CHEMICAL AND PHARMACOLOGICAL STUDIES OF *ARDISIA ELLIPTICA*: ANTIPLATELET, ANTICOAGULANT ACTIVITIES AND MULTIVARIATE DATA ANALYSIS FOR DRUG DISCOVERY

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List of publications and conference presentations

Publications

1. **J. Ching**, W.L. Soh, C.H. Tan, J.F. Lee, J.Y.C. Tan, J. Yang, C.W. Yap, H.L. Koh. Identification of active compounds from medicinal plant extracts using GC-MS and multivariate data analysis. Journal of Separation Science 2012, 35: 53-59.

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3. **J. Ching**, T.K. Chua, L.C. Chin, A.J. Lau, Y.K. Pang, J. Murti Jaya, C.H. Tan, H.L Koh. β -Amyrin from *Ardisia elliptica* Thunb. is more potent than aspirin in inhibiting collagen-induced platelet aggregation. Indian Journal of Experimental Biology 2010, 48:275-279.

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Conference presentations

Oral presentations

1. W.L. Soh, **J. Ching,** C.H. Tan, C.W. Yap, H.L. Koh. Novel Method Using Multivariate Data Analysis to Identify Antiplatelet Compounds from Medicinal Plant Extract. 1st PharmSci@Indonesia 2011 Symposium, Institute Technology of Bandung, Bandung, Indonesia, 11 June 2011 (Won best presentation award)

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7. L.C. Chin, **J. Ching**, HL Koh. Antiplatelet and anticoagulant effects of *Strobilanthes crispus*. 5th Congress of the Asian-Pacific Society on Thrombosis and Haemostasis, Grand Copthorne Waterfront Hotel, Singapore, 18-20 September 2008

8. **J. Ching**, L.C. Chin, C.H. Tan, H.L. Koh. A study of antiplatelet and anticoagulant activities in plants commonly found in Singapore. 3rd Medicinal Chemistry Symposium, National University of Singapore, 28 July 2008

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Summary

Medicinal plants have been important sources of novel therapeutics since time immemorial. Current antiplatelet and anticoagulant drugs used to treat cardiovascular diseases have numerous adverse effects. The objectives of this study are to investigate the potential antiplatelet and anticoagulant effects of a local medicinal plant, *Ardisia elliptica* Thunberg and to isolate and identify the active compound(s) responsible for the actives.

Ardisia elliptica is a local medicinal plant used in Malay traditional medicine for the treatment of pain in the region of the heart, parturition complications, fever, diarrhoea and liver poisoning. We hypothesised that *A. elliptica* possesses bioactive components that have antiplatelet and/or anticoagulant properties.

A 70% v/v methanol extract was obtained from the leaves of the plant and fractionated. HPLC and GC-MS were used for the analysis of the extract and fractions. Platelet aggregation assay was performed on the extract and fractions using a platelet aggregometer. Effects on plasma coagulation were studied by measuring the prothrombin time and activated partial thromboplastin time. The plant extract was found to have both antiplatelet and anticoagulant activities. From the most active fraction, β-amyrin was successfully isolated and purified by preparative and semi-preparative HPLC. α –amyrin co-eluted with another compound and was not successfully purified. The IC_{50} values for inhibition of collagen-induced platelet aggregation inhibition were 21.3 and 10.5 μM for α- and β-amyrin respectively. These values indicated that α- and β-amyrin are three and six times more active respectively than aspirin (IC_{50} value= 62.7 μM). Hence, α-

and β-amyrin are some of the active components in *A. elliptica* contributing to its antiplatelet activity. However α- and β-amyrin did not exhibit anticoagulant activity in the plasma coagulant assays, suggesting that other compounds are responsible for the anticoagulant activity in extracts of *A. elliptica*.

