THROMBOLYTIC AND BLEEDING TIME PROPERTIES OF ETHANOLIC EXTRACT OF *Carum copticum* AND *Nicotiana tabbacum*

A Thesis

By

MOST. NAIMA ISLAM
Registration No. 1305104
Semester: July- December, 2014
Session: 2013-14

MASTER OF SCIENCE (M.S.)
IN
PHARMACOLOGY

DEPARTMENT OF PHARMACOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

DECEMBER, 2014

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Submitted to the Department of Physiology & Pharmacology
Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh
In Partial fulfillment of the requirements
For the degree of

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DECEMBER, 2014

DEDICATED
TO MY
BELOVED PARENTS
AND
RESPECTED TEACHERS

ACKNOWLEDGEMENT

All praises are to the Almighty Allah, who kindly enables the author to complete the present research work successfully and to submit the thesis leading to Master of Science (MS)degree in Pharmacology.

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The Author

December, 2014

ABSTRACT

In this present study, the leaves extracts of tobacco leaves and azwain subjected to the thrombolytic activities were assessed by using human erythrocyte and the results were compared with standard streptokinase (SK). They have great impact on bleeding time and clotting time. An experiment was carried out in the laboratory of the department of physiology and pharmacology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, during the period of July-December/2014. Some plant and spices were collected from Dinajpur district. Primarily ethanol extract was prepared from those plant and spices. 20 micropipette was taken filled with 0.5 ml human blood and 3 micropipette was taken as excess micropipette for control. These two kind of micropipette were screened for assessment their comparative efficacy of clotted blood lysis. Ethanol extract was used at a dose of 100 micro ml/ micropipette. Then this micropipette was incubated at 37 degree centigrade temperature for 1hr 45 minutes. After incubation I found 4 plant and 2 spices has anticoagulant property. These plant are tobacco flower, Fern, Arum, Cannabis Tezpata and spices are Jowin and Pesta. Finally I was experimented with Jowin and tobacco *in-vivo* thrombolytic and bleed time property .Bleed time and thrombolytic property was experimented in laboratory compared with Streptokinase (SK). Streptokinase showed 88.13% efficacy after treatment on rabbit. Efficacy was recorded by compare among streptokinase control treated extract. Ethanol jawin (*Carum copticum*) extract lysis cloted blood 53.24% and ethanolic tobacco (*Nicotiana tabacum)* flower extract lysis cloted blood 46.28%.

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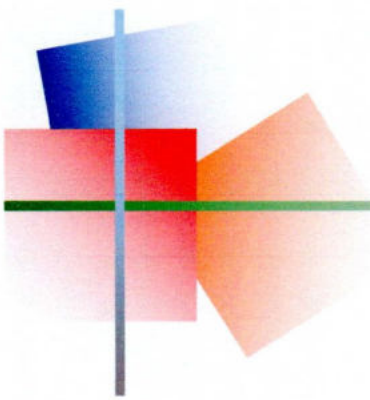
LIST OF ABBREVIATIONS

B.wt.	:	Body weight
BAU	:	Bangladesh Agricultural University
Conc.	:	Concentration
Cu mm	:	Cubic millimeter
d.w.	:	Drinking water
ESR	:	Erythrocyte Sedimentation Rate
et al.	:	Associates
Fig.	:	Figure
Gm	:	Gram
Hb	:	Hemoglobin
i.e.	:	That is
J.	:	Journal
Kg	:	Kilogram
Lit	:	Liter
Ltd.	:	Limited
Mg	:	Milligram
Ml	:	Milliliter
mm^3	:	cubic millimeter
No.	:	Number
PBS	:	Phosphate Buffer Solution
PCV	:	Packed Cell
PM	:	Population Mean
SE	:	Standard Error

LIST OF ABBREVIATIONS (contd.)

SM	:	Sample Mean
TEC	:	Total Erythrocyte Count
Vol.	:	Volume
µg	:	Microgram
%	:	Percent
&	:	And
@	:	At the rate of
<	:	Less than
>	:	Greater than
±	:	Plus minus
0^0C	:	Degree centigrade



Chapter 1

INTRODUCTION

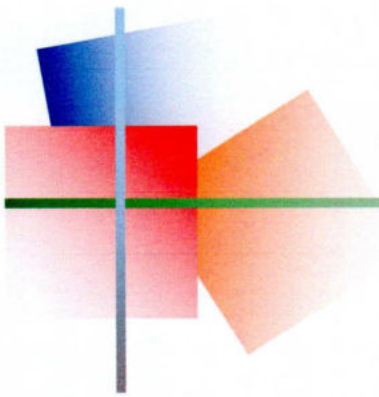
CHAPTER 1

INTRODUCTION

This study aims to investigate whether organic extracts possess thrombolytic properties with minimal or no toxicity. Coronary artery thrombosis has been treated by urokinase (UK), streptokinase (SK) or tissue plasminogen activators (t-PA) which are widely used clinical thrombolytic agent for the treatment of severe or massive deep venous thrombosis, pulmonary embolism, myocardial infarction, and occluded intravenous or dialysis cannulas. Although UK and SK are widely used in India, Bangladesh and other developing countries due to lower cost as compared to other thrombolytic drugs but, the use is associated with high risk of bleeding intracranial hemorrhage, severe anaphylactic reaction and lacks specificity. Moreover, these drugs are not used in patients who have undergone surgery or those with a history of nervous lesions, gastrointestinal bleeding or hypertension. However, as a result of immunogenicity multiple treatments with SK in a given patient are restricted. Blood clot or thrombus is a great disorder in the world. Most of the disease are blood clot, heart stroke, coronary artery block etc. synthetic drug like streptokinase, urokinase are available in market. These are expensive and have some side effects. In that situation scientist are searching new herbal drug which have no side effect and available for people, that's why my study was designed to evaluate thrombolytic and bleeding time properties of tobacco and ajawin extract.

In concern to that consideration my study was designed for the following objectives

- To evaluate the impact of Tobacco flower extract and Ajawin extract on bleeding time and clotting time.
- To compare thrombolytic activity of Ethanolic Tobacco flower and Zawain extract with Streptokinase.



Chapter 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Parvin, *et al.* (2013) Phytochemical screenings, thrombolytic activity and antimicrobial properties of the leaf extracts of *Lablab parpureus.* In this present study, the leaves extracts of *Lablab parpureus* were subjected to the thrombolytic activities were assessed by using human erythrocyte and the results were compared with standard streptokinase (SK). On the other hand, leaves extracts of *L. parpureus* revealed moderate antibacterial activity against some microorganisms used in the screening. Preliminary phytochemical investigation suggested the presence of reducing sugar group, tannins, saponins and alkaloids.

