IN VITRO ASSESSMENT OF COAGULATION ACTIVITIES IN HUMAN PLASMA TREATED WITH AJWA DATES (PHOENIX DACTYLIFERA L.) EXTRACTS

By

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DECLARATION

I hereby declare that this dissertation is based on my original work. I also declare that this dissertation has not previously or concurrently submitted by any other master student at USM or other institutions.

AUGUST 2015

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celcius
>	More than
<	Less than
g	Gravity
α	Alpha
g	Grams
σ	Sigma
δ	Delta
γ	Gamma
kg	Kilogram
µL	Microlitre
s	Seconds
○	Outliers
*	Extreme Outliers
a	Activated
mg	Milligram
h	Hours
min	Minutes
mL	Millilitre
RT	Room Temperature
PPP	Platelet-Poor Plasma
SPSS	Statistical Package for the Social Sciences
TMB	Tetramethylbenzidine
t_R	Retention Time
v/v	Volume per Volume
VKA	Vitamin K Antagonist

GC-MS	Gas Chromatography Mass Spectrometry
ET/EtOH	Ethanol
AQ	Aqueous
HREC	Human Research Ethics Committee
AMDI	Advanced Medical and Dental Institute
PDN	National Blood Centre
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
TMCS	Trimethylchlorosilane
RPM	Rotation per Minute
VTE	Venous Thromboembolism
PT	Prothrombin Time
APTT	Activated Partial Thromboplastin Time
TT	Thrombin Time
HPLC	High Performance Liquid Chromatography
F	Factor
TFPI	Tissue Factor Pathway Inhibitor
HMWK	High Molecular Weight Kininogen
CBC	Complete Blood Count
DIC	Disseminated Intravascular Coagulation
EDTA	Ethylene-Diamine Tetraacetic Acid
TIC	Total Ion Chromatogram
MI	Myocardial Infarction
CAM	Complementary and Alternative Medicine
RI	Retention Time Index
RBC	Red Blood Cell
Hb	Hemoglobin
PCV	Pack Cell Volume

ABSTRAK

Arteri dan vena trombosis telah menyumbang kepada peningkatan kadar penyakit dan kematian yang ketara. Pencegahan dan rawatan trombosis dapat direalisasikan dengan menggunakan ejen antitrombosis. Kurma (*Phoenix dactylifera* L.) telah dilaporkan mempunyai asid salisilik yang tinggi, iaitu sebatian yang bertindak sebagai vitamin K antagonis. Oleh itu, kurma berkemungkinan mampu bertindak sebagai antikoagulan. Matlamat kajian penyelidikan ini adalah untuk mengetahui pengaruh kurma Ajwa (*Phoenix dactylifera* L.) terhadap penilaian aktiviti pembekuan dalam plasma manusia secara *in vitro*. Plasma dari dua puluh tujuh penderma (n=27) dicampurkan dengan ekstrak kurma etanol dan akueus pada kepakatan berbeza iaitu 0.1, 0.5 and 1.0 g/mL. Tujuh kumpulan dibentuk bagi setiap plasma penderma: kontrol (saline normal), ET I (0.1 g/mL), ET II (0.5 g/mL), ET III (1.0 g/mL), AQ I (0.1 g/mL), AQ II (0.5 g/mL), AQ III (1.0 g/mL). Aktiviti pembekuan darah dianalisis melalui *prothrombin time (PT), activated partial thromboplastin time (APTT)* dan *thrombin time (TT)* bagi kedua-dua ekstrak. Tempoh pembekuan PT, APTT dan TT yang panjang dan sangat signifikan dilihat pada ET II dan ET III. Tempoh pembekuan PT dan TT yang panjang dan sangat signifikan dilihat pada AQ II dan AQ III. Tempoh pembekuan TT yang panjang dan signifikan dilihat pada AQ I. Pembekuan pada laluan luar dan laluan dalam serta tahap pembekuan terakhir dilihat pada kadar kepekatan ekstrak etanol yang sama. Laluan luar dan tahap pembekuan terakhir menunjukkan kadar pembekuan yang panjang dan signifikan bagi ekstrak akueus. Tahap pembekuan terakhir dilihat lebih terkesan berbanding laluan luar kerana pembekuan bermula pada AQ I. Sebagai kesimpulan, kurma Ajwa memberi kesan antikoagulan.

ABSTRACT

Arterial and venous thromboses contribute to significant morbidity and mortality rate. Antithrombotic agent is needed for prevention and treatment of thrombosis. Dates (*Phoenix dactylifera* L.) were reported to have high salicylic acid, a compound responsible for anticoagulant by acting as vitamin K antagonist. Thus, dates may have anticoagulant effect. The present study was design to assess coagulation activities in human plasma treated with Ajwa dates extracts *in vitro*. Platelet poor plasma (PPP) from twenty-seven donors (n=27) were treated with Ajwa dates ethanol and aqueous extracts at different concentrations (0.1, 0.5 and 1.0 g/mL). Seven groups were formed from each donor: control (normal saline), ET I (0.1 g/mL), ET II (0.5 g/mL), ET III (1.0 g/mL), AQ I (0.1 g/mL), AQ II (0.5 g/mL), AQ III (1.0 g/mL). *In vitro* coagulation activities of Ajwa dates were assessed by prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT). Very significant prolongation of PT, APTT and TT were observed for concentration ET II and ET III. Very significant prolongation of PT and TT were observed for concentration AQ II and AQ III. Significant prolongation of TT was observed in AQ I. Coagulation in extrinsic, intrinsic and final coagulation systems were prolonged at the same ethanol extract concentrations. Extrinsic pathway and final coagulation systems showed prolongation by aqueous extract. Final coagulation system was more affected compare to extrinsic pathway because prolongation at concentration AQ I was observed. In conclusion, Ajwa dates showed anticoagulation effect.