As the conventional process of repeated fractionation is a tedious process for the discovery of bioactive components, a platform method for drug discovery from plant extracts using multivariate data analysis (MVDA) was developed. The MVDA method independently predicted that α- and β-amyrin were active components in the plant extract for antiplatelet activity. The developed MVDA method is a more time-efficient and cost effective method than the conventional bioassay guided fractionation method.

The 70% v/v methanol extract and β-amyrin were subsequently studied in rats for their effects on tail bleeding, platelet aggregation and plasma coagulation. The extract and β-amyrin administered to rats orally were shown to prolong the tail bleeding times and inhibited platelet aggregation significantly. However anticoagulant activity was not observed at these dosages *in vivo*.

The pharmacokinetic profile of β-amyrin was then studied in rats. It was found that β-amyrin had a very long terminal elimination half-life ($t_{1/2\lambda z}$ = 10.2 ± 3.0 h) and slow clearance (Cl = 2.04 ± 0.24 ml min^{-1} kg^{-1}). The absolute oral bioavailability of β-amyrin in the crude plant extract was found to be generally low although slightly higher than that in the suspension of the pure form (3.83% *vs* 0.86%).

In conclusion, the results presented in this thesis provide some scientific evidence for the traditional uses of *A. elliptica*. Further work is warranted to develop the lead compounds into useful therapeutics.

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AA	Arachidonic Acid
ADP	Adenosine Diphosphate
APCI	Atmospheric-Pressure Chemical Ionisation Mass Spectrometry
APPI	Atmospheric-Pressure Photoionisation
aPTT	activated Partial Thromboplastin Time
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
AUC	Area Under Curve
CMC	Carboxymethylcellulose
COX	Cyclooxygenase
DMSO	Dimethylsulfoxide
ED_{50}	50% Effective Dose
EI	Electron Impact
ESI	Electrospray Ionisation
et al.	et alii/et alia
FT-IR	Fourier Transform Infrared Spectroscopy
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
GP	Glycoprotein
HIT	Heparin Induced Thrombocytopenia
HMWK	High Molecular Weight Kininogen
HPLC	High Performance Liquid Chromatography
I.P.	Intraperitoneal
IC_{50}	50% Inhibitory Concentration
IR	Infrared
kg	Kilogram
L	Liter
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
MOX	Methoxyamine Hydrochloride
MRT	Mean Residence Time
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MVDA	Multivariate Data Analysis
N. A.	Not Applicable
NaCl	Sodium Chloride
Na_2HPO_4	Sodium Hydrogen Phosphate
NCE	New Chemical Entity
ng	Nanogram
NMR	Nuclear Magnetic Resonance
^{o}C	Degrees Celsius
OPLS	Orthogonal Partial Least Squares

PAF	Platelet Activating Factor
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PKC	Protein Kinase C
PL	Phospholipids
PLS	Partial Least-Squares
PLS-DA	Partial Least Squares projection of latent structures-Discriminant Analysis
P.O.	*Per Os*
PT	Prothrombin Time
PXR	Pregnane X Receptor
QCAR	Quantitative Composition-Activity Relationship
RSD	Relative Standard Deviation
s	second
S.D.	Sprague-Dawley
SIM	Selective Ion Monitoring
TCM	Traditional Chinese Medicine
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TMS	Trimethylsilyl
TNBS	Trinitrobenzene Sulphonic Acid
TPA	12-O-tetradecanoylphorbol-13-acetate
TxA_2	Thromboxane A_2
UV	Ultraviolet
vWF	von Willebrand Factor
WE	Water Extract
WF	Water Fraction
WHO	World Health Organisation
W/W	Weight/Weight
K	Kilo
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Phosphate
VKORC	Vitamin K Epoxide Reductase Complex
V/V	Volume/Volume
α	Alpha
β	Beta
μ	Micro

CHAPTER 1

Introduction

1.1 Cardiovascular diseases and limitations of current treatments

Cardiovascular diseases such as coronary heart disease and stroke are the top killer of people globally, and by 2030 almost 23.6 million people are projected to die from cardiovascular diseases (WHO, 2010).