Narjis Hadi Mansoor (2013) In vitro study of the anticoagulant activity of some plant extracts examined by prothombin time, anticoagulant activity, red onion, garlic oil, grape oil.

Y., Sai Sandeep, *et al.* (2012) Evaluation of in vitro thrombolytic activity of phytochemicals in Bacopamonnieri Linn. Myocardial or cerebral infractions are the serious consequences in Atherothrombotic diseases leading to death and the side effects produced by consecutive use of thrombolytic agent like t-PA, Urokinase and streptokinase to treat these diseases has become a global concern. Bacopamonnieri has been used for centuries in Ayurveda system of medicine as a memory vitalizer and as a liver and heart tonic. The present study was carried out to evaluate the thrombolytic activity of ethanolic, methanolic, acetone and aqueous extract of different parts (root, stem and leaf) of B. monnieri by in vitro method. Extraction was carried out using Soxhlet apparatus. The leaf ethanolic extract showed highest thrombolysis followed by aqueous, methanol and acetone extract. Concentration of phytochemicals and incubation time were directly proportional to the clot lysis. Application of the present study may be accessible for greater section of the society for treatment of cardiovascular diseases.

Mohammad Shahadat Hossain, *et al.* (2012) In-Vitro Thrombolytic and Anti-inflammatory Activity of *Swertia Chirata.* Ethanolic extract of *Swertiachirata* was assessed for its thrombolytic, anti-inflammatory activity and phytochemical screening. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation. Aspirin was used as a standard drug for the study of anti-inflammatory activity. The ethanol extract of *Swertiachirata* showed mean inhibition of protein denaturation 45.31±0.000576 whereas, for control group it was found to be 50.00±0.00177. In thrombolytic activity using *in vitro* clot lysis assay method, the crude ethanol extract was found to have significant, thrombolytic test showed a maximum effect of 40.38% while the standard streptokinase showed 69.35ct.

Md. R. Al-Mamun*et al.* (2012) Thrombolytic activity of some spices and plants available in Bangladesh. Thrombolytic activities of some plants, namely *Tamarindusindica* (Fabaceae), *Flemingiacongesta* (Fabaceae), *Lawsoniainermis* (Lythraceae), *Mesuanagassarium* (Clusiaceae, andspices, namely *Coriandrum sativum* (Apiaceae), *Curcumalonga* (Zingibera ceae), *Cinnamomumta mala* (Laura ceae), *Nigellasativa* (Ranuncula ceae), *Eugenia aromaticum* (Myrtaceae), available in Bangladesh, were evaluated using an *in vitro* model. The thrombolytic activity in terms of percentage of weight loss of *in vitro* formed clots were found as *C. sativum* 43.25 ± 7.18%, *C. longa* 53.32 ± 4.96%, *C. tamala* 22.10 ± 3.18%, *N. sativa* 28.49 ± 3.72%, *E. aromaticum*32.18 ± 3.10%, *T. indica*28.91 ± 2.29%, *F. congesta*35.27 ± 7.35%, *L. inermis*62.40 ± 5.04%, *M. nagassarium* bark 39.54 ± 7.15% and *M. nagassarium* leaf 46.75 ± 3.97% with reference to the negative control distilled water 8.37 ± 1.18% and positive control streptokinase 84.63 ± 1.03%. Through our study, it was found that *L. inermis* and *C. longa* possess thrombolytic property that could lyse blood clots *in vitro.*

Md. Al Amin Sikder, *et al.* (2011) examined evaluation of ehrombolytic activity of four Bangladeshi medicinal plants, as a possible renewables for thrombolytic Compounds. Four Bangladeshi medicinal plants *Sansevieri atrifasciata*, *Justic agendarussa*, *Hydnocar puskurzii* and *Mesuana gassarium* have been investigated for their *in vitro* thrombolytic activity. The clot lysis activity was assessed by

addition of the test material to the pre-clotted blood and incubation for 90 min. at 37oC and was expressed as % lysis of clot. Each of the plant was extracted with methanol at room temperature and the concentrated methanolic extract was fractionated by the modified Kupchan partitioning method to provide pet-ether, carbon tetrachloride, chloroform and aqueous soluble fractions. Among the four plants the aqueous soluble fraction of *M. nagassarium,* carbon tetrachloride soluble fraction of *H. Kurzii,* aqueous soluble fraction of methanolic extract of *S. trifasciata* exhibited highest thrombolytic activity with clot lysis value of 50.86%, 47.50%, and 47.10% respectively. However, the pet ether and carbon tetrachloride soluble fraction of methanolic extract of *J. gendarussa* demonstrated significant thrombolytic activity as evident from 45.93% and 45.47% lysis of clot, respectively. Standard streptokinase was used as positive control which exhibited 61.50% lysis of clot while the negative control water revealed 2.56% lysis of clot.

J.H. Evangelista *et al,* (2012) examined Preliminary Assessment of In vitro Anticoagulant Activity vs. Heparin 1,000I.U. and Cytotoxicity of Selected Philippine Medicinal Plants. One main cause of mortality in developing countries is thromboembolic disorders such as pulmonary emboli, deep vein thrombosis and heart attacks. Several agents and interventions were available; however, there are still side effects that are acquired through these therapies. Herbal plants are popularly used nowadays in drug discovery due to their ancient medicinal use. Philippines, a tropical country, have a variety of herbal plants. The aim of the study was to assess the anticoagulant activity and cytotoxicity of both flesh and peels/seeds of selected plants available in the Philippines. The plants Allium sativum, Cucurma longa, Ananas comosus and Lycopersicum esculentum were used in the in vitro method using Heparin and water as controls. Percentage clot lysis of the plants were 18.30%, 21.77%, 21.85%, 35.91%, 15.67% and 24.52%, respectively. Only C. longa peel extracts and L. esculentum showed clot lysis beyond the negative control. C. longa peels showed a higher percentage clot lysis as compared with Heparin. Using one-way ANOVA, statistics showed the p value of 0.674574 from the clot lysis activity of all plant extracts. Cytotoxicity of the herbal plants was also determined using brine shrimp lethality assay (BSLA). The

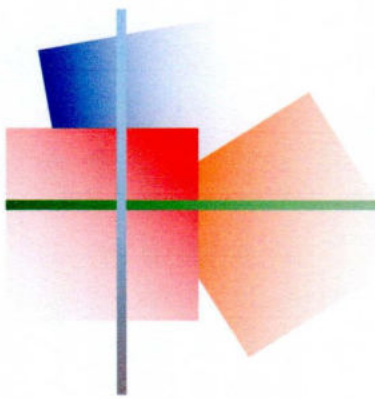
LC50 values of the herbal extracts ranges from 6.72 to 31.2µg/mL. A. sativum and C. longa flesh have the lowest values (most potent among the extracts). Descriptively, C. longa peels extract exhibited promising clot lysis activity, however further studies are still needed to strengthen the effectiveness of these Philippine plants as anticoagulant. Furthermore, it was also seen in this study that fruit and vegetable peels have prospective therapeutic application. It will not only benefit the industrial waste products reduction, but it can also serve as an alternative source of pharmacologic agents,

Jannat-e-Zereen and Gwyneth Ingram (2012) A Possible Involvement of *ACR4,* a Receptor Like Kinase, in Plant Defence Mechanism.