CHAPTER I

INTRODUCTION

1.1 Literature Review

1.1.1 Haemostasis

1.1.1.1 Definition

Haemostasis is a dynamic, ordered and well-regulated body process of blood fluids maintenance, vascular damage repairing and bleeding arrest (Zehnder 2012, p. 601). Haemostasis aims to prevent intravascular blood clot formation and to stop blood loss from blood vessel (Estridge & Reynolds 2008). Abnormality in normal haemostasis will disrupt the balance maintained in haemostasis mechanism and thus giving rise to two extreme and distinct conditions; obstructive intravascular clotting (thrombosis) or excessive bleedings (haemorrhage) (Zehnder 2012, p. 601; Sood, 2010). Haemostasis is governed tightly to prevent thrombosis and/or haemorrhage by substantial negative feedback mechanism of the endothelium and plasma (Shaz et al. 2013) as well as the delicate balance in procoagulant, anticoagulant and fibrinolysis process in haemostasis mechanism (Hoffbrand, Moss & Pettit 2006). Haemostasis involved five components that define haemostasis in an orderly events starting from blood vessels, platelets, coagulation factors, coagulation inhibitors and lastly fibrinolysis (Estridge & Reynolds 2008; Hoffbrand, Moss & Pettit 2006). Haemostasis is a multistep process that is divided into two; primary and secondary (Fowler & Perry 2015; Longo 2013). Primary haemostasis involved the action of the first two components of haemostasis, blood vessels and platelets while secondary haemostasis refers

to the action of coagulation factors and inhibitors as well as fibrinolysis (Fowler & Perry 2015). Contrary to Fowler & Perry (2015) statement, Longo (2013) stated that primary haemostasis describes the coagulation system while secondary haemostasis describes the other factors that contribute to haemostasis for example the blood vessel and platelets.

1.1.1.2 Coagulation in haemostasis

Coagulation is one of the components of haemostasis that follows the establishment of blood vessel and platelets action during bleeding (Figure 1.1). When bleeding occurs, damaged blood vessel and the regional small arteries and arterioles will constrict to reduce blood flow; thereby allowing contact activation of platelets and coagulation factors (Hoffbrand, Moss & Pettit 2006). If bleeding is too severe and critical, the cut edges of the bleeding site will retract and compressed by contracted skeletal muscles. During this retraction and contraction process, small capillary vessels will seal themselves when the cut edges touch to one another (Estridge & Reynolds 2008). Then, platelets circulating in the blood stream will adhere to the collagen of the exposed connective tissue and caused platelets to activate by releasing substances from their granules (Estridge & Reynolds 2008; Hoffbrand, Moss & Pettit 2006). Adhesion of platelets will be followed by platelet aggregation and subsequently blood coagulation in an extremely rapid manner depending on von Willebrand factor, fibronectin, divalent cations, calcium, magnesium and glycoprotein IIb/ IIIa complex in platelet membrane (Sood 2010). Coagulation of blood is determined by coagulation factors and inhibitors (Table 1.1). Coagulation factors include plasma proteins factor I till factor XIII (except ionized calcium; previously known as Factor IV) (Estridge & Reynolds 2008; Hoffbrand et al. 2006). These proteins are produce in the liver and remains in the blood in inactive form. However, when blood vessel is damaged, haemostasis signals the activation of coagulation factors, leading to the

formation of fibrin clot (Estridge & Reynolds 2008). On the other hand, coagulation inhibitors include tissue factor pathway inhibitor (TFPI), heparin cofactor II, α_2-Macroglobulins, α_2-antiplasmin, C_1 esterase inhibitor and α_1-antitrypsin (Hoffbrand, Moss & Pettit 2006). When fibrin clot is formed, bleeding will slowly stop as the site of injury is healing. At this point, fibrinolysis is needed to dissolve fibrin clot by the action of plasminogen and plasmin. Activation of plasminogen to plasmin is stimulated from injured vessel wall or from tissues (Hoffbrand, Moss & Pettit 2006). To sum it all, primary haemostasis takes place when sub-endothelial of collagen is exposed; signalling the initiation of platelet adhesion, granule secretion and initial aggregation. Following primary haemostasis, thrombin generation and fibrin clot formation marked the hallmark of secondary haemostasis (Fowler & Perry 2015).

1.1.1.3 Coagulation pathway

Coagulation process is best understood through coagulation pathway. Complex interaction between coagulation factors and inhibitors is shown through three pathways; intrinsic, extrinsic and common (Figure 1.1). Coagulation cascade describe the action of each factors in contributing to the final formation of fibrin clot while preventing thrombosis formation (Table 1.1 and Figure 1.1). In this cascade, the common pathway is activated by either the extrinsic or intrinsic pathway or by interaction between these two pathways (Estridge & Reynolds 2008). Tissue factor or alternatively known as FIII or thromboplastin does not present in the circulating blood, hence the name extrinsic pathway (Estridge & Reynolds 2008). Initiation of extrinsic pathway by TPFI occurs during vessel damages (trauma) (Estridge & Reynolds 2008). With the presence of calcium, FVII will be activated and subsequently activating FX (Estridge & Reynolds 2008). Intrinsic pathway is named because the factors involved in this pathway are circulating in the blood (Estridge &

Reynolds 2008). Intrinsic pathway is initiated when FXII or alternatively known as contact factor is activated by damaged surface (Estridge & Reynolds 2008). Activated FXII will then subsequently activate FXI and the subsequent activated FXI will activate FIX (Estridge & Reynolds 2008). Activated FIX will participate in the activation of FX that will convert prothrombin to thrombin (Estridge & Reynolds 2008). In common pathway, fibrinogen will be converted to fibrin by thrombin. With the aid of activated FXIII, fibrin clot will form and stabilized when fibrin cross-linked each other (Estridge & Reynolds 2008; Hoffbrand, Moss & Pettit 2006).