Patients of cardiovascular disease usually have myocardial infarction due to coronary artery thrombosis. Myocardial infarction is generally caused by platelets adhering onto the subendothelial matrix of the artery after it has been damaged by a ruptured artherosclerotheic plague. The aggregation of platelet at the site induces the formation of a prothrombotic surface which then induces a clot to form and subsequently vascular blockage (Michelson, 2010). Patients with cardiovascular diseases related to thromboembolism are usually treated with antiplatelets or anticoagulants like aspirin and warfarin to decrease the risk of recurrences of heart attack and stroke. Despite the efficacy of current drugs used in the treatment of such diseases, drugs like aspirin and warfarin are associated with numerous adverse effects, which will be elaborated later.

1.1.1 Antiplatelet drugs

Antiplatelet drugs used clinically are broadly classified into four classes: cyclooxygenase (COX) inhibitors, adenosine diphosphate (ADP) receptor antagonists, glycoprotein (GP) IIb/IIIa antagonists, and phosphodiesterase inhibitors (Michelson, 2010).

1.1.1.1 Cyclooxygenase inhibitors

There are two forms of COX: COX-1 and COX-2. COX-1 is constitutively expressed in the endoplasmic reticular membrane of all cells, such as gastric, vascular cell, kidney and platelets (Morita et al., 1995). It thus has varying roles such as maintenance of renal blood flow, gastric mucosal protection and platelet activation, though the generation of different prostaglandins (Smith, 1992). COX-2 exists in microvascular endothelial cells, which generates prostaglandin I_2 (McAdam et al., 1999) that has functions like decreasing platelet aggregation, vasodilation and inhibition of gastric acid secretion (Michelson, 2007). Aspirin is an example of drugs under the class of cyclooxygenase inhibitors. It works by inhibiting the catalytic activity of cyclooxygenase-1 (COX-1), thereby preventing the conversion of arachidonic acid into prostaglandin H_2, and eventually thromboxane A_2 (TXA_2) (Loll et al., 1995). When TXA_2 is not generated, platelets are prevented from activation via the thromboxane receptor. Because aspirin deactivates both COX-1 and COX-2, gastric mucosal erosion is a common adverse effect in patients taking the drug. Aspirin administration is associated with predisposition to *Helicobacter pylori* infections (Patrono et al., 2001). In addition, aspirin administration is also associated with Reye's syndrome, making it difficult for usage in susceptible individuals especially children and teenagers less than 18 years old (Glasgow, 2006).

1.1.1.2 ADP receptor antagonists

The second class of antiplatelet drugs are the ADP receptor antagonists. ADP activates platelet aggregation by increasing the concentration of free cytoplasmic calcium via the G_q-coupled $P2Y_1$ receptor and the inhibition of the G_i-coupled $P2Y_{12}$ receptor, which inhibits adenylyl cyclase. Both the receptors need to be activated

