A PRELIMINARY STUDY ON BLOOD COAGULATION ACTIVITIES OF MALAYSIAN *Mikania cordata* LEAVES

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UNIVERSITI SAINS MALAYSIA

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A PRELIMINARY STUDY ON BLOOD COAGULATION ACTIVITIES OF MALAYSIAN *Mikania cordata* LEAVES

by

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DECLARATION

I hereby declare that I am the sole author of this thesis entitled "A Preliminary Study on Blood Coagulation Activities of Malaysian *Mikania cordata* Leaves". I declare that this thesis is being submitted to Universiti Sains Malaysia (USM) for the purpose of the award of Master of Science in Transfusion Science. This dissertation is the result of my own research under the supervision of Dr Badrul Hisham Yahaya except as cited in the references. The dissertation has been accepted for the study performed and is not concurrently submitted in candidature of any other degree.

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LIST OF SYMBOLS AND ABBREVIATIONS

LIST OF ABBREVIATIONS

AMDI : Advanced Medical & Dental Institute

APTT : Activated Partial Thromboplastin Time

BSTFA : N,O-Bis(trimethylsilyl)trifluoroacetamide

CVA : Cerebral vascular accident

DIC : Disseminated Intravascular Coagulopathy

GCMS : Gas Chromatography Mass Spectrometry

HPLC : High Performance Liquid Chromatography

HMWK : High-molecular-weight kininogen

MSD : Mass Selective Detector

NIST : National Institute of Standards and Technology

NRCS : Natural Resources Conservation Service

PPP : Platelet Poor Plasma

PT : Prothrombin Time

TFPI : Tissue factor inhibitor

TT : Thrombin Time

LIST OF SYMBOLS

%	: percentage
µm	: micrometer
dH_2O	: Distilled water
g	: Gram
m	: meter
ml	: Mililiter
mm	: millimeter
NaCl	: Sodium Chloride
ºC	: Degree Celcius
rpm	: rotation per minutes
w/v	: weight / volume
µg	: Microgram

ABSTRAK

Ejen antikoagulan dan antifibrinolitik yang sedia ada, dilaporkan mempunyai kesan sampingan yang boleh mengancam nyawa. Oleh itu pencarian ejen baru dari sumber semulajadi sangat diperlukan pada masa kini. *Mikania cordata*, juga dikenali sebagai selaput tunggul telah digunakan secara tradisional oleh golongan tua untuk merawat pelbagai jenis jangkitan dan penyakit. Tujuan kajian ini adalah untuk mengkaji kesan *M. cordata* ke atas aktiviti pembekuan darah dan menganalisis sebatian bioaktif yang ada di dalam ekstrak daun yang mungkin mempunyai kesan ke atas aktiviti pembekukan darah. Aktiviti pembekuan darah dikaji secara *in vitro* menggunakan ujian masa prothrombin (PT), pengaktifan separa masa tromboplastin (APTT) dan masa thrombin (TT) yang diukur mengunakan plasma yang mengandungi antikoagulan sitrat daripada penderma sukarela yang sihat. Plasma dicampurkan dengan ekstrak daun mengikut kepekatan yang berbeza (0.78, 1.56, 3.13, 6.25, 12.50 dan 25.00 mg/mL) dan keputusan menunjukkan bahawa masa APTT dan TT berpanjangan berbanding dengan kontrol. Namun begitu, ujian PT menunjukkan keputusan aktiviti prokoagulan pada kepekatan 3.13mg/mL dan aktiviti antikoagulan pada kepekatan 12.5 dan 25.0 mg/mL. Keputusan analisis kromatografi gas - spektrometri jisim menunjukkan kehadiran sebatian bioaktif dalam ekstrak akueus daun *M. cordata* seperti asid caffeic, bis (trimethylsilyl) ester O (trimethylsilyl) - asid malic; 2,3,4,5- tetrakis-O (trimethylsilyl) - arabinose; 1,2,3,5- tetrakis-O (trimethylsilyl) - arabinofuranose; octakis (trimethylsilyl) melibiose; dan octakis (trimethylsilyl) - maltosa yang menyumbang kepada aktiviti antikoagulan. Sebagai kesimpulan, penemuan ini menunjukkan bahawa *M. cordata* berpotensi menjadi produk antikoagulan berasaskan tumbuhan untuk keperluan klinikal masa hadapan.

ABSTRACT

Existing anticoagulants and antifibrinolytic agents have been reported to have life-threatening side effects. Therefore, a search for novel agents of natural origin is demanded nowadays. *Mikania cordata* also known as 'selaput tunggul' was traditionally used by folks to treat various infections and diseases. In this study the aimed was to investigate the effect of *M. cordata* in blood coagulation activities and to analyse the bioactive compounds in the leaves extract which might have significant effects on coagulation activities of the blood. The *in vitro* blood coagulation activities such as prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) assay were measured on citrated plasma from healthy volunteer donors. The plasma was spiked with different concentration of aqueous leaves extract (0.78, 1.56, 3.13, 6.25, 12.50 and 25.00 mg/mL) and the results showed that the prolongation of APTT and TT in concentration-dependent manner ($p<0.01$). However in the PT assay, the results showed procoagulant activities at concentration 3.13 mg/mL and anticoagulant activities at concentration 12.5 and 25.0 mg/mL. The result of gas chromatography mass spectrometry (GCMS) analysis showed the presence of bioactive compounds in aqueous extract of *M. cordata* such as caffeic acids, bis (trimethylsilyl) ester O–(trimethylsilyl)- malic acid; 2,3,4,5- tetrakis-O-(trimethylsilyl)- arabinose; 1,2,3,5- tetrakis-O- (trimethylsilyl)- arabinofuranose; octakis (trimethylsilyl) melibiose; and octakis (trimethylsilyl)- maltose are known contributing compounds for anticoagulant effects. As a conclusion, these findings suggest that *M. cordata* may provide a potential plant based anticoagulant products for future clinical use.