1.1.1.4 Haemostatic screening assay

Defects in haemostasis may arise from vascular disorders, thrombocytopenia or platelet function disorder as well as problems in blood coagulation. Due to these reasons, screening test for blood coagulation disorders are platelet count, thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen quantitation (Estridge & Reynolds 2008; Fowler & Perry 2015; Hoffbrand, Moss & Pettit 2006). With regard to assessment of coagulation activity; PT, APTT and TT are used to evaluate common and extrinsic pathway; common and intrinsic pathway; and final coagulation stage respectively (Estridge & Reynolds 2008; Fowler & Perry 2015; Hoffbrand, Moss & Pettit 2006; Torres-Urrutia et al. 2013). Battery of test is compulsory when assessing coagulation abnormalities because no single test is capable to sufficiently detect such abnormalities (Sood 2010). PT, APTT and TT served as initial screening for clotting factor activity (Longo 2013) and also regarded as functional tests because they monitored the formation of clot. PT measures fibrinogen (Hoffbrand, Moss & Pettit 2006), FII, FV, FVII and FX while APTT measures FX, FV, FII, fibrinogen (Hoffbrand, Moss & Pettit 2006), FVIII, FIX and FXI (Fowler & Perry 2015; Hoffbrand, Moss & Pettit 2006).

Concerning haemostatic studies, samples collected for evaluation is highly sensitive and susceptible to even smallest changes in samples integrity (Bennett, Lehman & Rodgers 2007; Kitchen, Olson & Preston 2009). Therefore, good performance of haemostatic assay will requires consistency, reproducibility and continuous accurate result over time with critical attention to the preservation of samples integrity (Kitchen, Olson & Preston 2009). Knowledge on pre-analytical variables and the consequences in determining precise result is at utmost importance in coagulation laboratory (Kitchen, Olson & Preston 2009). Factors that compromised samples integrity include patient's condition (Kitchen, Olson & Preston 2009), anticoagulant concentration, container materials, collection technique, centrifugation and storage (Bennett, Lehman & Rodgers 2007). Among all coagulation tests available, PT has long been the most frequently used test in evaluating coagulation activity since the first establishment of this test on 1983 (Kitchen, Olson & Preston 2009). Worldwide, PT is regarded as the sensitive test for most coagulation factors depressed by vitamin K antagonist (VKA) (Bennett, Lehman & Rodgers 2007). When coupled with PT and APPT assay, differential diagnosis of bleeding disorders is achievable with the addition Complete Blood Count (CBC) test that reveals the number of platelet count and rather a very useful parameter in diagnosis of haemostasis disorders (Bennett, Lehman & Rodgers 2007). However, haemostasis screening test (PT, APTT, fibrinogen level and platelet count) has inadequate clinical correlation with actual bleeding risk (Fowler & Perry 2015).

Table 1.1 Coagulation Factors and Inhibitors (Hoffbrand, Moss & Pettit 2006)

Coagulation Factors		Coagulation Inhibitors
Factor No.	Descriptive name	
I	Fibrinogen	TFPI Heparin cofactor II α2-Macroglobulins α2-antiplasmin C1 esterase inhibitor α1-antitrypsin Protein C Protein S Antithrombin III
II	Prothrombin	
III	Tissue factor	
V	Labile factor	
VII	Proconvertin	
VIII	Antihaemophilic factor	
IX	Christmas factor	
X	Stuart-Prower factor	
XI	Plasma thromboplastin antecedent	
XII	Hageman (contact) factor	
XIII	Firbrin-stabilizing factor Prekallikrein (Fletcher factor) High Molecular Weight Kininogen (HMWK)	

1.1.1.5 Disorders of defective haemostasis

Defects in any of haemostasis components will eventually caused haemostasis disorders (Sood 2010). Haemostasis disorders are grouped based on the aetiology factors. Disorders of haemostasis include those of platelets and vessel walls; coagulation; arterial and venous thrombosis; and pulmonary thromboembolism (Longo 2013). Coagulation-related disorders derived from congenital or acquired abnormalities; with acquired-coagulation disorders dominating most of the cases (Longo 2013; Sood 2010). Coagulation disorders are tabulated as follows (Table 1.2).