M AtiarRahman, Rabeya Sultana, Talha Bin Emran, M Saiful Islam, M Ashiqur Rahman, Joti Sankhar Chakma, Harun-ur Rashid and **Chowdhury Mohammad Monirul Hasan (2013)** Effects of organic extracts of six Bangladeshi plants on *in vitro* thrombolysis and cytotoxicity, *BMC Complementary and Alternative Medicine*.

Md. Rakib Al-Mamun1,2, Nabiha Amrin2, Jahura Begum2 and Md. Abdul Mazid1 (2012) Thrombolytic activity of some spices and plants available in Bangladesh.

Sweta Prasad, Rajpal Singh Kashyap, and Hatim F Daginawala (2011) Effect of *Fagonia Arabica* (Dhamasa) on in vitro thrombolysis, BioMed Central. Atherothrombotic diseases such as myocardial or cerebral infarction are serious consequences of the thrombus formed in blood vessels. Thrombolytic agents are used to dissolve the already formed clots in the blood vessels; however, these drugs have certain limitations which cause serious and sometimes fatal consequences. Herbal preparations have been used since ancient times for the treatment of several diseases. Herbs and their components possessing antithrombotic activity have been reported before; however, herbs that could be used for thrombolysis has not been reported so far. This study's aim was to investigate whether herbal preparations (aqueous extract) possess thrombolytic activity or not.



Chapter 3

MATERIALS & METHODS

CHAPTER 3

MATERIALS AND METHODS

The experiment was conducted at the department of Physiology and Pharmacology, Hajee Mohammad Danesh Science & Technology University, Dinajpur, during the period from January-June, 2014 to complete the research work following steps were followed:

3.1 Collection of the plant and spices:

Some plant are collected from the HSTU campus, Dinajpur and spices sample was collected from Bahadur Bazar Market, Dinajpur. Plants are Fern, Auram, Tobacco, spices are cardamon, Tezpat, Zira, Zowin, Masted oil, Small alach etc.

3.2 Collection and processing of plant material

Fern, Auram, Tobacco leaves, Cardamon, Tezpat, Zira, Zowin, Masted oil, Small alach were selected for effectiveness as thrombolytic agent on Cardiovascular system. Mature and disease free Fern, Auram, Tobacco leaves, Cardamon, Tezpat, Zira, Zowin, Masted oil, Small alach leaves were collected from HSTU campus.

3.3 Preparation of Ethanol Extract:

3.3.1Ethanol

Ethanol also called ethyl alcohol pure alcohol, grain alcohol, or drinking alcohol, is a volatile, flammable, colorless liquid with the structural formula CH_3CH_2OH, often abbreviated as C_2H_5OH or C_2H_6O. Ethanol is a psychoactive drug and is one of the oldest recreational drugs still used by humans. Ethanol can cause alcohol intoxication when consumed. Best known as the type of alcohol found in alcoholic beverages, it is also used in thermometers, as a solvent, and as a fuel. In common usage, it is often referred to simply as alcohol or spirits.

Ethanol is a 2-carbon alcohol with the empirical formula C_2H_6O. Its molecular formula is CH_3CH_2OH. An alternative notation is $CH_3–CH_2–OH$, which indicates that the carbon of a methyl group ($CH_3–$) is attached to the carbon of a methylene group ($–CH_2–$), which is attached to the oxygen of a hydroxyl group (–OH). It is a constitutional isomer of dimethyl ether.