before platelets can aggregate (Michelson, 2007). When the $P2Y_1$ receptor is activated, platelets undergo shape change and as well as a rapid reversible shape change. When the $P2Y_{12}$ receptor is activated, platelets aggregate in a slow, sustained, progressive fashion that is not preceded by shape change (Michelson, 2010). Currently only $P2Y_{12}$ receptor antagonist are studied clinically. Examples include ticlopidine, clopidogrel and prasugrel (Michelson, 2010). Ticlopidine is an irreversible antagonist of the $P2Y_{12}$ receptor. It has adverse effects like bleeding, gastrointestinal toxicity (heartburn, indigestion, nausea and vomiting), rash, neutropaenia and rare cases of thrombotic thrombocytopaenic purpura (Michelson, 2007; Michelson, 2008). Because of its numerous adverse effects, ticlopidine has been largely replaced by clopidogrel. Clopidogrel has a better adverse effect profile compared to ticlopidine as it does not show gastrointestinal toxicity (Matetzky et al., 2004; Sabatine et al., 2005; Michelson, 2007; Snoep et al., 2007; Michelson, 2010). However clopidogrel has a slow onset of action and shows interindividual variability where poor inhibition of platelet response was seen in some patients (Matetzky et al., 2004; Sabatine et al., 2005; Snoep et al., 2007). Prasugrel is another antagonist of the $P2Y_{12}$ receptor being introduced and it does not show the adverse effects exhibited by both ticlopidine and clopidogrel (Michelson, 2010). Prasugrel is also more potent than clopidogrel (Payne et al., 2007; Wiviott et al., 2007; Michelson et al., 2009), but according to Wiviott et al. (2007), the TRITON-TIMI 38 (Trial to assess Improvement in Therapeutic outcomes by optimizing platelet inhibition with prasugrel–Thrombolysis In myocardial Infarction 38), a Phase III trial on patients with acute coronary syndromes, patients with prasugrel has more haemorrhagic adverse effects. There were more patients in the prasugrel group than clopidogrel

group experiencing major bleeding and the rate of life-threatening bleeding was also higher.

1.1.1.3 GP IIb/IIIa antagonists

The third class of antiplatelet drugs are the GPIIb/IIIa antagonists. There are three FDA approved GPIIb/IIIa antagonists, which includes abxicimab, eptifibatide and tirofiban (Michelson, 2010). These drugs target the final pathway of platelet aggregation, where fibrinogen, or under conditions of high shear stress, von willibrand factor (VWF), binds to GPIIb/IIIa (Michelson, 2010). All the three drugs require intravenous administration, and show adverse effects like bleeding and thrombocytopaenia (Michelson, 2007; Michelson, 2010). Numerous clinical trials have been conducted on the use of GPIIb/IIIa antagonists (Table 1.1).

These clinical trials show varying results. While many of the trials showed a positive effect, there were also some trials which showed disappointing results. For example in GUSTO-IV, there was no significant reduction in the number of acute coronary syndromes in patients treated with either abciximab or placebo (Simoons et al., 2001).

Table 1.1 Clinical trials conducted on the use of GPIIb/IIIa antagonists.

Clinical trial	Reference
CAPTURE (C7E3 Anti-Platelet Therapy in Unstable Refractory Angina)	Simoons et al., 1997
EPIC (Evaluation of c7E3 for Prevention of Ischemic Complications)	Califf et al., 1994
EPILOG (Evaluation of PTCA to Improve Long- Term Outcome with Abciximab GPIIb-IIIa Blockade)	Topol et al., 1997
EPISTENT (Evaluation of Platelet Inhibition in Stenting)	Lincoff et al., 1999
ESPRIT (Enhanced Suppression of the Platelet IIb- IIIa Receptor with Integrilin Therapy)	Tcheng et al., 2000
GUSTO-IV (Global Use of Strategies to Open Occluded Coronary Arteries-IV)	Simoons et al., 2001
IMPACT II (Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis II)2	Tcheng et al., 1997
PRISM (Platelet Receptor Inhibition in Ischemic Syndrome Management)	Bazzino et al., 1998
PRISM-Plus (Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Angina)	Bazzino et al., 1998
PURSUIT (Platelet IIb-IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy)	Harrington et al., 1998
RESTORE (Randomized Efficacy Study of Tirofobanvfor Outcomes and Restenosis)	Hanrath et al., 1997
TARGET (Do Tirofoban and ReoPro Give Similar Efficacy Trial)	Topol et al., 2001

