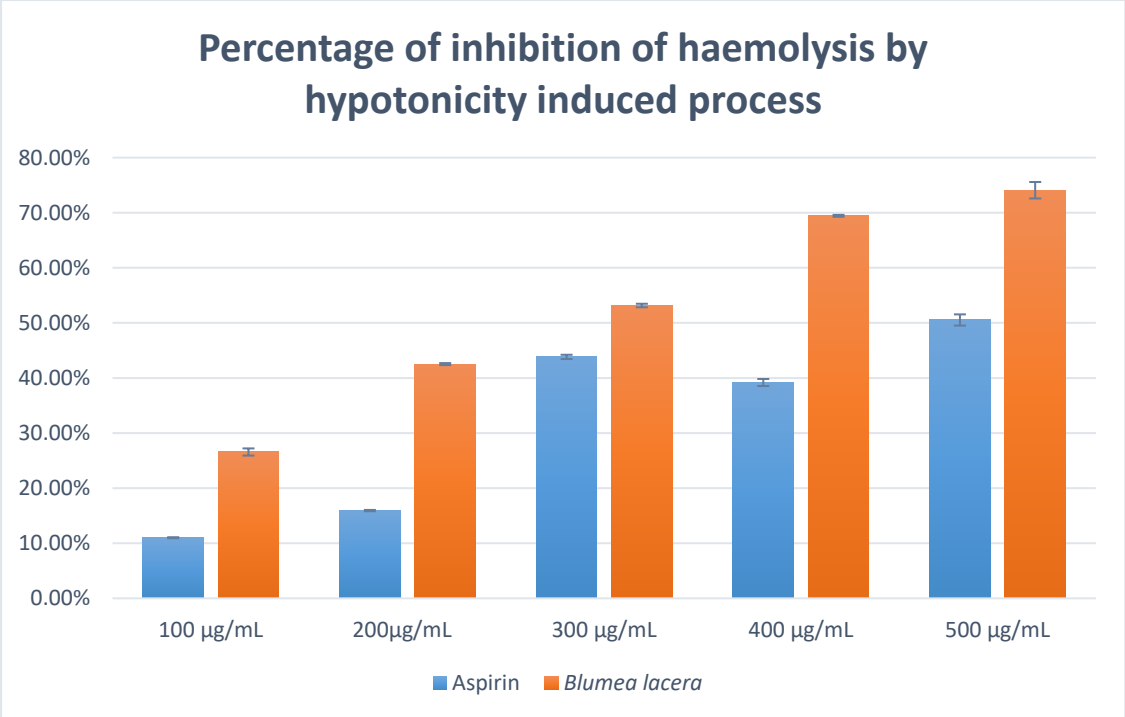

Figure 11: Percentage of haemolysis inhibition concentration (µg/mL) Vs. percentage of inhibition

3.3 Thrombolytic activity

To find out cardio protective drugs, anti-coagulant, anti-platelets and fibrinolytic activity from natural sources the extractives of *Blumea lacera* were assessed for thrombolytic activity and the results are presented in Table-1. Addition of 100µl SK, a positive control (30,000 I.U.) to the clots and subsequent incubation for 90 minutes at 37°C, showed 49.894% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot 5.407%. The mean difference in clot lysis percentage between positive and negative control was found very significant. In this study, the suspension of *Blumea lacera* exhibited highest thrombolytic activity 61.787%.

Table 9: Thrombolytic activity

Group	Percentage of lysis
Streptokinase	49.894%
Blumea lacera	61.787%
Distilled water	5.407%