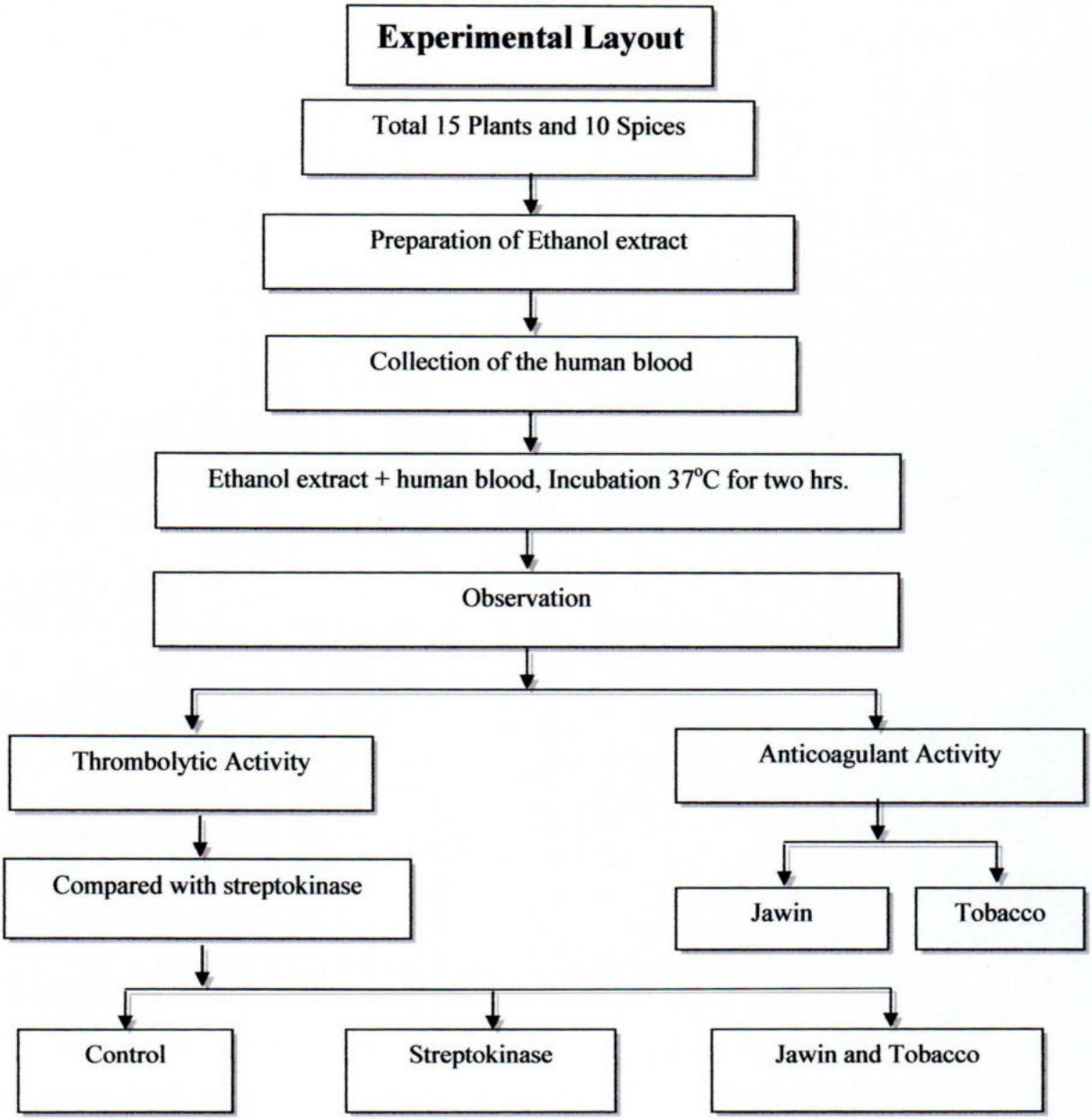


Fig. 3.1 Layout of the experiment

3.3.2 Extract

Extract is a solution of the essential constituents of a herbal agent. It is prepared by boiling the plant material in water and then evaporating the strained decoction to a desired concentration. Extracts harbour the more active principles of the medicinal plants, allowing the less active principles to be removed as a dross. Various solvents may be used including alcohol, water and glycerin. Extracts should always be prepared at the lowest temperature possible that is compatible with good extraction of the healing principles. If care is not taken, some of the lighter and more volatile oils and other important ingredients may be lost. In addition, the vessel in which the extract is prepared should always be tightly covered until such time as the extract has reached a sufficiently cool temperature (i.e., body temperature). Fluid Extract is an alcohol or glycerin preparation of herbal extract containing the active constituents in a definite ratio of plant material to solvent. There are several types of fluid extracts:

- Heated extracts are prepared by boiling (but preferably simmering) a herbal agent in water and then evaporating the strained decoction to a desired concentration — this yields a more concentrated herbal remedy.
- Fluid extract is made by evaporating an already prepared fluid (such as an infusion or decoction) to the desired concentration.
- Cold extract is similar to an infusion. It is prepared by taking twice as much herb as is desired for an infusion and then letting it sit in an enamel or non-metallic pot for eight to twelve hours. It is then strained and taken as one would take an infusion.
- Freeze drying is also used to get active ingredients out of plants and preserve them. The technique is used in the coffee industry as well as for herbs.
- Fresh Plant Extracts are becoming commercially popular. In these cases, fresh plants are used to make the tincture rather than dried material. Sometimes this results in a more potent preparation, other times less

potent. The properties of a fresh plant extract will also vary from dried plant extracts, both positively and negatively.

3.3.3 Plant extract

A plant extract must, by definition, be obtained from a solid-liquid extraction. Solid-liquid extraction is defined as an operation to separate elements contained in a solid body by solubilization with a solvent, and it may be followed by purification. The extract is contained in the solvent. If the solvent is an edible solvent, it is not necessary to dissociate it from the extract. If the solvent is not an edible solvent, separation allows obtaining a dry extract. Selection of raw materials, choice of solvent, process utilization and equipment performance are determining factors. These multiple parameters must be professionally combined. Only their proper combination allows obtaining high-quality plant extracts.

3.3.4 Preparation of leaves extract

For the preparation of extract, the leaves were dried in sun for 10 days and followed by oven at 55-60°C for 2 days. The dried leaves were pulverized with a blender. A 25 (unit) mesh diameter sieve was used to obtain the fine dust, after then dust was preserved in airtight plastic container until they were directly used for screening and preparation of water extract. 10g each leaves powder was added to 70ml of distilled water and was shaking overnight at room temperature, filtered and distilled water was added up to 100ml to make 10% extract.

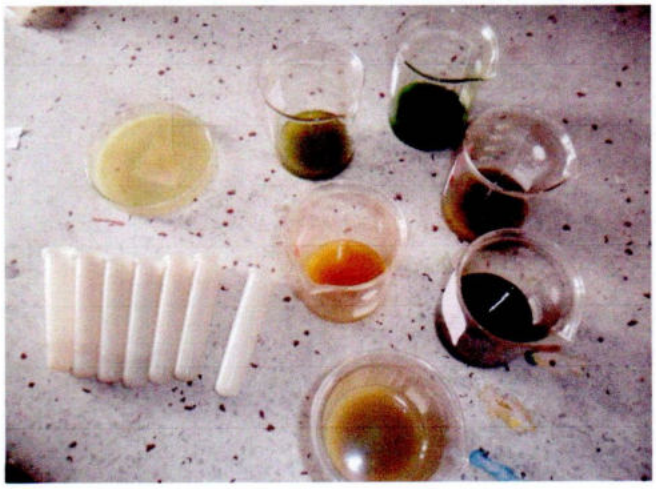


Fig 1: Preparation of ethanol plant extract

3.3.5 Preservation of Plant Extract

The collected plant material (plant parts: stems, leaves, flowers, roots, bark etc.) is dried in a ventilated oven at 45°C for 24 H, and subsequently milled to a fine powder by means of an IKA Universal Mixer M20 (or other type). An amount of 20.0 g of the dried plant powder is weighed in an Erlenmeyer of 100 ml to which 70 ml of hexane (purity grade 99 %) is added (the plant sample has to be submerged with solvent) for pre-extraction. The Erlenmeyer is placed in a sonicator-bath (Branson 8210 or some other type) and sonicated at a temperature 40 ° C during 30 minutes. The mixture is filtered using paper filter, followed by washing the Erlenmeyer with 20 ml of hexane and then with 50 ml of hexane. The filtrate is poured in a round-bottomed flask and the solvent is concentrated in vacuo (at about 11 mm Hg) up to 5-10 ml by means of rotavapor, utilizing a water bath at 40°C. This residue is brought in a 30-ml vessel to let the solvent evaporate. The open vessel is left overnight in a well-ventilated hood in order to evaporate the last traces of the solvent in the hexane pre-extract. The solids, collected on the filter, are broken up and dried in the air overnight in the hood. The dried material is extracted in the same way with methanol-water (90:10). The dried material from the filters placed in an Erlenmeyer of 100 ml to which 70 ml of 90 % methanol is added. The mixture is sonicated as above at 4^0C during 30 minutes, after which it is filtered, followed by washing the Erlenmeyer with 20 ml of 90 % methanol. The filtrate is poured in a round-bottomed flask and the solvent is evaporated in vacuo completely. The dry 90% methanol extract is dissolved in as little as possible 100 % methanol by using the sonicator-bath and poured in a 30-ml vessel to let it evaporate overnight in the hood.