1.1.1.4 Phosphodiesterase inhibitors

The fourth class of drugs belong to the family of phosphodiesterase (PDE) inhibitors. The majority of the PDEs found in platelets are PDE3 and PDE5, which utilises mainly cyclic AMP (cAMP) and cyclic GMP (cGMP) as substrates respectively (Hasalam et al., 1999). Phosphodiesterase inhibitors work by different pathways, including the inhibition of cyclic nucleotide phosphodiesterase and adenosine uptake blockage. This results in the increase in cAMP and cGMP levels in the platelet, which inhibits signal transduction leading to platelet aggregation (Michelson, 2007). Two examples of the phosphodiesterase inhibitors are dipyridamole and cilostazol. Dipyridamole inhibits cGMP PDE5 in the platelets while cilostazol is selective for cAMP PDE3. Dipyridamole was reported to cause headache, dizziness, hypotension, flushing, gastrointestinal toxicity (nausea, vomiting, diarrhoea and abdominal pain) and rash (Sacco et al., 2008; Michelson, 2010). Cilostazol was reported to cause bleeding, headache, diarrhoea, palpitations, dizziness, rash and pancytopaenia (Lee et al., 2007; Michelson, 2010). The adverse effects of cilostazol led to approximately 15% of patients to discontinue use of the drug (Lee et al., 2007).

1.1.2 Anticoagulation drugs

The process of blood coagulation is complex. Briefly, there are three stages of plasma coagulation: initiation, propagation and fibrin formation (Rang et al., 2003; Weitz and Bates, 2005). The initiation step can occur by two pathways, the intrinsic pathway and the extrinsic pathway (Figure 1.1). The coagulation cascade starts with the formation of tissue factor (TF)/ factor VIIa (FVIIIa) complex at the site of tissue injury via the extrinsic pathway. The intrinsic pathway is initiated by the interaction of factor XII, high molecular weight kininogen (HMWK) and prekallikrein to form

XIIa. XIIa in turn converts XI to XIa which activates IX to IXa. In the extrinsic pathway, factor X is also converted to Xa via factor VIIa, tissue factor, and cofactors like calcium and phospholipids. Propagation of the coagulation cascade occurs at this step, where factor X is activated. The activated factor X then converts prothrombin to thrombin. The final stage, fibrin formation occurs when fibrinogen is converted to fibrin by thrombin (Rang et al., 2003; Weitz and Bates, 2005). When screening for haemostasis, tests including prothrombin time (PT) and activated partial thromboplastin time (aPTT) are commonly used. PT is a reflection of the extrinsic and final common pathways of the plasma coagulation cascade, while aPTT reflects the intrinsic and final common pathways (Kamal et al., 2007).