CHAPTER 1

INTRODUCTION

1.1 Blood coagulation

Basic coagulation is a major defence mechanism (Lee et al., 2013) and the blood flow must be regulated in order to avoid bleeding by balancing the bleeding (haemorrhage) and clotting (thrombosis) process. Haemostasis is defined as process of blood clotting, which is followed by a process of dissolving or lysing the clotted blood (Harmening, 2001). Many inherited or acquired conditions can disturb its function. The components involved in haemostasis are vasoconstriction process, the platelet adhesion, activation and aggregation, activation of blood coagulation cascade and fibrinolytic system (Hoffbrand et al., 2006).

Vasoconstriction is the process of reducing the diameter of blood vessels to reduce the blood flow at the site of injury and minimise the loss of blood at injury site. Then the platelet adhesion, activation and aggregation take place simultaneously with activation of blood coagulation cascade. Platelet activation will lead to the platelet accumulation at the injury sites and prevent the blood loss. The blood coagulation will continue by production of fibrin to entrap the blood at the injury sites and form blood clots (Ciesla, 2012, Hoffbrand et al., 2006).

Coagulation is divided into two major systems which are primary and secondary haemostasis systems. The primary haemostatic system consists of vasoconstriction and platelet function meanwhile the secondary haemostatic system involved in activation of coagulation cascade proteins, platelet phospholipids, and substrates in a series of delicately balanced enzymatic reactions that lead up to in fibrin formation and reinforce the formation of platelet plug until the healing process complete. The conversion of the soluble fibrinogen into insoluble fibrin clot is accompanied by the thrombin's action. Thrombin is a powerful coagulant factor that formed from prothrombin, a precursor of circulating protein. The process then followed by dissolution of platelet plug and the fibrin clot meshwork is achieved by fibrinolysis process (Ciesla, 2012).

Blood coagulation system is a enzymatic cascade that initiated the response upon the tissue damage (Hoffbrand et al., 2006, Karim et al., 2013). An immediate response of vasoconstriction of the injured vessels is responsible for an initial slowing the blood flow to the area of injury. Cascade of circulating precursor protein are the coagulation factors enzymes which is culminates in the generation of the thrombin and convert the soluble plasma fibrinogen into fibrin. Fibrin will entrap the platelet aggregates at the sites of vascular injury and converts the unstable primary platelets plugs to firm, definitive and stable haemostatic plug (Harmening, 2001, Hoffbrand et al., 2006). Thrombin also responsible for the feedback activation of other blood coagulation factors and it is considered as important factors in blood coagulation (Guglielmone et al., 2001).