3.4 Collection and management of Human Blood

1oml Human blood were collected from Vein by 10ml syrenge. This blood were kept into 20 micropippet. Each micropippet contain 0.5ml blood. After few minutes blood automatically was clotted and serum was separated from the tube. After then blood containing pippet kept into pippet stand.

3.5 Different plant extract mixing with clotted blood

100 micromililitre ethanol plant extract measured by micropippet syringe and mixed with specific plant extract with specific test tube. Three blood test were preserved for control.

Fig 2: Tobacco extract

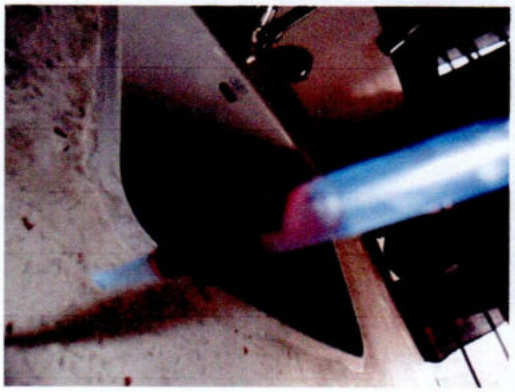


Fig 3: Clot lysis by tobacco leave ethanolic extract

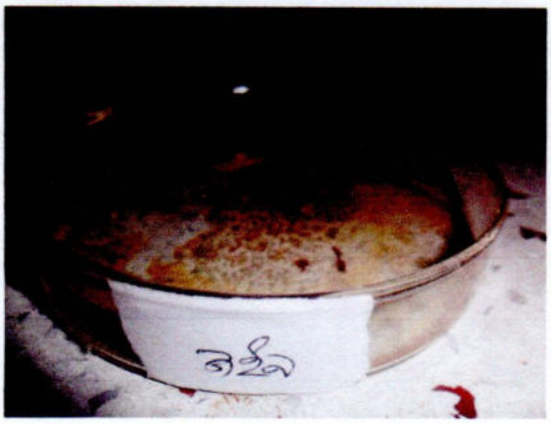


Fig 4: Ajawin extract

Fig 5: Clot lysis by Ajawin ethanolic extract

Fig 6: Different plant extract mixing with clotted blood

Fig 7: During Centrifuge

Fig 8: Clot lysis by strepkinase

Fig 9: Control

3.6 Evaluation of the efficacy of Fern, Auram, Tobacco, spices are cardamon, Tezpat, Zira, Ajawin, Masted oil, Small alach leaves extract as Cardiovascular Disease on thrombosis.

3.7 Anticoagulant

3.7.1 Anticoagulants (antithrombics, fibrinolytic, and thrombolytics) are a class of drugs that work to prevent the coagulation (clotting) of blood. Such substances occur naturally in leeches and blood-sucking insects. A group of pharmaceuticals called anticoagulants can be used *in vivo* as a medication for thrombotic disorders. Some anticoagulants are used in medical equipment, such as test tubes, blood transfusion bags, and renal dialysis equipment.

These oral anticoagulants are derived from coumarin, which is found in many plants. A prominent member of this class is warfarin (Coumadin). It takes at least 48 to 72 hours for the anticoagulant effect to develop. Where an immediate effect is required, heparin must be given concomitantly. These anticoagulants are used to treat patients with deep-vein thrombosis (DVT), pulmonary embolism (PE) and to prevent emboli in patients with atrial fibrillation (AF), and mechanical prosthetic heart valves.

3.7.2 Adverse effects

Patients aged 80 years or more may be especially susceptible to bleeding complications, with a rate of 13 bleeds per 100 person-years. These oral anticoagulants are used widely as poisons for mammalian pests, especially rodents.

(For details, see rodenticide and warfarin.) Depletion of vitamin K by Coumadin therapy increases risk of arterial calcification and heart valve calcification, especially if too much vitamin D is present.

3.7.4 Heparin and derivative substances

Heparin is a biological substance, usually made from pig intestines. It works by activating antithrombin III, which blocks thrombin from clotting blood. Heparin can be used *in vivo* (by injection), and also *in vitro* to prevent blood or plasma clotting in or on medical devices. Invenipuncture, Vacutainerb and blood collecting tubes containing heparin usually have a green cap.

3.7.5 Major pharmaceutical heparin recall due to contamination

In March 2008, major recalls of heparin were announced by pharmaceutical companies due to a suspected and unknown contamination of the raw heparin stock imported from China. The contaminant was later found to be a compound called oversulfated chondroitin sulfate. The US Food and Drug Administration was quoted as stating at least 19 deaths were believed linked to a raw heparin ingredient imported from the People's Republic of China, and they had also received 785 reports of serious injuries associated with the drug's use. According to the New York Times: 'Problems with heparin reported to the agency include difficulty breathing, nausea, vomiting, excessive sweating and rapidly falling blood pressure that in some cases led to life-threatening shock'.

3.7.6 Low molecular weight heparin

Low molecular weight heparin, a more highly processed product, is useful as it does not require monitoring of the APTT coagulation parameter (it has more predictable plasma levels) and has fewer side effects.

3.7.7 Synthetic penta saccharide inhibitors of factor Xa

- Fondaparinux is a synthetic sugar composed of the five sugars (pentasaccharide) in heparin that bind to antithrombin. It is a smaller molecule than low molecular weight heparin.
- Idraparinux

3.7.8 Antithrombin protein therapeutics

The antithrombin protein itself is used as a protein therapeutic that can be purified from human plasma or produced recombinantly (for example, Atryn, which is produced in the milk of genetically modified goats.)

Antithrombin is approved by the FDA as an anticoagulant for the prevention of clots before, during, or after surgery or birthing in patients with hereditary antithrombin deficiency.