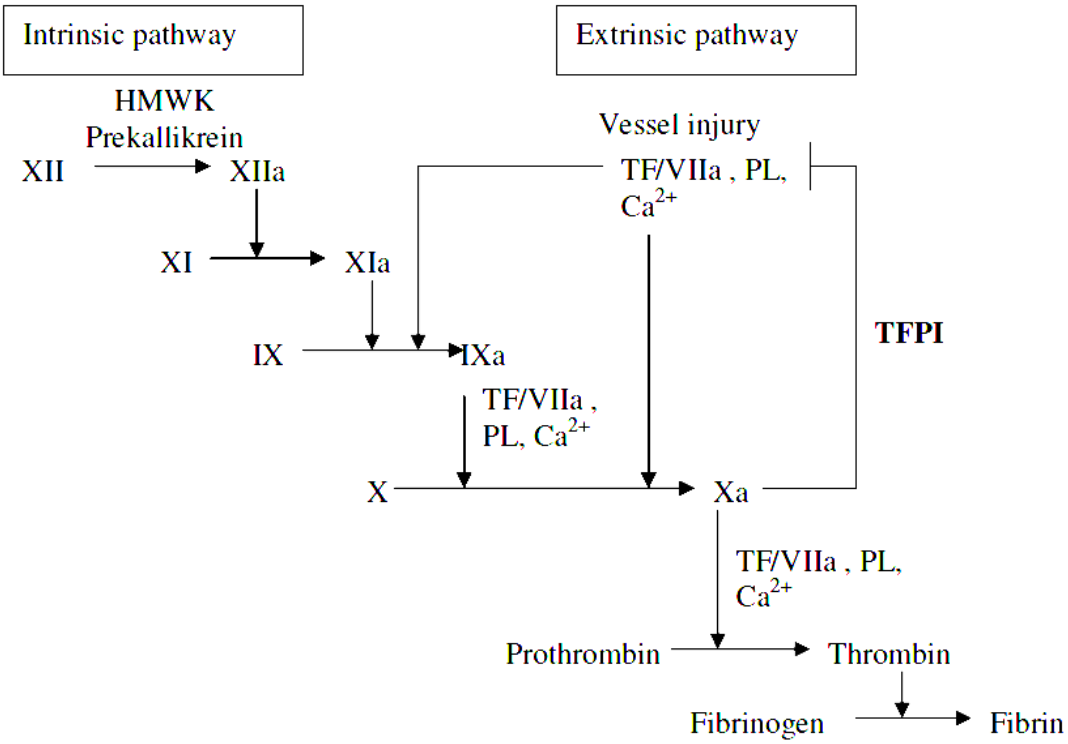


Figure 1.1 The coagulation cascade shown in conjunction with the participation of the tissue factor pathway inhibitor (TFPI). (PL, negatively charged phospholipids; TF, tissue factor; HMWK, high molecular weight kininogen.)

Warfarin had been the most common oral anticoagulant drug used for over 60 years since its introduction in the 1940s (Ahrens et al., 2010). Warfarin prevents coagulation by inhibiting the γ-carboxylation of the vitamin K dependent coagulation factors II, VII, IX and X, by inhibiting the vitamin K epoxide reductase complex subunit 1 (VKORC1) (Whitlon et al., 1978). Despite the efficacy of warfarin, patients taking the drug are at risk of serious or fatal bleeding. Warfarin also has an unpredictable pharmacokinetic profile, which is dependent on genetic variability (Ahrens et al., 2010). Genetic factors influencing warfarin's pharmacokinetic profiles include polymorphisms in the VKORC1 and CYP2C9 genes (Rettie and Tai, 2006). CYP2C9 is a major hepatic enzyme required for metabolic clearance of warfarin. Therefore mutations in the CYP2C9 gene will lead to decreased clearance of the drug, causing a prolonged half-life and over anticoagulation. A polymorphism in the VKORC1 gene also puts patients at risk of overdosage. Individuals with the VKORC1*2 polymorphism requires less warfarin than those with the wildtype gene. This puts patients at risk of overdosage leading to excessive bleeding (Ahrens et al., 2010). Non-genetic factors causing problems associated with varying drug responses in different patients include body mass index, age and drug history. Another widely used anticoagulant, heparin is generally safe to use. However heparin has to be injected, which limits its use to not more than two weeks (Melnikova, 2009). Like warfarin, adverse effects of heparin therapy include haemorrhage and heparin-induced thrombocytopenia (HIT type II), which occurs in 3% of patients (Melnikova, 2009). HIT can cause thrombosis, leading to limb gangrene or death (Rang et al., 2003; Thong and Kam, 2005).

In view of the short comings of the currently used antiplatelet and anticoagulation drugs, development of better drugs with fewer adverse effects is necessary.

1.2 Medicinal plants

Herbal medicines have been used since antiquity. There are currently three major types of herbalism being practiced: Asian, European and indigenous herbalism. Among Asian herbalism, the most famous would include those from China (as part of Traditional Chinese Medicine or TCM) and India (also known as Aryuveda). TCM and Aryuveda have been practiced for thousands of years, and their remedies usually comprise of mixtures of plant and/or animal parts. The combination of different components acts in such a way that one component will work in complementary with another, and enhance the therapeutic effects of the mixture (Elvin-Lewis, 2001). European herbalism has its origins from Mediterranean civilisations. It was believed in the Middle Ages that the shape and colour of the plant would imply what it is useful for. This was stated in the Doctrine of Signatures, a philosophy which helped decide how plants were selected for treatment of diseases, for example, a heart-shaped leaf and yellow plant parts would be good for treating heart and hepatitis conditions respectively. Compounds from these plants were eventually being isolated or synthesised for usage. Indigenous herbalism is very diverse and practiced among cultures that are still intact. The different types of herbalism vary among regions, are usually based on anecdotal information and have widely accepted efficacy and safety.