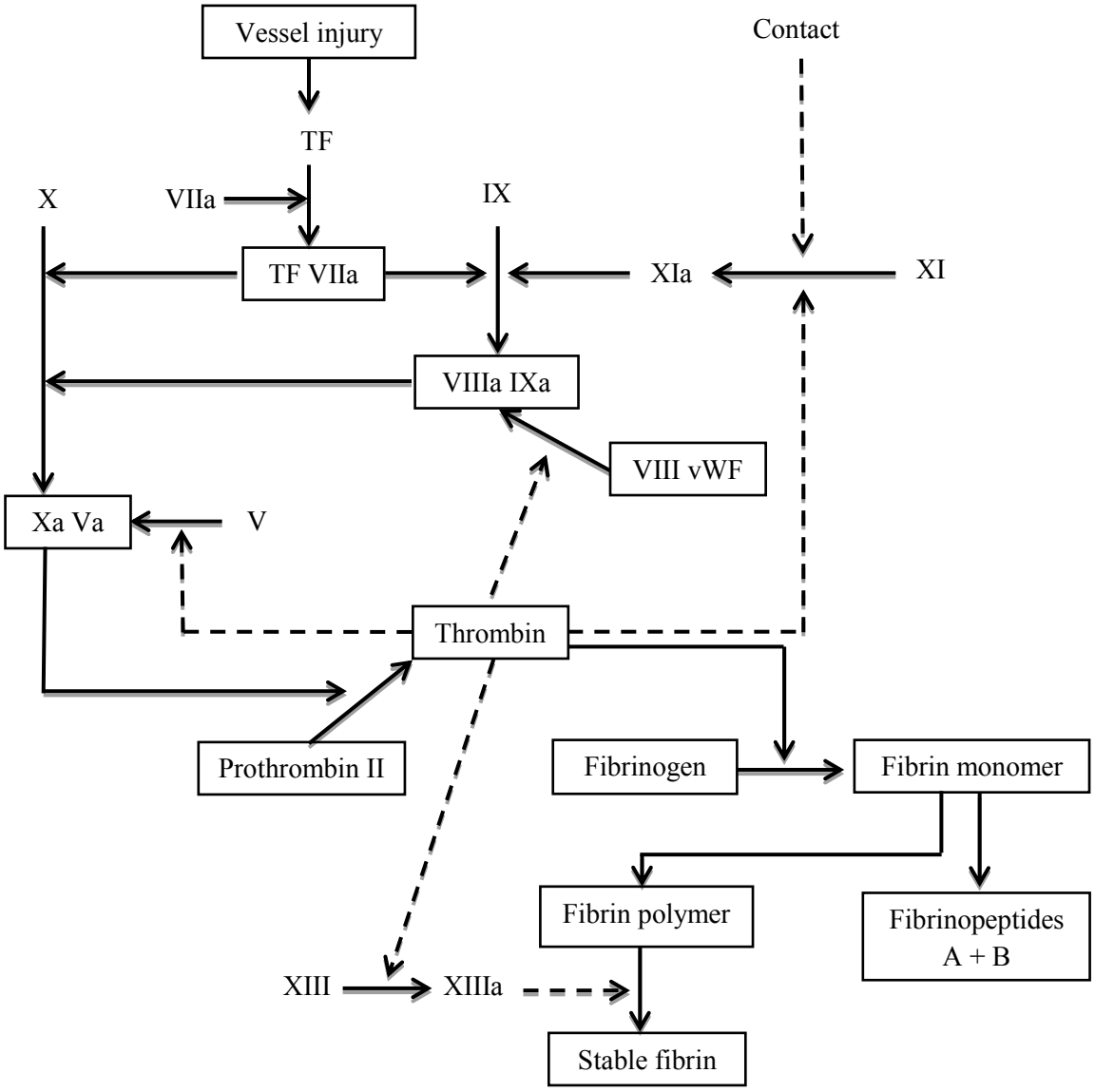
Majority of blood coagulation factors involved are pro-enzyme, which is needed to be activated sequentially, one after another in the blood coagulation cascade (Hoffbrand et al., 2006). Blood coagulation system involves three pathways which are extrinsic, intrinsic and common pathways. Although it has been traditional and useful for *in vitro* laboratory testing to divide the coagulation system into extrinsic and intrinsic pathways, such a division actually does not occur *in vivo.* This is because the tissue factor - factor VIIa complex is a potent activator for both factor IX and factor X. Both intrinsic and extrinsic pathways require initiation that leads to subsequent activation of various coagulation factors in cascading, waterfall or domino effects and both share common pathways (Harmening, 2001).

1.1.1 Extrinsic pathway

The principal of initiating pathway *in vivo* blood coagulation is the extrinsic pathway, which involve the components of blood and vascular elements. The crucial component is the tissue factor which an intrinsic membrane protein composed of a single polypeptide chain. This protein functions as a cofactor to factor VIII in intrinsic system, and to factor V in the final common pathway. Tissue factor inhibitor (TFPI) is a protein that in association with factor Xa which is inhibits the tissue factor - factor VII complex (Colman et al., 2006). The extrinsic pathway is initiated with the release of tissue thromboplastin that has been expressed after the damage to a blood vessel. Factor VII, tissue thromboplastin and calcium are later formed as a complex which converts factor X into Xa. Factor Xa with the help of factor V then converts the prothrombin to

thrombin. Furthermore the thrombin causes the conversion of fibrinogen to fibrin. This entire process normally takes between 10 and 15 seconds to complete (Ciesla, 2012).

Figure 1.1: Blood coagulation cascade



(Adapted from Essential Haematology, 6th Edition, 2011, © A. V. Hoffbrand & P.A.H. Moss)

1.1.2 Intrinsic pathway

Intrinsic pathway also known as contact pathway is initiated upon the contact of blood to the negatively charged foreign substance such as collagen, endothelial surface, or phospholipids. Intrinsic pathway consist of factors I, II, V, VIII, IX, and XII (Hoffbrand et al., 2006). The vascular trauma induces the changes that initiate a cascading sequence of contact activation and results in the activation of factor IX by a novel dimeric serine protease factor Xia, providing a pathway independent of factor VII for blood coagulation. However, an important difference exists between these two pathways in the clotting cascade whereas the activation of factor IX by XIa requires only in the presence of ionised calcium. The activation of factor IX by VIIa requires the protein cofactor, tissue factor, calcium that embedded in a lipid bilayer cell membrane (Ciesla, 2012, Colman et al., 2006).

The role of the contact system proteins in initiation of intrinsic pathway of coagulation in haemostasis is questionable because only a deficiency of factor XI is associated with a haemorrhagic tendency. These proteins participate instead in the initiation of the inflammatory response, angiogenesis, complement activation, fibrinolysis, and kinin formation. The previous studies show that kininogen is an anticoagulant protein *in vivo*. The mechanism may be due to the inhibiting the binding of low concentrations of thrombin to platelet GP Ib/IX (Colman et al., 2006).