3.7.9 Food and herbal supplements

Foods and food supplements with blood- thinning effects include nattokinase, lumbrokinase, beer, bilberry, celery, cranberries, fish oil, garlic, ginger, ginkgo, ginseng, green tea, horse chestnut, licorice, niacin, onion, papaya, pomegranate, red clover, soybean, St. John's wort, turmeric, wheatgrass, and willowbark. Many herbal supplements have blood-thinning properties, such as danshen and feverfew. Multivitamins that do not interact with clotting are available for patients on anticoagulants.

However, some foods and supplements encourage clotting. These include alfalfa, avocado, cat's claw, coenzyme Q10, and dark leafy greens such as spinach. Their intake should be avoided whilst taking anticoagulants or, if coagulability is being monitored, their intake should be kept approximately constant so that anticoagulant dosage can be maintained at a level high enough to counteract this effect without fluctuations in coagulability.

Grapefruit interferes with some anticoagulant drugs, increasing the amount of time it takes for them to be metabolized out of the body, and so should be eaten only with caution when on anticoagulant drugs.

3.7.10 Laboratory use

Laboratory instruments, blood transfusion bags, and medical and surgical equipment will get clogged up and become nonoperational if blood is allowed to clot. In addition, test tubes used for laboratory blood tests will have chemicals

added to stop blood clotting. Apart from heparin, most of these chemicals work by binding calcium ions, preventing the coagulation proteins from using them.

- EDTA strongly and irreversibly binds calcium. It is in a powdered form. Full Form of EDTA is Ethylene Diamine Tetra Acetic Acid. It chelates calcium ion to prevent blood from clotting.
- Citrate is in liquid form in the tube and is used for coagulation tests, as well as in blood transfusion bags. It binds the calcium, but not as strongly as EDTA. Correct proportion of this anticoagulant to blood is crucial because of the dilution, and it can be reversed with the addition of calcium. It can be in the form of sodium citrate or acid-citrate-dextrose.
- Oxalate has a mechanism similar to that of citrate. It is the anticoagulant used in fluoride oxalate tubes used to determine glucose and lactate levels.

3.8 Streptokinase (SK)

3.8.1 Streptokinase (SK) is an enzyme secreted by several species of streptococci that can bind and activate human plasminogen. SK is used as an effective and inexpensive thrombolysis medication in some cases of myocardial infarction (heart attack) and pulmonary embolism. Streptokinase belongs to a group of medications known as fibrinolytics, and complexes of streptokinase with human plasminogen can hydrolytically activate other unbound plasminogen by activating through bond cleavage to produce plasmin. There are three domains to Streptokinase, denoted α (residues 1–150), β (residues 151–287), and γ (residues 288–414). Each domain binds plasminogen, although none can activate plasminogen independently.

3.8.2 Mechanism of action

Plasmin is produced in the blood to break down fibrin, the major constituent of blood thrombi, thereby dissolving clots once they have fulfilled their purpose of stopping bleeding. Extra production of plasmin caused by streptokinase breaks down unwanted blood clots, for example, in the lungs (pulmonary embolism). The usual activation of Plasminogen (Plgn) is by proteolysis of the Arg561—Val562 bond. The amino group of Val562 then forms a salt-bridge with Asp740, which

triggers a conformational change producing the active protease Plasmin (Pm). When (SK) is present, it binds to Plgn to form a complex (SK. Plgn) that converts substrate Plgn to Pm. Residues 1–59 of SK regulate its capacity to induce an active site in bound Pg by a nonproteolytic mechanism and to activate substrate Pg in a fibrin-independent manner. This complex subsequently rearranges to an active complex although the Arg561–Val562 bond remains intact. Therefore another residue must substitute for the free amino group of Val562 and provide a counterion for Asp740 in this active complex. Two candidates for this counterion have been suggested: Ile1 of streptokinase and Lys698 of Plgn. Deletion of Ile1 of SK markedly inhibits its capacity to induce an active site in plasminogen, which supports the hypothesis that establishment of a salt bridge between Ile1 of SK and Asp740 of plasminogen is necessary for SK to induce an active site in plasminogen by a nonproteolytic mechanism. In contrast with the Ile1 substitutions, the Lys698 mutations also decreased the dissociation constant of the SK complex by 15 to 50 fold. These observations suggest that Lys698 is involved in formation of the initial SK· Plgn complex.

3.8.3 Administration

If PCI is not available within 90 minutes of first contact, streptokinase is given intravenously as soon as possible after the onset of a heart attack to dissolve clots in the arteries of the heart wall. As Streptokinase is a bacterial product, the body has the ability to build up an immunity to it. Therefore, it is recommended that this medication should not be used again after four days from the first administration, as it may not be as effective and can also cause an allergic reaction. For this reason, it is usually given only for a person's first heart attack. Further thrombotic events could be treated with Tissue plasminogen activator (tPA). Overdose of streptokinase or tPA can be treated with aminocaproic acid.

3.9 Plant material collection

Ajawin (Carum copticum) and tobacco flower (*Nicotiana tabacum)* were collected from local market Bahadur Bazar at Dinajpur in Bangladesh. The seed were washed with fresh water and dried under shade at room temperature. The seed

were sinked into ethanol at two days. The seed were grinded wit morter and pestle and squeezed out the liquid portion with hand and centrifuged at 1000 rpm. Then dried out the water from the centrifugal material and powdered and stored. 60g of powdered drug was extracted separately with methanol, petroleum ether by continuous hot percolation in soxlet apparatus and with water by cold maceration for 3 days respectively. All the extracts were filtered and evaporated using a rotary evaporator. Dried extracts were stored at 20°c until used.

3.10 Study population

Blood samples obtained from my dear batchmates, were used to assess the anticoagulant effects. Participants were 25-26 years old. They had been chosen for this study according to the following criteria: having normal prothrombin time, not suffering from any cardiovascular diseases (hypertension, congestive heart failure, coagulation disorders such as, Hemophilia A or B) or diabetes, not recently using nonsteroidalanti inflammatory drugs, not obese or smokers and free from dyslipidemic disorders.

3.11 Collection of blood samples

The blood samples were obtained from normal individuals by using sterile syringes, withdrawn from vein of right arm of each individual and placed separately in containers containing tri-sodium citrate to prevent the clotting process. Centrifugation (15 minutes at rate 3000 rpm) was carried out to separate the blood cells from plasma in order to obtain pure platelet plasma (ppp) for prothrombin time test. The obtained plasma sample of each individual were poured separately in plane containers using automatic pipette and stored at room temperature.