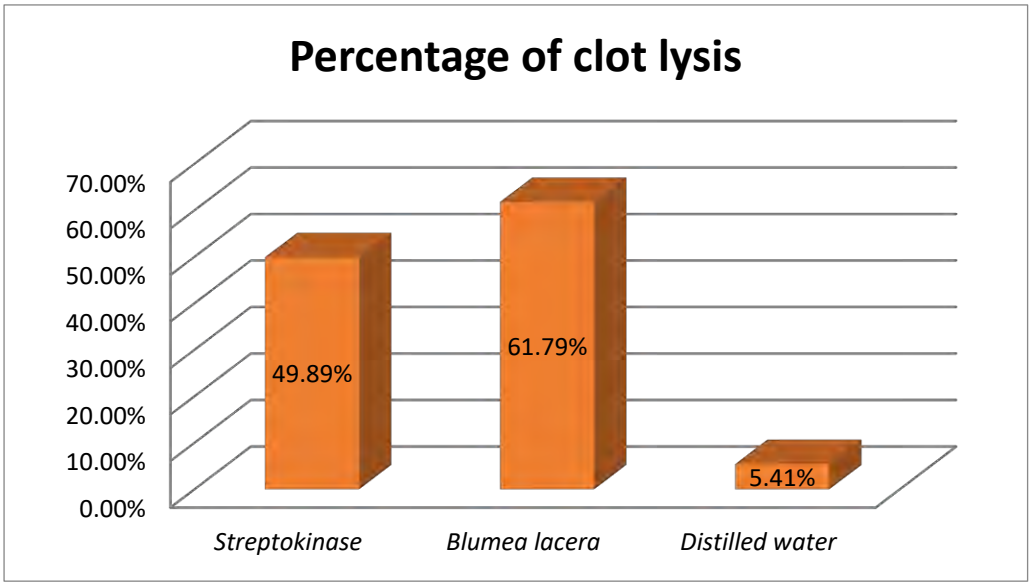

Figure 12: Percentage of clot lysis of Streptokinase, distilled water and methanolic extract of Blumea lacera

Table 10: Clot Lysis of methanolic extract of Blumea lacera

Sl. no Volunteer	Weight of blank tube	Weight of clot before lysis	Weight of clot after lysis	Weight of clot	Percentage of lysis	Average of lysis percentage
1.	0.836	0.303	1.018	0.182	60.06%	61.78%
2.	0.843	0.285	0.024	0.181	63.51%	

Table 11: Clot lysis of Streptokinase (positive control)

Sl. no Volunteer	Weight of blank tube	Weight of clot before lysis	Weight of clot after lysis	Weight of clot	Percentage of lysis	Average of lysis percentage
1.	0.858	1.125	0.996	0.138	51.68%	49.89%
2.	0.833	1.123	0.973	0.140	48.11%	

Table 12: Clot lysis of distilled water (negative control)

Sl. no Volunteer	Weight of blank tube	Weight of clot before lysis	Weight of clot after lysis	Weight of clot	Percentage of lysis	Average of lysis percentage
1.	0.814	1.116	0.853	0.012	4.363%	5.41%
2.	0.817	1.065	0.833	0.016	6.451%	

Chapter 4

Discussion

In the present study from the heat induced haemolysis process, the extract of *Blumea lacera* is effective in inhibiting cell lysis at different concentration. The percentage of membrane stabilization of methanolic extract of *Blumea lacera* by heat induced were done at 100, 200, 300, 400 and 500 µg/mL concentrations and the lysis inhibition percentage were 30.21%, 48.33%, 55.89%, 61.03% and 72.80% respectively. It is clear that anti-inflammatory activity of the extract by haemolysis were concentration dependent. From the result, it has been found that the concentration of 400 µg/mL and 500 µg/mL have shown remarkable inhibition rate and those are 61.03% and 71.80% respectively, compared to the standard drug (aspirin).

On the other hand, from the hypotonicity induced haemolysis procedure the extract again showed in the heat induced procedure. The most significant result showed at the highest 500 µg/mL concentration which is 74.08% of erythrocyte membrane lysis. At other concentrations the percentage of cell lysis inhibition activity are 100 µg/mL – 26.57%; 200 µg/mL – 42.52%; 300 µg/mL – 53.15% and 400 µg/mL – 69.43%. Here, the anti-inflammatory activity is also dose dependent. So from both of the experiments regarding anti-inflammatory property investigation it has been found that the methanolic extract of *Blumea lacera* has a significant potential to be used as an anti-inflammatory agent.