According to Ciesla (2012), the factor XII auto activates to factor XIIa in the presence of the protein prekallikrein meanwhile the activation of factor XI to factor Xia requires the presence of another protein which is cofactor of HMWK. The factor XIa activates the factor IX to factor IXa, which is then, converts the factor X to factor Xa in the presence of factor VIIIa and platelet phospholipid factor PF3. Calcium is requires in rapid activation of factor X. The reaction then enters the common pathway of blood coagulation cascade (Ciesla, 2012).

1.1.3 Common pathway

The common pathway is the point at which the intrinsic and extrinsic pathways merge and combined together, where the factors I, II, V and X are measured. Prothrombin time (PT) and activated partial thromboplastin time (APTT) do not detect the quantitative or qualitative of platelet disorders or factor XIII deficiency. Factor XIII also known as the fibrin stabilizing factor, is responsible for stabilizing a soluble fibrin monomer into insoluble fibrin clot. A patient with factor XIII deficiency cannot stabilize the clot and lead to the bleeding to occur (Bennett et al., 2007, Hoffbrand et al., 2006).

1.1.4 Role of thrombin in fibrinogen

The activation of plasma fibrinogen by a protease enzyme, thrombin will results in a stable fibrin clot and visible proof of fibrin formation. Thrombin also participates in factor XIII-XIIIa activation, which occurs when thrombin cleaves a peptide bond from each of the two alpha chains. It combined with the calcium ions and caused the

inactivation of the factor XIII. This process enables the factor XIII to dissociate to factor XIIIa. If the thrombin are allowed to circulates in its active form (factor Ia), uncontrolled clotting would occurred. Therefore the thrombin circulates in its inactive form, prothrombin (factor II). Thrombin cleaves fibrinogen (factor I), which results in a stable fibrin monomer and fibrinogen peptides A and B. These initial monomers polymerise end-to-end because of the hydrogen bonding (Ciesla, 2012, Hoffbrand et al., 2006).

According to the Ciesla B. (2012), the fibrin formation can occurs in three phases including proteolysis, polymerisation and stabilisation. Proteolysis occurs when protease enzyme thrombin cleaves the fibrinogen and resulting in a fibrin monomer, A and B fibrinopeptides. Polymerisation occurs spontaneously when fibrin monomers line up end-to-end because of hydrogen bonding meanwhile the stabilisation occurs when the factor XIIIa covalently links with the fibrin monomers into fibrin polymers and forming an insoluble fibrin clot. This clot will enclosed the injury sites and heal the wound (Ciesla, 2012, Harmening, 2001).

1.1.5 Haemostasis screening assays

In normal screening test of coagulation pathways to detect the blood coagulation disorder, PT, APTT and thrombin time (TT) are used as measurements. According to Bennett et al. (2007), the PT assay measuring the extrinsic (tissue factor) and common pathways meanwhile the APTT measures the intrinsic and common pathways. The PT and APTT assays are useful to distinguish between the effects of the test agents including leaves extract, fruit extract and other natural compounds on the intrinsic and

extrinsic pathways. The TT assay are important in evaluating disorders of thrombin, haemostasis as well as the presence of oral anticoagulant heparin (Brown, 1988).

1.1.5.1 PT assay

The PT assay has two purposes which are; to screen for acquired or inherited as well as deficiencies in the extrinsic and common pathways of blood coagulation cascade. As we know, each pathway has specific coagulation factors such as factor VII, X, II (prothrombin), I (fibrinogen) and V involve in extrinsic pathway and factor X and V involve in common pathway (Hoffbrand et al., 2006).

The PT assay is affected by decreased level of extrinsic factors. Since the prothrombin, factor VII and X that measured by PT assay are vitamin K-dependent protein, therefore PT assay are useful for detecting vitamin K deficiency from any causes including warfarin therapy, liver disease or malnutrition. The PT assay also frequently used to follow oral anticoagulant therapy such as warfarin which is inhibiting the factors X, IX, VII, and II (Dey and Bhakta, 2012). However, the PT assay does not measure the factor XIII activity or other factors of the intrinsic pathway.

The PT is performed by mixing the citrated plasma with the commercial tissue factor, thromboplastin and calcium which resulting the activation of the factors VII, X, V, fibrinogen and prothrombin. Thromboplastin which is a commercial factor that derived from animal tissue or recombinant methods. Tissue factor in the preparation of

thromboplastin will binds to the factor VII in citrated plasma and initiate the coagulation process. The clotting time is measured in seconds using instruments with photo-optical or mechanical endpoints that detect the formation of fibrin in plasma. The normal values range for PT is 10 – 15 seconds depending on normal range of the laboratory (Bennett et al., 2007, Brown, 1988, Dey and Bhakta, 2012, Hoffbrand et al., 2006).

In general the PT assay is more sensitive in detecting the low levels of factors VII and X as compared to low levels of prothrombin, fibrinogen or factor V. In particular the different thromboplastin reagents may exhibits sensitivities of variable to these deficiencies factors. The PT assay are reported less affected by heparin when compare to APTT assay (Bennett et al., 2007). Shortened the PT values may result from poor quality of venipuncture, deficiency or inhibition of one or more of the following factors: VII, X, V, II and fibrinogen, liver disease, warfarin therapy, disseminated intravascular coagulopathy (DIC) disease or cold activation of sample which is *in vitro* activation from factor XII activation by factor VII that occurs if the citrated plasma sample is stored at cold temperatures above freezing for several hours (Bennett et al., 2007, Brown, 1988, Hoffbrand et al., 2006).