Although traditional medicines had been used for centuries, the use of herbal medicines for treatment of illnesses was slowly phased out. Allopathic practitioners

viewed herbal medicine as not potentially useful or even harmful as many herbal concoctions did not have proof of safety and efficacy. As such, western medicine became the mainstream healthcare system in most parts of the world. However, there had been a revival of the "back to nature" belief among people. Many practitioners believed that 'primary active ingredients in herbs are synergized by secondary compounds, and secondary compounds mitigate the adverse effects caused by primary active ingredients' (McPartland and Pruitt, 1999). In addition to that, a revival of interest in the traditional screening of drugs from plants has been observed following success stories of discovery of blockbuster drugs from natural sources. Examples are such as paclitaxel (Taxol®) from the Pacific Yew tree, huperzine from *Huperzia serrata* and the more recent Tamiflu®, whose active compound was synthesised from shikimic acid isolated from a Chinese herb *Illicium anisatum* or star anise.

1.2.1 Natural products in drug discovery

While the impact of natural products on drug discovery is apparent, pharmacognosy is not favoured by the pharmaceutical industries. This is because making use of synthetic chemical libraries and combinatorial synthesis is deemed to be more convenient, faster and simpler. Although such techniques are preferred, some combinatorial libraries have very low hit rates or even no hits at all (Koehn and Carter, 2005). A review of the drugs introduced since 1994 showed that approximately 50% of the drugs are either natural product or natural product-derived compounds (Newman and Cragg, 2007). Examples of these are anticancer drugs vincristine and vinblastine from the Madagascar periwinkle (*Catharanthus roseus*) introduced during the 1960s, and an antibacterial drug daptomycin (derived from a bacterium, *Streptomyces roseosporus*) introduced in 2003. In addition, of the 1184

new chemical entities (NCEs) analysed between years 1981 to 2006, only 30% was made by totally synthetic routes (Newman and Cragg, 2007). The other NCEs are natural products or related to natural products, derived biologically, modified from natural products, or having pharmacophores from natural compounds. These statistics show that many NCEs are derived naturally. This is so because being secondary metabolites from natural sources, they could have exhibited more characteristics that resemble other existing drugs when compared to totally synthetic compounds (Koehn and Carter, 2005). Thus they are easier to use as lead compounds for modification into drugs.

Research is actively ongoing for natural products for treatment of different diseases. For example, lipoic acid which is naturally occurring in both plants and animals, is being studied as a treatment for Alzheimer's disease (Bonda et al., 2010). For cancer treatment, natural products such as polyphenol from green tea (Araujo et al., 2011; Chen et al., 2011; Siddiqui et al., 2011) and curcumin from turmeric (Sato et al., 211; Sharma et al., 2011) are popularly studied. A search on the database Web of Science using the keywords "natural product*" generated over 100,000 hits from years 1991 to 2010. It can be seen from Figure 1.2 that the number of papers published has increased steadily from 2 in 1991 to over 9600 in 2010. From the current situation, natural products will definitely continue to be an important source of prototypic chemical structures for new drugs in years to come.