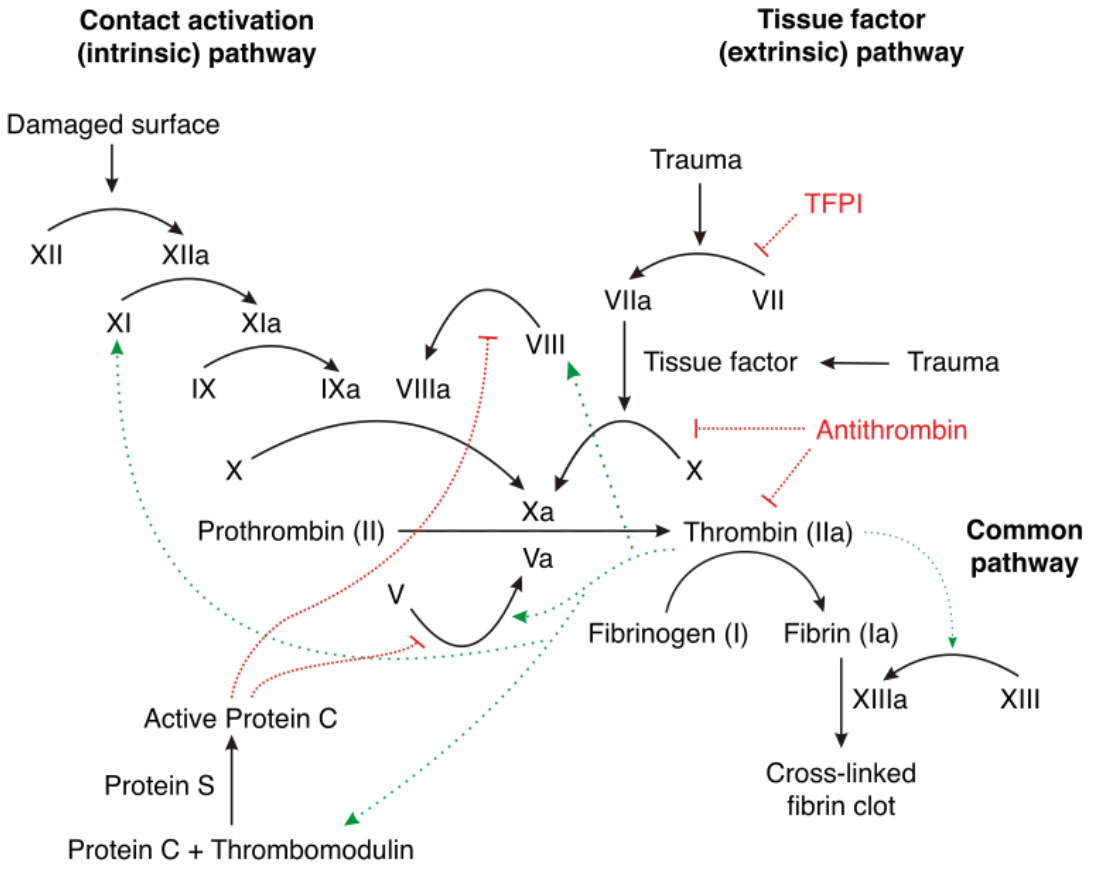


Figure 1.1 The more in-depth version of the coagulation cascade (Joe Dunckley 2007). TFPI; tissue factor pathway inhibitor, a; activated. Red line represents negative feedback and green line represents positive feedback.

Since the heart of coagulation mechanism is regulated by coagulation factors, the coagulation-inhibitory and fibrinolytic systems are maintained to obtain delicate balance in preventing accidental intravascular clotting while simultaneously sustained the patency of vascular lumen post-intravascular clotting (Sood 2010). Table 1.2 shows how one factor deficiency or other acquired factors may cause disorder with bleedings as the major outcome (Longo, 2013; Sood 2010). In diagnosing bleeding disorders, comprehensive understanding of haemostatic mechanism is a must (Zehnder 2012, p. 601). While diagnosis of coagulation disorders depends mostly on the clinical evaluation, laboratory results of coagulation test will confirm the diagnosis (Sood 2010). Eventhough each

disorder has specific treatment and management; correction of coagulation abnormality by drugs tends to be more specific to certain limited disorders while replacement therapy with whole blood, fresh frozen plasma or factor concentrates has more wide indications (Sood 2010).

Table 1.2 Coagulation disorders derived from congenital or acquired abnormalities.

Aetiology	Coagulation disorders	Reference
Congenital	Haemophilia A (FVIII) and Haemophilia B(FIX) (most common; X-linked recessive) Deficiency in FII, FV, FVII, FX, FXI, FXIII and fibrinogen (rare; autosomal recessive) FXII (Hageman factor deficiency) Fletcher factor (pre-kallikrein deficiency) Fitzgerald factor (high molecular weight kiningen deficiency)	Longo 2013 Sood 2010
	Von Willebrand's Disease	Sood 2010
Acquired	Haemorrhagic diathesis of liver disease Disseminated Intravascular Coagulation (DIC) Vitamin K deficiency	Longo 2013 Sood 2010
	Anticoagulant drugs Acute primary fibrinolysis Massive transfusion of stored blood Circulating inhibitors of coagulation causing bleeding in recognized DIC in wide variety of disorders	Sood 2010

1.1.1.6 Anti-coagulant drugs

Imbalanced haemostatic activity may resulted in thrombosis or bleeding (Sood 2010; Zehnder 2012, p. 601). Thrombosis formation in blood vessel is harmful when blood coagulation cascade is unchecked by inoperative coagulation inhibitor, blood flow and fibrinolysis (Hoffbrand, Moss & Pettit 2006). Significant morbidity and mortality events accounted by arterial and venous thromboses (Weitz 2013, p. 277). Arterial thrombosis commonly causes acute myocardial infarction (MI), ischemic stroke and limb gangrene, which can progress to fatal pulmonary embolism and postphlebitic when deep-vein thrombosis occurs (Weitz 2013, p. 277). Venous thrombosis is rare but occurs at vascular disruption site, post-surgical trauma and indwelling venous catheters (Weitz 2013, p. 277). Prevention and treatment of thrombosis can be realized by the use of antithrombotic agents that are grouped into three; antiplatelets, anticoagulants and fibrinolytic drugs (Weitz 2013, p. 277). Antithrombotic agents have been used in clinical settings for more than 50 years and becoming one of the most frequently prescribed medications (Patriquin & Crowther 2013, p.477). Antiplatelet agents include aspirin, thienopyridines, dipyridamole, glycoprotein IIB/IIIA receptor antagonist; anticoagulant agents include heparin, low-molecular-weight heparin, fondaparinux, warfarin, dabigatran, rivaroxaban, apixaban; and fabrinolytic agents include streptokinase, anisteplase, urokinase, alteplase, tenecteplase and reteplase (Weitz 2013, p. 277). Antithrombotic agents can be taken orally or parenterally for various indications (Patriquin & Crowther 2013, p.477). Antiplatelets along with anticoagulants and fibrinolytic agents are mostly used to inhibit or treat arterial thrombosis since the pathophysiology of arterial thrombosis comprises of high amount of platelets due to the fleece-injured arteries. On the other hand, anticoagulants alone are the backbone for prevention and treatment of venous thromboembolism (VTE) due to the fact that fibrin predominantly contributes to such condition while fibrinolytic drugs served as therapeutic