1.1.5.2 APTT assay

The APTT assay is one of the most common assays in the clinical coagulation laboratory. This assay is useful for screening the acquired or inherited deficiencies of intrinsic pathway, for detecting the lupus anticoagulant and for monitoring heparin

therapy. The APTT assay measures the factors VIII, IX, XI and XII in addition to fibrinogen, prothrombin, and factor X and V. This assay is affected by the decreased levels of intrinsic pathways components as well as decreased levels of common pathway components; however factors VII and XIII are not measured (Hoffbrand et al., 2006, Harmening, 2001, Bennett et al., 2007).

In general, the APPT is performed by adding the citrated patient plasma to the APTT reagent which is contact activator and its need preincubation period to initiate the activation of factors in intrinsic pathway. This will involves the activation of factors XII and XI in the presence of cofactors, prekallikrein and high-molecular-weight kininogen. The activated factor XI is then converts the factor IX to activated factor IX. After that, the calcium is then added to the preincubation mixture which results in the factors IXa/VIII activation of factor X and then the activation of prothrombin into thrombin followed up by conversion of fibrinogen into soluble fibrin that polymerises into stable fibrin. The formation stable fibrin is the endpoint as the clotting time for APTT assay which detected by using instruments with photo-optical and mechanical (Bennett et al., 2007). The normal values range for APTT assay is approximately 30 – 40 seconds depends on the laboratory normal values ranges.

Prolongation of clotting time in the APTT assay suggest a possibility of the disturbance in the complex coagulation cascade that involve wider range of coagulation factors which indicated the deficiency or inhibition of one or more of the following factors: VII, X, V, II and fibrinogen (Karim et al., 2013). The most common cause of disorder

are Hemophilia A (factor VIII) and Christmas disease also known as Hemophilia B (factor IX). The APTT assay also will be affected in the presence of circulation inhibitors such as antithrombin, protein C and protein S towards the inhibition of the calcium ion or phospholipids action and coagulation factors (Laffan and Manning, 2010). Prolongation of APTT and PT also can be seen with the low levels of fibrinogen (Karim et al., 2013).

1.1.5.3 TT assay

The TT assay is performed by adding the thrombin that usually harvested from bovine or human origin, purified and lyophilised into the citrated plasma either with or without added calcium. This assay measures the amount and quantity of fibrinogen and the rate of conversion of fibrinogen into stable fibrin (Goodnight and Hathaway, 2001). Thrombin is a protease protein produced in the activation of plasma prothrombin. Thrombin function is to cleave the *fibrinopeptides* A and B from α and β polypeptide monomer of circulating fibrinogen. Upon the cleavage, the individual fibrinogen molecules polymerise and forming insoluble fibrin, the protein in the visible clot (Corriveau and Fritsma, 1988).

In general, TT is performed by adding citrated plasma as a source of fibrinogen and if the fibrinogen is available in the plasma, quantitatively and qualitatively, a fibrin clot is established once the standard concentration of thrombin has been added into the plasma. The reference range is 11 to 15 seconds depends on the laboratory environment and

analyzer. A prolonged TT assay may indicate the deficiency of normal fibrinogen usually <100 mg/dL, as normally seen in the patients with congenital hypofibrinogenemia or afibrinogenemia. The TT assay may rarely be prolonged in conditions with abnormally high levels of fibrinogen (inflammation) or more commonly, qualitatively abnormal fibrinogen (hereditary dysfibrinogenemia, cirrhosis, hepatocellular carcinoma, and newborn infants) (Bennett et al., 2007).

Substances those interfering with the thrombin-induced fibrinogen conversion to a fibrin are associated with a prolonged TT including heparin, antithrombin antibodies after exposure to bovine thrombin, proteolytic products of fibrin, and fibrinogen, procainamide-induced anticoagulant, systemic amyloidosis and abnormal serum proteins. Combinations of these mechanisms producing an increased TT are seen in renal disease and DIC (Corriveau and Fritsma, 1988, Goodnight and Hathaway, 2001).

1.1.6 Blood coagulation disorders

Platelet and blood coagulation factors are the two important components that involve in the formation of the blood clot. Any deficiencies and defect involves in blood coagulation factors will cause the bleeding disorders. According to Hoffbrand et al. (2006), bleeding disorder can be acquired or inherited. Inherited blood disorder such as hemophilia A (deficiency of factor VIII), hemophilia B (deficiency of factor IX) and von Willebrand's disease (deficiency of vW factor). Meanwhile, the acquired disorders are liver disease, vitamin K deficiencies, drugs such as warfarin, DIC and others.

In blood coagulation disorders such as hemophilia A, any injuries will cause the bleeding disorder such as prolonged the bleeding after dental extractions, surgery, giving birth, spontaneous haematuria and haemorrhage. These conditions can lead to death in severe conditions. Therefore the patients need to be treated with some drugs such as with antifibrinolytic (tranexamic acid for mild bleeding) or procoagulant treatment to improve the efficacy of the coagulation cascade (Hoffbrand et al., 2006, Rang et al., 2007).