Another study was on investigation of thrombolytic activity by the methanolic extract of *Blumea lacera.* Thrombosis is a diseased condition of blood clots inside the blood stream due to failure of hemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death. Mostly used thrombolytic agents are tissue plasminogen activator (tPA) and urokinase (UK), streptokinase (SK). Here for this study commercially available streptokinase

(STK) was taken as standard thrombolytic agent. The test drug (*Blumea lacera*) showed the significant average percentage of clot lysis (61.77%) compared to the standard (49.89%). So *Blumea lacera* extract has a positive thrombolytic property.

Chapter 5

Conclusion

In this study, it can be claimed that *Blumea lacera's* methanolic extract has significant therapeutic potency of inflammation and possess significant thrombolytic activity. Various *in-vitro* study has been conducted on *Blumea lacera* although the study regarding anti-inflammatory property of *Blumea lacera* was yet to be done. After completion of the study, it is evident that concentration of *Blumea lacera* extract exhibit significant anti-inflammatory property than the standard aspirin especially at high concentration by preventing higher percentage erythrocyte lysis (Table- 6 & 9). Due to the presence of active compounds like alkaloids, steroids, terpenoids, flavonoids, glycosides, tannins and phenolic compounds may responsible for this activity.

Another experiment regarding the investigation of thrombolytic property of *Blumea lacera* where streptokinase was used as the positive thrombolytic agent and distilled water as non-thrombolytic agent. From the comparative study with the streptokinase and distilled water, *Blumea lacera* showed the positivity in this result. Positive results of thrombolytic test led us to interference that the plant extract may have the bioactive compounds that may aid for ongoing cardiovascular drug discovery from the herbal resources. It is clear that *Blumea lacera* extraction shows better thrombolytic activity than the standard thrombolytic agent (Table- 10). However, some unpredictability regarding effectiveness and efficiency of the plant extract may urge as an issue in treatment purpose; less side effects, non-toxicity, isolated phytochemical and purified plant extract can be a great deal against inflammatory disease and thrombosis.

Chapter 6

Future prospects

Further studies on this plant (*Blumea lacera*) can also be carried out-

- Isolation of individual phytochemical constituents.
- Investigate anti-inflammatory activity by *in-vivo* study.
- Determine the actual mechanism by which *Blumea lacera* shields red blood cells to protect against autoimmune conditions.
- Other biological investigations like anxiolytic, analgesic, antipyretic activity etc.

References

Bhuiya, N. M. M. A., Hasan, M., Mahmud, Z. Al, Qais, N., Kabir, M. S. H., Ahmed, F., & Uddin, M. M. N. (2017). In vivo analgesic, antipyretic and anti-inflammatory potential of leaf extracts and fractions of Eria javanica. *Journal of Complementary and Integrative Medicine*, *14*(1). https://doi.org/10.1515/jcim-2016-0040

G, L., & S, M. D. (2011). Invitro Anti-Inflammatory Activity of Methanol Extract of Enicostemma Axillare. *International Journal of Drug Development and Research*, *3*(3), 189–196.

Hossain, M. I., & Mahmood, A. Al. (2015). *Study on in-vitro thrombolytic activity of methanolic extract of Mesua ferrea leaves*. 52–55.

Hossain, M., Islam, M., Azad, A. K., & Faruq, A. Al. (2016). in-Vivo Investigation of Analgesic , Antipyretic Anti-Diarrheal and Anxiolytic Activity of Blumea Densiflora Dc . *EUROPEAN JOURNAL OF PHARMACEUTICAL Research Article AND MEDICAL RESEARCH*, *3*(11), 50–55.

Khair, A. (2014). Pharmacological Activities of Blumea lacera (Burm. f) DC: A Medicinal Plant of Bangladesh. *British Journal of Pharmaceutical Research*, *4*(13), 1677–1687. https://doi.org/10.9734/bjpr/2014/10001

Ramjan, A., Hossain, M., Runa, J. F., Md, H., & Mahmodul, I. (2014). Evaluation of thrombolytic potential of three medicinal plants available in Bangladesh, as a potent source of thrombolytic compounds. *Avicenna Journal of Phytomedicine*, *4*(6), 430–436.