agent for patients with VTE (Weitz 2013, p. 277). The main goal of anticoagulant therapy is to prevent unwanted clot formation in patient with artificial heart valve, phlebitasis, circulatory problems and joint replacement (Estridge & Reynolds 2008). Heparin and warfarin are the infamous antithrombotic agents most frequently used in clinical settings (Patriquin & Crowther 2013, p.477). However, current anticoagulant therapy demonstrated the use of recently-developed multiple novel agents such as dabigatran, rivaroxaban and apixaban that tackles issues associated with traditional agents; for example dosing variability, difficult monitoring, drug-drug and drug-environment interactions (Patriquin & Crowther 2013, p.477). Data on adverse drugs events showed warfarin and antiplatelets agents as one of the main factors contributing to emergency hospitalization in older patients (Budnitz et al. 2011). Anticoagulant therapy requires meticulous and details monitoring in providing the utmost therapeutic benefits but maintaining safe balance between preventing thromboembolic events and side effects (Patriquin & Crowther 2013, p.477). Two serious issues associated with antithrombotic therapy that often being brought up in medicolegal proceedings are failures to prescribe anticoagulants when clinically indicated and insufficient monitoring once therapy started (Patriquin & Crowther 2013, p.477).

1.1.2 Dates

1.1.2.1 Overview

Dates (*Phoenix dactylifera* L.) belongs to the Arecaceae family and has been cultivated for over 6000 years ago (Alkaabi et al. 2011) and the most common and vital fruit crop in Middle East (Mohamed et al. 2014). Dates are best categorized into the stage of ripening; Kamiri (stage 1), Khalal (stage 2), Rutab (stage 3) and Tamar (stage 4) (Alkaabi et al.

2011). There are various types of dates; Ajwa, Munifi, Hilali, Ruthana, Khodry, Khalas, Sukkary, Sefri and Segae with each of them have roles in diverse disease prevention (Rahmani et al 2014). In terms on nutrients, dates have high carbohydrate content (70–80%), fat (0.20–0.50%), protein (2.30–5.60%), dietary fiber (6.40–11.50%), minerals (0.10–916 mg/100 g dry weight), and vitamins (C, B1, B2, B3 and A) with very little or no starch (Mohamed et al. 2014). The phytochemical constituents of dates include alkaloids, flavonoids, steroids, tannins, estertepens, vitamins and phenolic acids (Onuh et al. 2012). The health benefits derived from dates are diverse and expanding including antihyperlipidemic, anticancer, gastroprotective, hepatoprotective, nephroprotective properties (Mohamed et al. 2014); anti-oxidant, anti-inflammatory, anti-bacterial (Rahmani et al 2014); and used for anaemia, stroke and tooth ache treatment, weight-gain and anti-aging (Onuh et al. 2012). Onto the medicinal value of specific type of dates; Ajwa, the Saudi Arabia and/or Madinah Al-Munawara-cultivated dates posed significant value in several types of diseases cure as well as protective effect in hepatic toxicity (Rahmani et al 2014).

1.1.2.2 Dates rich in salicylic acid

Dates have very high concentration of salicylic acid, which is more than 1 mg/100g (What is salicylic acid 2014). To our knowledge, there is one available scientific study that reported the salicylic acid content in dates (Swain, Dutton & Truswell 1985). Swain & Dutton (1985) studied the concentration of salicylic acid using High Performance Liquid Chromatography (HLPC) on 333 foods. Swain, Dutton & Truswell (1985) reported that fresh dates have 3.75 mg/100g salicylic acid while dried dates have 4.50 mg/100g salicylic acid. Salicylic acid is the compound that has the ability to block the action of vitamin K during coagulation pathway activation and act as a vitamin K antagonist (Roncaglioni et al.

1988). Previously, oral anticoagulant therapy was synonym to oral Vitamin K antagonist (VKA) especially in North America where warfarin is referred as VKA (Patriquin & Crowther 2013, p.477). This marked the importance of VKA action as an anticoagulant. Thus, dates may have anticoagulant effect. In an animal study, salicylates at moderate dose may prevent thrombus formation while limiting bleeding complications (Roncaglioni et al. 1998). Roncaglioni and team demonstrated that salicylates have anticoagulant effect comparable to warfarin when the animals administered with 175 mg/kg salicylates were compared to animal administered with 0.1-0.2 mg/kg warfarin.

1.1.2.3 Dates and hemostatic activities

To our knowledge, there is no scientific report on the relationship between dates and haemostatic activities. On a closely related study, Onuh et al. (2012) had reported a study on the effect of dates extracts on peripheral blood parameters using animal model. Their study concluded a dosage dependent significant increase in absolute values, red blood cell (RBC), Hemoglobin (Hb), Pack Cell Volume (PCV), reticulocytes and platelets. In relation to haemostatic activities, platelets count were seen significantly increased in rats treated with both ethanolic and aqueous crude dates extracts for a continuous 112 days. Increment in platelet counts suggests pro-coagulant effect on haemostatic activity. While there is no available scientific data on the correlation between dates and haemostatic activities, many scientific data has been published on antithrombotic effects of fruits, vegetables and spices as published by Torres-Urrutia et al. (2013).