Blood clots also known as thrombus can develop in the blood vessels and circulatory system because of imbalance between clotting and bleeding will lead to blockage of vascular. This condition can be serious and responsible to health threatening results. Thrombosis are divided into two type which are arterial thrombus which arise and form in the arteries, meanwhile the venous thrombus are the thrombus that form in the veins of blood vessels (Jain et al., 2014). There are various diseases that arising from the blood clots problem such as deep vein thrombosis, pulmonary embolism, atherosclerosis, diabetic complications, cerebral vascular accident (CVA) and myocardial infarction which are the major life threatening disease lead to morbidity and mortality (Jain et al., 2014, Sherwani et al., 2013).

Thrombolysis process is a complex mechanism that interacts with the clot components and surrounding plasma which involve the component of plasminogen, fibrin, plasminogen activator and plasmin. Antithrombolytic agents are used in order to treat the diseases and dissolve the blood clots in the blood vessels. Anticoagulant therapy

attempts to impede thrombus formation in individuals who have predisposing factors for a clot formation or who are predisposed by virtue of a medical event without the threat of morbidity or mortality from hemorrhage. Warfarin is an oral anticoagulant, which means it must be ingested. It was discovered accidentally at the University of Wisconsin in 1939 after a farmer found that his cattle were haemorrhaging to death for no apparent reason. The cattle grazed in a field of sweet clover, which contains dicumarol (actually, bishydroxycoumarin) causing the cattle to bleed (Ciesla, 2012).

Aspirin and heparin are markedly effective in treating the patient with thrombolytic disease by activating the lysis and preventing the reocclusion. There are also several compounds of coumadin including indanedione, dicumarol, and warfarin. Dicumarol works too slowly in the patients and indanedione has many side effects. Warfarin, or 4-oxycoumarin, is the most commonly used oral anticoagulant. Warfarin works by inhibiting the y-carboxylation step of clotting and the vitamin K-dependent factors (Ciesla, 2012, Karim et al., 2013). Therefore all the anticoagulant and antifibrinolytic have their own deleterious side effect that need to be replaces by others natural product which have no side effects.

1.2 *Mikania cordata*

1.2.1 Structure

Mikania cordata plant is a smooth vine, climber shrub that widely grow in Malaysia (Ab. Patar and Yahaya, 2012), have serrated leaves, many white flowers and known as common obnoxious weed to Bangladesh (Nayeem et al., 2011). *M. cordata* is a fast growing, perennial vine, creeping or twining, and branches stems. The leaves are slender and long-petioled, ovate heart-shaped or deltoid-ovoid with 4 -10 centimeters long, with pointed tip, rounded heart-shaped or truncate base and toothed margins. The matured leaves, the stem and the branches are easily form the roots when the leaves come contact with the soil (Bulbul et al., 2013). The heads of plant are 4-flowered with cylindrical, 6-9 millimeter long and borne in compound inflorescences. Achenes are smooth, glandular and linear-oblong with 2.5-3 millimeters long. The pappus of this plant is composed of one series, 40-45 bristles, about 4mm long, whitish at first and salmon coloured (reddish) afterwards. Maybe distinguished by the following characteristics: 40 to 45 reddish pappus bristles, corollas white and heads 7 to 7.5 mm long.

Figure 1.2: *Mikania cordata*

1.2.2 Classification

- **Kingdom:** Plantae
    - **Subkingdom:** Tracheobionta
        - **Superdivision:** Spermatophyta
            - **Division:** Magnoliophyta
                - **Class:** Magnoliopsida
                    - **Subclass:** Asteridae
                        - **Order:** Asterales
                            - **Family:** Asteraceae
                                - **Genus:** *Mikania*
                                    - **Species:** *Mikania cordata*

(Adapted from NRCS, United States Department of Agriculture)

Mikania cordata (Burm.f) B.L. Robinson, are locally known as 'akar lupang', "selaput tunggul' and 'ulam tikus' among Malaysia and in Indonesia known as 'sembung rambat'. This plant also known as Taralata, Asamlata, Chinese creeper and Germalata (Nayeem et al., 2011, Bulbul et al., 2013), Dubainna lota (Rashid et al., 2012), Refugee (Biswas et al., 2011) is a traditional plant that commonly used among folks.

1.2.3 Geographical distribution

M. cordata are found throughout of tropical regions in India, Bangladesh (Paul et al., 2000), Philippines, Malaysia (Ab. Patar and Yahaya, 2012), Brazil, Africa and South America including Argentina, Paraguay, and Uruguay (Bulbul et al., 2013).

1.2.4 Active compounds

Acetic acid can be found in herbs leaves such as *M. cordata* leaves. The previous study has found that acetic acid compound was the third most compound composition of total of *M. cordata* leaves (6.27%). Furthermore, a lot of chemical compounds were reported such as hydroquinone, stigmasterol, deoxyspergualin, glycerin and squalene (Ab. Patar and Yahaya, 2012). In *Mikania micrantha*, the main compounds were sesquiterpenoids, flavonoids, coumarins, diterpenes, pytosterols, polyphenols, terpenoids, mikanolide, miscandenin derivatives, sequiterpenes lactones and halohydrocarbon (Gasparetto et al., 2010, Li et al., 2013, Ahmed et al., 2001).