3.12 In-vitro thombolytic activity of *Carumcopticum (Ajawin)*

***Blood sample*:** Blood (n=6) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

***Thrombolytic activity*:** The thrombolytic activity of all extracts was evaluated by the method developed by Daginawala (2006) and slightly modified by Kawsar *et al.* (2011) using streptokinase (SK) as the standard.

Table 1: Thrombolytic activity of different fractions of *Carum copticum (Ajawin)*

Sample	Thrombolytic Activity (% of lysis)
SK	88.13%
Water	3.9%
ESF	53.24 %
TFE	46.68%

SK= Streptokinase, TFE=Tobacco flower extract and ESF= Ethanolic soluble fraction of the seed extracts of *Carum copticum (Ajawin)*

3.12.1 Phytochemical screening

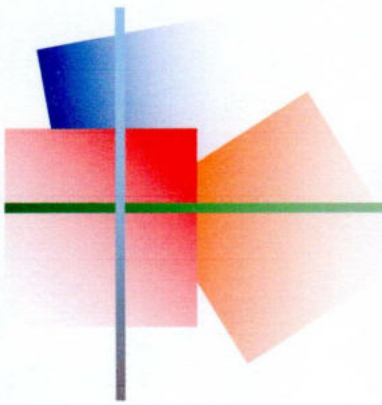
Dried extracts were subjected for the presence of different phyto constituent like alkaloid, steroid, flavonoid, tannin, glycoside etc.

3.12.2 Collection of blood and Plasma re-calcification

0.2 ml plasma, 0.1 ml of crude extract of different concentration and different volume of $CaCl_2$ (25 *m*M) were added together in a clean fusion tube and incubated at 37^0C in water bath. For control experiment extract solution was replaced by same volume of 0.9% saline water. The clotting time was recorded with stopwatch by tilting the test tubes every 5 seconds. This time is called the prothrombin time.

***Blood sample*:** Blood (n=6) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

***Thrombolytic activity*:** The thrombolytic activity of all extracts was evaluated by the method developed by Daginawala (2006) and slightly modified by Kawsar *et al.* (2011) using streptokinase (SK) as the standard.



Chapter 4

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

This experiment was conducted to study the Thrombolytic activity of ethanol plant extract that may act on cardiovascular thrombus.

This experiment was held under the Department of Physiology & Pharmacology, Faculty of Veterinary and Animal Science.

Finally five plant and spices have thrombolytic activity after first trial. I was carrying on my experiment these five plant and spices such as jawin, pesta, fern, auram (kochu) and tobacco plant.

The experimental units were kept in the laboratory. At first I weighted micro test tubes. The weight of each test tube is 0.792. Fresh and clean water was made available at all the times. The experiment was conducted according to the completely randomized design.

extractives of *Carum copticum (Ajawin)* 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 91.07% lysis of clot. At the same time, distilled water was treated as negative control which exhibited negligible lysis of clot (4.74%). In this study, the carbon tetrachloride soluble fraction (CTCSF) exhibited highest thrombolytic

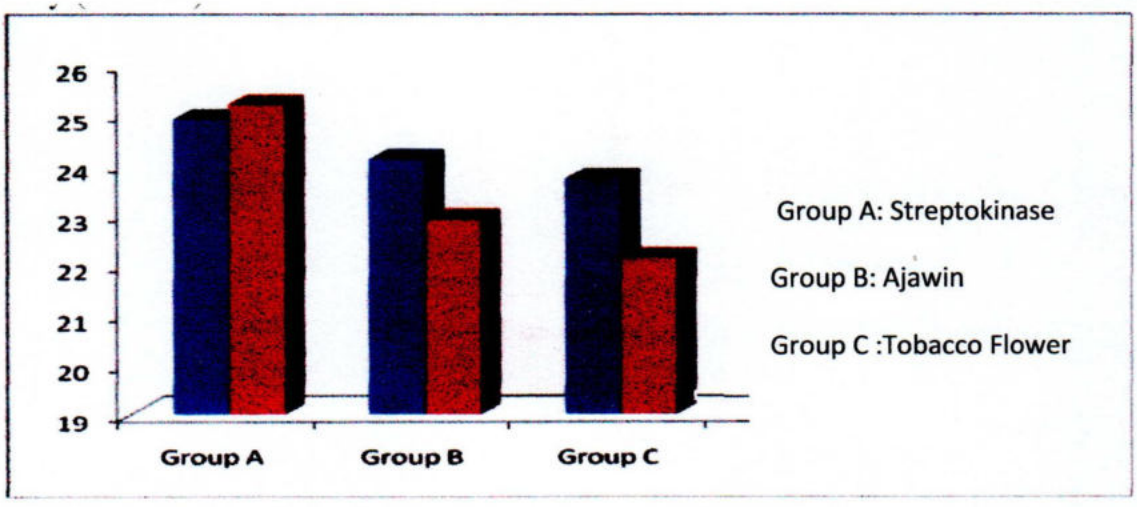


Fig: Thrombolytic activity

Table 2: Lysis of clotted blood by Tobacco flower, Ajawin and Streptokinase at standard Thrombolytic agent.

Treated Grpup	Sl. No.	Weight of Blood Before Lysis (gm)	Weight of blood (After lysis)	Result (amount of lysised blood) gm	Percentage	Average
Tobacco flower extract	1st	0.69	0.36	0.33	47.8%	46.28%
	2nd	0.67	0.35	0.32	42.6%	
	3rd	0.68	0.39	0.29	42.6%	
	4th	0.66	0.35	0.31	46.9%	
	5th	0.69	0.37	0.32	46.4%	
Ajawin extract	1st	0.31	0.15	0.16	51.6%	53.24%
	2nd	0.36	0.16	0.20	55.5%	
	3rd	0.34	0.17	0.17	50%	
	4th	0.35	0.15	0.20	57.1%	
	5th	0.38	0.18	0.20	52.6%	
Streptokinase	1st	0.39	0.02	0.37	94.87%	88.13%
	2nd	0.44	0.04	0.40	90%	
	3rd	0.45	0.04	0.41	91%	
	4th	0.46	0.07	0.36	84.78%	
	5th	0.45	0.09	0.36	80%	

Note: From above data we can see. Tobacco flower extract has 46.28% of thrombolytic activity and Ajawin extract has 53.24% thrombolytic activity shown.