1.1.3 Salicylic acid

1.1.3.1 Salicylic acid in human

Salicylic acid (2-hydroxybenzoic acid) is produced when acetylsalicylic acid (2-acetoxybenzoic acid) hydrolized (Figure 1.2). In human liver and blood, aspirin (acetylsalicylic acid) will be immediately converted to salicylic acid once ingested into the body (Duthie et al. 2005). Salicylic acid bound to plasma proteins and circulated in the blood and distributed to all tissues in the body (Duthie et al. 2005) especially synovial cavity, central nervous system and saliva (Hare, Woodside & Young 2003). Apart from serum, salicylic acid may be found in the urine. A study found that diet rich in salicylic acid by vegetarians surprisingly showed overlapping serum and urinary salicylates concentration in comparison to those taking low-dose aspirin; indicating prominent absorption of salicylic acid from ingested fruits and vegetables (Battezzati 2006; Duthie et al. 2005). Aspirin (acetylsalicylic acid) is analgesic, anti-thrombotic and anti-inflammatory (Scotter et al. 2007). Other than possible antithrombotic effect, acetylsalicylates may benefit human health by preventing colon cancer, pregnancy induced pre-eclampsy and worked as prophylactic agent against coronary heart disease at concentration as low as 30 mg/day (Venema et al. 1996).

1.1.3.2 Salicylic acid in foods and dates

Salicylic acid presents in many foods including fruits, vegetables, herbs, spices and vary in concentration (Battezzati 2006). Data on the salicylates content of foods are scarce and contradictory (Scotter 2007; Venema et al. 1996). On a study reported by Venema et al. (1996), low salicylic acid and no acetylsalicylic acid was detected in foods thought to have

high salicylates as published in the literature by fellow researchers. Salicyclic acid in food is often associated as natural blood thinner referring to food that has the ability to reduce blood. Salicylic acid has been reported to act as vitamin-K antagonist by lowering plasma levels of vitamin-K dependent clotting factors as well as inhibiting vitamin-K carboxylation in the liver (Roncaglioni et al. 1988). Therefore, a diet rich in acetylsalicylic acid should have an antithrombotic effect (Venema et al. 1996). Since dates have been claimed to have very high salicylic acid, it may suggest that dates might influenced haemostatic activity. However, low level of salicylates in normal diet probably insufficient to give physiological effect in vivo (Venema et al. 1996) and subsequently affect disease risk (Janssen et al. 1997). Determination of salicylic acid in foods (Swain, Dutton & Truswell 1985; Venema et al. 1996) and soups (Baxter et al. 2001) implied the use of HPLC in earlier studies. HPLC was also used in the determination of salicylates in biological samples such as serum (Blacklock et al. 2001), urinary salicylates (Janssen et al. 1996) and plasma (Dadgar et al. 1985). Application of gas-chromatography mass spectrometry (GCMS) was later applied in the determination of salicylic acids in plants (Deng et al. 2003; Engelberth et al. 2003) and human serum (Battezzati 2006). Scotter et al. (2007) came with an optimised method of salicylic acid determination in 76 foods using GCMS. A highly specific, rapid and cost effective method was developed for quantification of salicylic acid and acetylsalicylic acid (Scotter et al. 2007). GCMS is preferable because HPLC is lengthy and likely to be affected with interference especially when UV detection method is used (Scotter et al. 2007). Eventhough HPLC with fluorescence detection method is able to successfully determined salicylic acid in plants; samples need to undergo complex purification procedure to isolate salicylic acid from other fluorescent compounds of the same plants (Engelberth et al. 2003). Thus, GCMS provide a dependable method for separating, identifying and quantifying salicylic acid from samples that has undergo carboxylic acids derivatization (Engelberth et al. 2003).

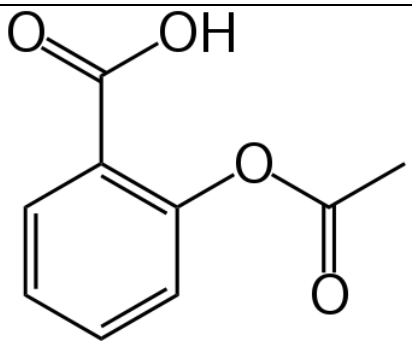
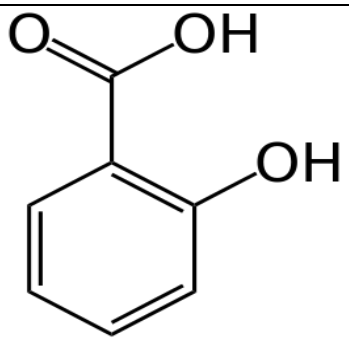
Acetylsalicylic Acid ($C_9H_8O_4$)	Salicylic Acid ($C_7H_6O_3$)
	

Figure 1.2 Skeletal formulas of acetylsalicylic acid and salicylic acid (Fvasconcellos 2008).

1.1.3.3 Mechanism of action

Understanding of salicylic acid as vitamin K antagonist requires comprehensive knowledge on vitamin K mechanism of action. History of vitamin K as antihaemorrhagic dated since more than half century ago when vitamin K was discovered to be able to correct dietary-induced bleeding in chicks (Vermeer & Schurgers 2000). Thereafter, vitamin K showed another profound effect as antihaemorrhagic by prolonging clotting times in obstructive jaundice patients and correcting bleeding in newborn with hemorrhagic disease (Kitchens, Kossler & Konkle 2013). Vitamin K then has established role in managing excessive anticoagulation due to the administration of warfarin, warfarin rodenticides and other oral anticoagulants (Kitchens, Kossler & Konkle 2013). The active role of vitamin K in coagulation cascade is originally to synthesis prothrombin (FII), however the role of vitamin K is expanding and discovered to be essential in functioning FII, FVII, FIX, FX as well as protein C, protein S (Bolan & Klein 2013, p.496; Longo 2013; Sood 2010;