The previous study showed that *M. cordata* leaves extract have the compounds of α-cubene (21.3%), spathulenol (3%), γ-curcumene (6.3%), β-pinene (4.1%), α-cedrene (4.9%), copaene (4.1%), caryophyllene oxide (10.1%) and α-bisabolol (6.6%) (Aguinaldo et al., 2003a). In others study using flower oils extract of *M. cordata* reported that various bioactive compounds such as such as β-pinene (14.9%), zingiberene (6%), α-bergamotene (5.6%), β-caryophyllene (5.6%), γ-curcumene (11.7%) and α-cubebene (12.4%) (Chowdhury et al., 2007, Aguinaldo et al., 2003b).

Moreover, the mikamicranolide, dihydromikanolide, 2 – cubebene, γ – elemene, 2 – copaene, deoxymikanolide and mikanokryptin were reported in *M. micrantha* using aerial parts. In *M. micrantha* was reported the presence of 3,4-di-O-caffeoylquinic acid n-butyl ester, eupalitin, eupafolin, luteolin, and 3,5-di-O-caffeoylquinic acid n-butyl ester (Herz et al., 1967, Huang et al., 2009).

1.2.5 Medicinal values

The leaves of *M. cordata* has been proven to treat several illness such as antiulcer activity (Paul et al., 2000), analgesic effect (Ahmed et al., 2001), effect on nervous system (Bhattacharya et al., 1988), antibacterial (Sekendar A.Shaiful et al., 2011). The Tonchongya Tribe in Bandarban District of Bangladesh has used the leaves of *M. cordata* (Burm.f) and *Chromolaena odorata* (L.) King & H.Rob. by crushing together and applied to wounds and cuts to stop the bleeding instantly (Rashid et al., 2012). The crashed leaves are used to stop bleeding from cuts and wounds, against jaundice, septic sore and snake bite among Bangladesh's folks (Nayeem et al., 2011, Biswas et al., 2011) and traditionally used to stop the bleeding among Malaysian's folks (Ab. Patar & Yahaya, 2012).

Among Bangladesh's folk, the infusion and decoction of leaves are used for colds, influenza, fever and bronchitis in children. Besides that, the decoction of flower also is used for coughs and diabetes. The whole plants are rich sources of vitamin A, B and C and also used for fish poisoning. It is easily to get this plant because it is a very rapidly growing perennial vine and wildly grow on the ground. In the previous study Ab. Patar

and Yahaya (2012) shown that the *M. cordata* leaves consist of bioactive compounds which is known as acetic acid. Acetic acid was the third most abundant in this plants and might have a potential effect in blood coagulation system. This was proven by Victor Fernandez-Duenas et al. (2008) which reported that the acetic acid has clinical significant in blood coagulation study as a procoagulant because it reduced the bleeding time in mice. However, because of the problem in sample size of the subjects, the study was inconclusive in the present time (Ab. Patar and Yahaya, 2012). Considering the effect of *M. cordata* leaves, it is crucial to look into this plant in term of bioactive compound as well as the blood coagulation activities. Therefore in this study, the main objective was to investigate the blood coagulation compounds of the Malaysian *M. cordata* leaves that lead to blood coagulation activities of human plasma *in vitro.*

1.3 Rationale of study

Anticoagulants and antifibrinolytics play vital roles in the clinical medicine as agents for prevention and treatment of thromboembolic and blood coagulation disorder. Anticoagulants including heparin and warfarin meanwhile, antifibrinolytic including tranexamic acids have been used widely for more than five decades. Although the efficacy of these drugs still remains undisputed, the deleterious life-threatening side effects have been well documented and led to high morbidity and mortality rate (Jain et al., 2014, Manicam et al., 2010).

Considering the life-threatening side effects, the novel anticoagulant must be developed to overcome the morbidity and mortality rate in patients. Since the production of

developing this novel coagulant and anticoagulant is costly; therefore a cheaper yet effective alternative would be a great welcome in clinical settings. This limitations has led to the exploitation of plants as an alternative method to overcome the side effects, since the plants is considered as a reliable source for new invention of a novel coagulant and anticoagulant agents (Jain et al., 2014, Felix-Silva et al., 2014, Guglielmone et al., 2001). Hence, more studies pertaining to the use of plants as therapeutic agents should be emphasised, especially those related to blood coagulation activities.

1.4 Objectives

1.4.1 General objective

- To investigate the effects of Malaysian *Mikania cordata* leaves extracts on human blood coagulation activities *in vitro*.

1.4.2 Specific objectives

- To study the effects of *in vitro* blood coagulation activities in different concentration of aqueous extract of Malaysian *Mikania cordata* leaves using prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) assays.

- To determine the composition that involves in blood coagulation activities of *Mikania cordata* leaves in aqueous and ethanolic extracts by using Gas Chromatographic Mass Spectrometry (GCMS).

1.5 Hypothesis

H_A : Malaysian *Mikania cordata* leaves enhance the blood coagulation activities

H_O : Malaysian *Mikania cordata* leaves have no effects on blood coagulation activities

1.6 Expected outcome

- There are blood coagulation activities in aqueous extract of *Mikania cordata* using PT, APTT and TT Assay *in vitro*. This finding is expected to benefits in clinical treatment as the nature coagulant, either procoagulant or anticoagulant. If the plant acts as procoagulant, it can be used as antifibrinolytic for treatment of thromboembolic, treat the wound healing and stop the bleeding. If the plant acts as anticoagulant, it can be used for treatment of blood coagulation disorder.