Sreelekha, K. P., Krishna T. P. A., Krishna, T. P. A., Deepa, P. E., Darsana, U., Juliet, S., Nair, S. N., Ravindran, R. (2017). Pharmaco-chemical characterization of leaves of Blumea mollis (D. Don) merr. from Western Ghats of wayanad region of Kerala, India.

Journal of Pharmacognosy and Phytochemistry, *6*(4), 319–323.

Blumea balsamifera Sembong, Capa, Telinga kerbau. (1897).

Khatri, S., Phougat, N., Chaudhary, R., Singh, B., & Chhillar, A. K. (2016). International journal of pharmacy and pharmaceutical sciences. *International Journal of Pharmacy and Pharmaceutical Sciences*, *8*(8), 313–319. Retrieved from

Pang, Y., Wang, D., Fan, Z., Chen, X., Yu, F., Hu, X., … Yuan, L. (2014). Blumea balsamifera- A phytochemical and pharmacological review. *Molecules*, *19*(7), 9453–9477. https://doi.org/10.3390/molecules19079453

Pharmaco-chemical characterization of leaves of Blumea mollis (D. Don) merr. from Western Ghats of wayanad region of Kerala, India. (2017). *Journal of Pharmacognosy and Phytochemistry*, *6*(4), 319–323.

Ibrahim, M., Ahsan, Q., Homa, Z., Ruhul Kuddus, M., Khair, A., Bin Rashid, R., & Abdur Rashid, M. (2014). Pharmacological Activities of *Blumea lacera* (Burm. f) DC: A Medicinal Plant of Bangladesh Phytochemistry View project Agricultural growth in Bangladesh View project Pharmacological Activities of *Blumea lacera* (Burm. f) DC: A Medicinal Plant of Bangladesh. *Original Research Article British Journal of Pharmaceutical Research*, *4*(13), 1677–1687. https://doi.org/10.13140/2.1.3054.7206

Kannan, S. (2006). Free radical theory of autoimmunity. *Theoretical Biology and Medical Modelling*, *3*, 1–16. https://doi.org/10.1186/1742-4682-3-22

Murugananthan, G., G, S. K., P, S. C., & Mohan, S. (2017). *Anti-Arthritic and Anti-Inflammatory Constituents from Medicinal Plants*. (April 2013). https://doi.org/10.7324/JAPS.2013.3429

Phyu Myint, P., Nyein Sann, A., Mar Soe, N., & Phyu Phyu Myint, C. (2017). Screening of phytochemicals, antioxidant and antimicrobial activities of *Blumea lacera* (Burm. f.) DC. Leaf and Root. ~ *31 ~ Journal of Medicinal Plants Studies*, *5*(4), 31–35. Retrieved from http://www.plantsjournal.com/archives/2017/vol5issue4/PartA/5-3-56-134.pdf

Salisu, A., Veronica, E., Ogechi, N., Uwem, A., & Olakunle, F. (2015). *A comparative study of the phytochemical , antibacterial and scavenging effects of methanolic leaves extract of Blumea lacera (Burm . F .) DC and Blumea aurita (Linn F .) DC*. *2*(6), 264–270.

Singh, A., Malhotra, S., & Subban, R. (2008). Anti-inflammatory and analgesic agents from Indian medicinal plants. *International Journal of Integrative Biology*, *3*(1), 57–72.

Williams LAD, Connar AO, Latore L, Dennis O, Ringer S, Whittaker JA, Conrad J, Vogler, B, Rosner H, Kraus W. The in vitro anti-denaturation effects induced by natural products and nonsteroidal compounds in heat treated (immunogenic) bovine serum albumin is proposed as a screening assay for the detection of anti-inflammatory compounds, without the use of animals, in the early stages of the drug discovery process. West Indian Med J. 2008; 57: 327–331

Sree Kumari, C., Yasmin, N., Hussain, M. R., & Babuselvam, M. (2015). In vitro anti-inflammatory and anti-arthritic property of Rhizopra mucronata leaves. *International Journal of Pharma Sciences and Research (IJPSR)*, *6*(3), 482–485. Retrieved from http://www.ijpsr.info/docs/IJPSR15-06-03-052.pdf