Vermeer & Schurgers 2000), protein Z (Vermeer & Schurgers 2000) and osteocalcin (Bolan & Klein 2013, p.496; Vermeer & Schurgers 2000). Two enzymes involved during metabolism and regeneration of vitamin K are γ-glutamylcarboxylase and epoxide reductase (Bolan & Klein 2013, p.496; Longo 2013). Vitamin K is needed as cofactor for γ-glutamylcarboxylase during formation of γ-carboxyglutamic acid residues on coagulation proteins (Bolan & Klein 2013, p.496; Longo 2013). In turn, γ-glutamylcarboxylase will catalyze epoxide reductase for regeneration of reduced vitamin K (Longo 2013). In coagulation cascade, vitamin K- dependent coagulation proteins bind to the calcium and phospholipids of activated platelets with the help of γ-carboxyglutamate (Gla) residues and eventually improve thrombin formation by speeding up the reaction rate at several orders of magnitude (Vermeer & Schurgers 2000). Since Gla has stable divalent anionic charges, interaction of Gla and calcium ions allow localization of clotting factors to take place as well as building up internal calcium channels (Bolan & Klein 2013, p.496). Warfarin act as VKA by blocking the action of epoxide reductase and thus inhibit vitamin K effect (Longo 2013). Worldwide, VKA is used for the treatment of patient with developing thrombosis risk to inhibit blood coagulation (Vermeer & Schurgers 2000). In clinical practice, marked correction of PT test within 6-24 hours post-parenteral vitamin K administration confirmed the deficiency of vitamin K (Sood 2010).

1.2 Problem Statement / Research Question

Dates were reported to have high concentration of salicylic acid. Salicylic acid is the compound that has the ability to block the action of vitamin K during coagulation pathway activation and act as a vitamin K antagonist (Roncaglioni et al. 1988). Thus, dates may have anti-coagulation effect. However, an *in vivo* study by Onuh et al. (2012) on the effects of dates extract on blood parameters showed significant increment of platelets count;

which may suggest pro-coagulant activity of dates. In addition, literatures have reported that dates reduce postpartum haemorrhage (Khadeem et al. 2007); suggesting dates having antihaemorrhagic activity. In view of antiplatelet and anticoagulant drugs; aspirin is the infamous and mostly used antiplatelet drug (Weitz 2013, p. 277) while warfarin is the most widely used oral anticoagulant in the world (Krynetskiy & McDonnel 2007; Patriquin & Crowther 2013, p. 477). However, some conventional antiplatelet and anticoagulant drugs are associated with adverse events and therapeutic failure. Side-effects of anticoagulant drugs include internal bleeding, prolonged bleeding time, palpitation, gastrointestinal symptoms and haemorrhage. Aspirin along with other oral anti-platelet drugs account for 13.3% while warfarin accounts for 33.3% emergency hospitalization for adverse drug events in older American (Budnitz et al. 2011). At present time, no ideal anticoagulant drugs have ever existed since all anticoagulants and fibrinolytic will increase bleeding risk (Zehnder 2012, p. 601). These two claims of dates having high salicylic acid and the medicinal value to reduce postpartum haemorrhage as well as significant platelet count increment seem to contradict to each other. Therefore, the studies of coagulation activities of dates as well as analysis of chemical composition of dates are of great interest. Natural products are beneficial remedy because they are cheap and readily available without complication and side effects (Rahmani et al. 2014). General supportive measure is needed in coagulation disorders (Shaz et al. 2013). Since salicylates enhanced oral anticoagulant therapy (Sood 2010), the study of dates as a dietary supplement for patient with coagulation problem is highly attentive. On another side, supplements have potential of altering haemostatis (Markham & Dog 2013, p. 595); therefore, knowledge on dates as pro-coagulant or anti-coagulant food is beneficial in improving human health with coagulation-related problem.

1.3 Research Objectives

1.3.1 General

To assess coagulation activities in human plasma treated with Ajwa dates (*Phoenix dactylifera* L.) extracts *in vitro*.

1.3.2 Specific

1. To determine coagulation activities in human plasma treated with Ajwa dates (*Phoenix dactylifera* L.) extracts at different concentration (0.1, 0.5 and 1.0 g/mL).
2. To screen chemical compounds of Ajwa dates (*Phoenix dactylifera* L.) qualitatively using gas chromatography-mass spectrometry (GCMS) from ethanol and aqueous extracts.

1.4 Research Hypothesis

H_0: Ajwa dates *(Phoenix dactylifera* L.) do not have coagulation effect

H_A: Ajwa dates *(Phoenix dactylifera* L.) have coagulation effect

1.5 Rationale of Study / Expected Outcome

1. This research concerned about the potential role of dates as anti-coagulant.
2. This research focused on the improvement of human health by introducing dates as pro- or anti-coagulant food.

Chapter II

METHODOLOGY

2.1 Study design

This research employed experimental study design with control and treatment groups. Dates were extracted with single extraction method using ethanol and aqueous. Venous blood was withdrawn from twenty-seven donors (n=27) to be collected into K_2-EDTA and trisodium-citrate tubes for platelet and coagulation studies respectively. Platelet poor plasma (PPP) were treated with Ajwa dates ethanol and aqueous extracts at different concentrations (0.1, 0.5 and 1.0 g/mL). Seven groups were formed from each donor: control (normal saline), ET I (0.1 g/mL), ET II (0.5 g/mL), ET III (1.0 g/mL), AQ I (0.1 g/mL), AQ II (0.5 g/mL), AQ III (1.0 g/mL). *In vitro* coagulation activities of Ajwa dates were assessed by prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT). Data was analyzed statistically using SPSS Version 22.0 (Figure 2.1). Following extraction, both ethanol and aqueous extracts were analyzed qualitatively using Gas Chromatography – Mass Spectrometry (GCMS).