- There are compositions that enhance the blood coagulation activities in *Mikania cordata* that can be identified in aqueous and ethanol extracts by using GCMS.

The overview of the research is visualised in the Figure 1.3.

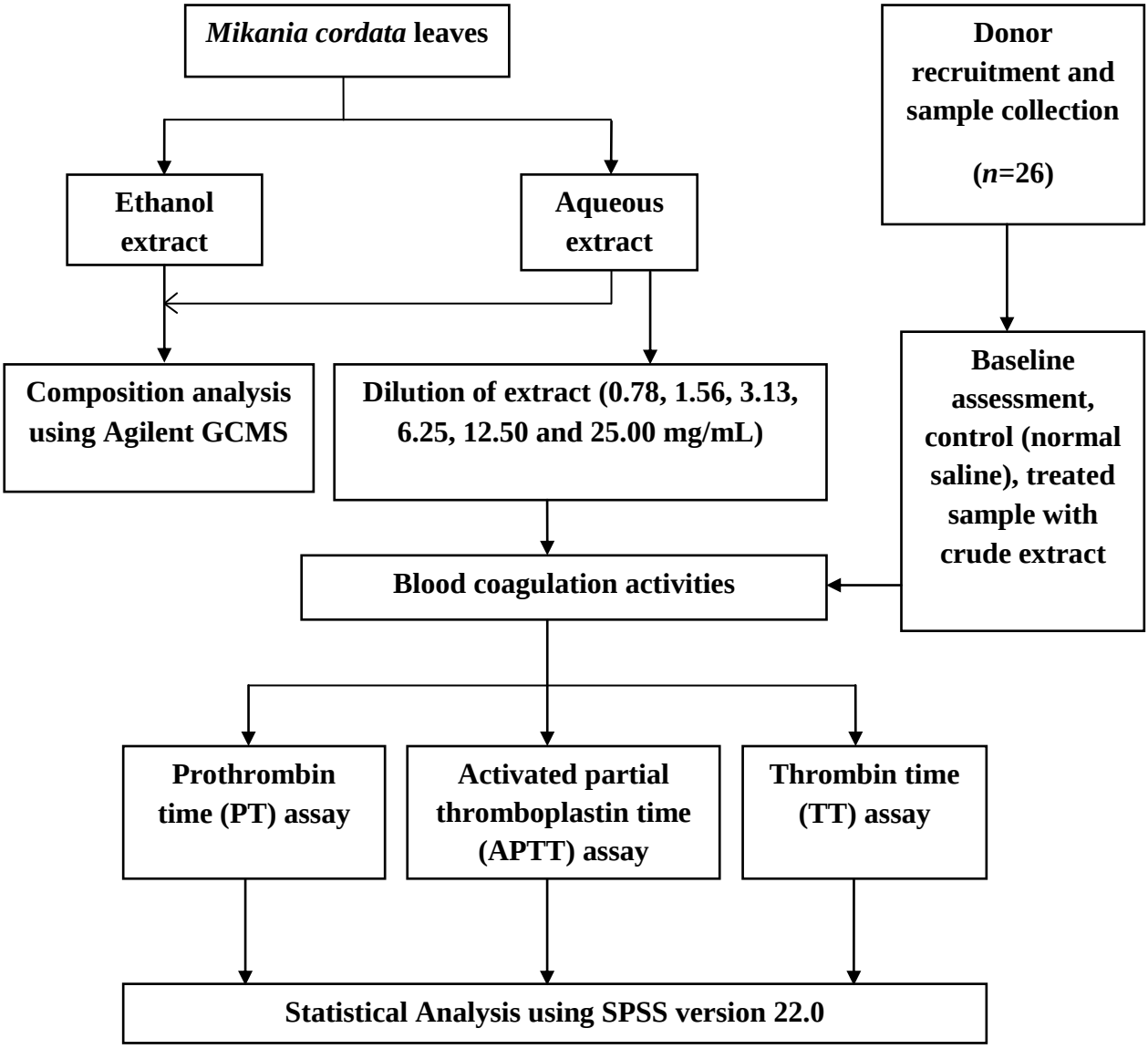


Figure 1.3: Flow chart of study

CHAPTER 2

MATERIALS AND METHOD

2.1 Materials

2.1.1 Preparation of 0.9 % normal saline (0.9% NaCl)

0.9 mg of sodium chloride powder (NaCl) (Sigma-Aldrich, United States) was weighted and dissolved in volumetric flask (1 ml) that containing 1000 ml of distilled water (dH_2O) to produce 0.9% of normal saline. The solution was mixed until all the powder dissolved. Then, the 0.9% normal saline solution was aliquots in small Schott's bottle for storage at room temperature until further use.

2.1.2 Preparation of STA Reagents (Diagnostica Stago, S.A.S France)

2.1.2.1 Preparation of STA - Coag Control N+P reagent

1 ml of distilled water (dH_2O) was added in both STA - Coag Control N vial and STA - Coag Control P vial (Diagnostica Stago, S.A.S France). The reconstituted materials were allowed to stand at room temperature (18 – 25 °C) for 30 minutes. After that, the reagents were mixed well by swirling the vial without creating any bubbles before use.