Our standard Thrombolytic agent is streptokinase that is active 88.13%. Ajawin extract is effected in thrombolytic activity greater than Tobacco flower extract. These two extract is significantly effected but streptokinase is higher than Tobacco flower extract and Ajawin extract.

Table 3: Lysis of clotted blood by Ajawin Ethanol Extract

Sl. No.	Weight of Blood Before Lysis (gm) (mean ± SEmean)	Weight of blood (After lysis) (mean ± SEmean)	Result (amount of lysised blood)gm (mean ± SEmean)
A=1st Day	0.3600 b ± 0.0071	0.1540 b ± 0.0075	0.1646 a ± 0.0358
B=2nd Day	0.4140 a ± 0.0121	0.1840 a ± 0.0098	0.2090 a ± 0.0465
C=3rd Day	0.4360 a ± 0.0186	0.1900 a ± 0.0114	0.2064 a ± 0.0472
Probability value	0.0014	0.0479	0.00
LSD value	0.04358	0.01378	0.1307
CV %	7.27	12.32	50.30

Note: Values followed by same superscripts in the same Colum are not statistically significant ($p>0.05$), different superscripts indicate that difference is significant ($p<0.05$). In this other tables, A=Total amount of blood, B= Weight of blood (After lysis), C= Result (amount of lysised blood) gm, Control no change.

Clotting time and Bleeding time:

Firstly I take the normal clotting time and bleeding time of rabbit five times then I inserted the streptokinase in one rabbit. Tobacco flower ethanolic extract and ajawin ethanolic extract inserted on other two rabbit. After two hour I take the bleeding time and clotting time (on microscope) five times. These results are given below:

Table 4: Bleeding time and Clotting time on Rabbit by inserting Tobacco flower ethanolic extract, Zawain ethanolic extract and Streptokinase

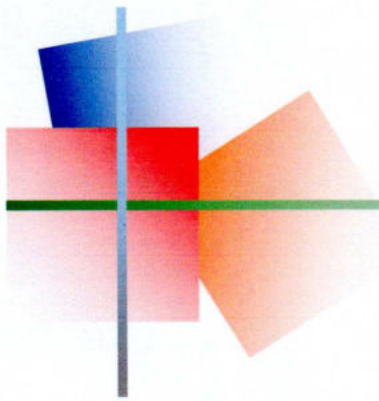
Name of the plant extract/drug	Cloting Time		Bleeding time	
	Normal	Treated	Normal	Treated
Tobacco flower ethanolic extract	2 min. 15 sec.	2 min. 35 sec.	55 sec.	1 min. 13 sec.
	2 min. 17 sec.	2 min. 30 sec.	57 sec.	1 min. 12 sec.
	2 min. 14 sec.	2 min. 32 sec.	54 sec.	1 min. 10 sec.
	2 min. 18 sec.	2 min. 36 sec.	56 sec.	1 min. 14 sec.
	2 min. 15 sec.	2 min. 30 sec.	54 sec.	1 min. 13 sec.
Zawain ethanolic extract	2 min. 17 sec.	2 min. 35 sec.	50 sec.	1 min. 13 sec.
	2 min. 14 sec.	2 min. 40 sec.	44 sec.	1 min. 08 sec.
	2 min. 15 sec.	2 min. 30 sec.	54 sec.	1 min. 15 sec.
	2 min. 18 sec.	1 min. 55 sec.	52 sec.	1 min. 12 sec.
	2 min. 16 sec.	2 min. 25 sec.	45 sec.	1 min. 15 sec.
Streptokinase	2 min. 15 sec.	4 min. 6 sec.	1 min. 5sec.	1 min. 13 sec.
	2 min. 14 sec.	3 min. 55 sec.	1 min. 9sec.	1 min. 35 sec.
	2 min. 15 sec.	4 min. 03 sec.	1 min. 3sec.	1 min. 33 sec.
	2 min. 14 sec.	4 min. 10 sec.	1 min. 5sec.	1 min. 25 sec.
	2 min. 16 sec.	4 min. 16 sec.	1 min. 6sec.	1 min. 40 sec.

Note: From above data, we can see Zawain ethanolic extract and Tobacco flower ethanolic extract is delay the bleeding time and clotting time little more but Streptokinase is delay the bleeding time and clotting time than Zawain ethanolic extract and Tobacco flower ethanolic extract.

Table 5: Analysis of Result: Bleeding time and Clotting time on Rabbit by inserting ethanolic tobacco flower and zawain extract

Name of the plant extract/drug	Cloting Time (minute)		Bleeding time (minute)	
	Normal (Mean ± SE mean)	Treated (Mean ± SE mean)	Normal (Mean ± SE mean)	Treated (Mean ± SE mean)
Tobacco ethanolic extract	2.16a ± 0.00629	2.33b ± 0.01249	0.55b ± 0.00583	1.12 b ± 0.00678
Zawainethanolic extract	2.16a ± 0.00707	2.17b ± 0.15700	0.49c ± 0.01949	1.13 b ± 0.01288
Streptokinase	2.15a ± 0.00374	3.98a ± 0.10968	1.06a ± 0.00980	1.29 a ± 0.04716
LSD value	0.014	0.340	0.044	0.087
Probability value	0.380	0.00	0.00	0.002
CV %	0.65	8.77	4.17	5.40

Note : Values followed by same superscripts in the same Colum are not statistically significant ($p>0.05$), different superscripts indicate that difference is significant ($p<0.05$). In this other tables, A= Normal clotting time, B= Treated Bleeding time, C= Result (amount of lysised blood) gm, Control no change.



Chapter 5

SUMMARY AND CONCLUSION

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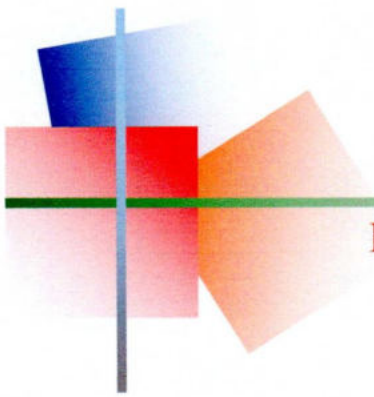
SUMMARY AND CONCLUSION

From my study, In comparison of thrombolytic activity, Streptokinase showed 88.13% as a standard agent, where Ethanolic ajawin extract 53.24% and ethanolic tobacco flower extract 46.28% also showed significant result.

In bleeding time properties there was no change in case of ethanolic tobacco flower extract and ethanolic ajawin extract but streptokinase showed significant result.

Further studies are necessary to in vivo analysis or chemical compositions analysis of tobacco flower extract and ajawin extract.



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