2.2 Extraction

2.2.1 Ajwa Dates Collection

This method of dates collection was adapted from Biglari et al. (2007) with few modifications. Ajwa dates were purchased from local distribution centre (Yusuf Taiyoob Sdn. Bhd., Pulau Pinang, Malaysia). Dates weighed about 4–8g per fruit and

for each extraction, approximately 100 g (~16 dates) of dates were used. Dates were properly selected in terms of size, colour, ripening stage and quality. The edible part of dates (100 g) were pitted and oven-dried for 7 days.

2.2.2 Ethanol Extraction

Pitted dates (100 g) were dry-blended with an analytical mill (IKA, A11 Basic). Grounded dates were extracted two times with 200 ml 99.7%, ethanol (QreC) at room temperature (20°C, 1H) using ultrasonic cleaner (WiseClean, WUC-A10H). The extracts were centrifuged (6000rpm, 10min) and filtered. The supernatant was concentrated under reduced pressure (40°C, 3H) using a rotary evaporator (EYELA, N1100) to obtain dates ethanolic crude extracts. The crude extracts were kept at 4°C until used for analysis.

2.2.3 Aqueous Extraction

This method of aqueous extraction was adapted from Vayalil (2002) with few modifications. Grounded dates were extracted two times with 200ml distilled water at room temperature (20°C, 1H) using ultrasonication (WiseClean, WUC-A10H). The extracts were centrifuged (6000rpm, 10min) and filtered. The supernatant was collected and lyophilized using freeze dryer (Christ, Alpha 1-4 LSC) to obtain aqueous crude extracts. The crude extracts were kept at 4°C until used for analysis.

2.2.4 Yield Percentage (%) Calculation

The percentage of extraction yield was calculated using the following formula (Zhang, Bi & Liu 2007).

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried extract} \times 100}{\text{Weight of original sample}}$$

2.3 GC-MS analysis

2.3.1 Crude Extracts Derivatization

This method of chemical derivatization was adapted from Scotter et al. (2007) with few modifications. A 1 mL aliquot of crude extract solution (1 mg/mL) was transferred to a 2 ml glass vial and solvent was removed under a stream of N_2 on an evaporator system (Glas-Col) at 35°C. The vial was removed as soon as the residue solvent dried. A 200 µl of BSTFA/TMCS derivatising agent (Supelco) was added to the vial. The vial was capped immediately and heated at 60°C for 1 H with occasional swirling. The solution was cooled to room temperature prior to analysis by GC–MS (Agilent, 7890A).

2.3.2 Analysis of Compounds

This method of GC-MS analysis was adapted from Deng et al. (2003). Analysis was done using HP-5ms capillary column (30m, 0.25mm, 0.25µm) equipped with MSD 5975C detector and spitless injection system. Helium gas (99.999%) was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was set at 100°C for 2 min then programmed at 15° C/min to 300°C which was maintained for 10 min. The results

were analyzed qualitatively in full-scan acquisition mode with a mass range of 45–500 a.m.u. The compounds were identified using NIST database. Identification was made by comparing mass spectra of each compound with the compound in NIST database for matching quality.

2.4 Assessment of Coagulation Activities

2.4.1 Sample Size Calculation

By using Power and Sample software (Dupont & Plummer 1997) which based on paired *t*-test formula; result showed as follows: $\alpha = 0.05$, $\delta = 0.345$ (detectable difference at 60%), $\sigma = 0.575$ (based on coagulation activity result (Torres-Urrutia et al. 2011), power = 0.8 (80%), n = 27 (plus 10% drop out rate) for subject group.

2.4.2 Donor Recruitments

Twenty-seven donors (n=27) were recruited at Clinical Trial Complex, Advanced Medical and Dental Institute (AMDI), USM. All donors were informed about the objective of this study and had signed informed consent form prior to blood collection. The protocol was authorized by Human Research Ethics Committee (HREC), USM. Inclusion criteria include all the criteria for blood donation as stated by National Blood Centre (PDN). Inclusion criteria includes male or female; age range between 18-60 years old; healthy and free from clinical disorders; body weight of more than 45 kg; had taken food before donating blood; not pregnant, not breast-feeding, menstrual period on day-5 onwards and do not involved in any risk activities. Exclusion criteria include had

taken antiplatelets/anticoagulant/fibrinolytic drugs, vitamin/mineral supplements or medication for at least 7 days before recruitment.

2.4.3 Blood Collection

For platelet count, 3 mL blood was collected in K_2-ethylene-diamine tetraacetic acid (EDTA). For coagulation studies, 6 mL blood was collected in trisodium citrate tube (3.2%) in a 9:1 ratio. Blood was centrifuged (6000rpm, 10min) to obtain PPP (platelet-poor plasma) and analyzed immediately.

2.4.4 Platelet Count

Platelet count was determined using automated hematology analyzer (Sysmex, KX-21). The result of platelet count was expressed in number of platelets per microlitre of blood.

2.4.5 Measurement of coagulation factors

This method of anticoagulant activity study was adapted from Karim, Noor & Seman (2013). PT, APTT and TT tests were conducted using an automated coagulation analyzer (Diagnostica Stago, STA Compact). The PPP was pre-incubated (37°C, 7min) with crude extract solutions at 1:1 (v/v) with concentration 0.1 g/mL, 0.5 g/mL and 1 g/mL. The results of coagulation tests were expressed in seconds (s).

2.4.6 Sample Disposal

Excess samples from this research were not used for other reasons and were destroyed with the consent from Human Research Ethics Committee USM (HREC).

2.5 Statistical Analysis

Statistical analysis was performed using SPSS Version 22.0 (IBM Corp.). The data was expressed as median with minimum and maximum range of duplicate determination. Kruskal-Wallis with Bonferroni correction was used to compare significance difference between control and each treatment group. Values were considered significantly different when $p<0.05$ and very significantly different when $p<0.001$.