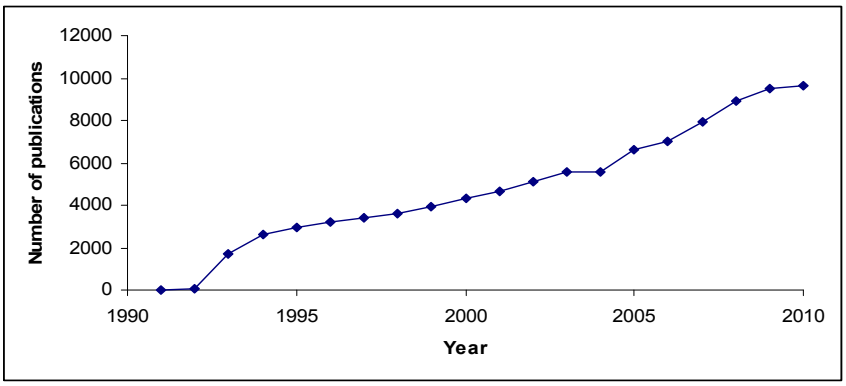


Figure 1.2 Number of publication hits generated by Web of Science using keywords "natural product*" from 1991 to 2010.

1.2.2 Antiplatelet and anticoagulant compounds from medicinal plants

Medicinal plants are a rich source of antiplatelet and anticoagulant compounds. The commonly used antiplatelet, aspirin (acetylsalicylic acid) for example, had its origins from salicin obtained from the willow plant (*Salix* spp.). Willow leaves were used to treat pain since the times of ancient Assyrians and Egyptians (Levesque and Lafont, 2000). Similarly, the anticoagulant drug warfarin (3-phenyacetyl ethyl, 4-hydroxycoumarin) had its origins from a medicinal plant, the sweet clover. The effect of sweet clover was first observed in cattle suffering form haemorrhage when the plant was used as cattle-feed (Mueller and Scheidt, 1994). Chua and Koh (2006) had reviewed 55 medicinal plants and 136 phyto-constituents with antiplatelet and anticoagulant activities (Chua and Koh, 2006).

From garlic, an antiplatelet compound, methyl allyl trisulfide, had been isolated and was found to inhibit platelet aggregation induced by arachidonic acid, collagen, thrombin, ADP, PAF and U46619 (Lim et al., 1999). Kaempferol 3-O-β-glucopyranoside and kaempferol 3-O-β-neohesperidoside extracted from the wild garlic *Allium ursinum* also inhibited platelet aggregation induced by collagen (Carotenuto et al., 1996). Other anti-platelet compounds have been discovered from

well-known plants such as *Ginkgo biloba* (Diamond et al., 2000) and green tea (Son et al., 2004).

Anticoagulant compounds were also isolated from numerous medicinal plants. Quercetin 3-acetyl-7,3′,4′-trisulphate and quercetin 3,7,3′,4′-tetrasulphate isolated from *Flaveria bidentis* exhibit anticoagulant activity at a concentration of 1 mM (Guglielmone et al., 2002), prolonging PT and aPTT. Quercetin 3,7,3′,4′-tetrasulphate was found to inhibit platelet aggregation induced by thromboxane B_2 formation due to collagen or arachidonic acid (Guglielmone et al., 2005).

Reports of active compounds from medicinal plants with antiplatelet or anticoagulant activities from the year 2006 onwards are listed in Table 1.2.

Table 1.2 List of reports of active compounds from medicinal plants with antiplatelet or anticoagulant activities

	Plant	Mechanism	Reference
Antiplatelet compounds			
Andrographolide, 14-deoxy-11, 12-didehydroandrographolide	*Andrographis paniculata*	Inhibit thrombin-induced aggregation	Thisoda et al., 2006
α-amyrin, β-amyrin	*Ardisia elliptica*	Inhibit collagen-induced aggregation	Ching et al., 2010
Eugenol, amygdalactone, 2-methoxycinnamaldehyde, coniferaldehyde	*Cinnamomum cassia*	Inhibit AA, U46619 and epinephrine-induced platelet aggregation	Kim et al., 2010
Cinnamic alcohol, 2-hydroxycinnamaldehyde,	*Cinnamomum cassia*	Inhibit AA and U46619-induced aggregation	Kim et al., 2010
Coumarin, cinnamaldehyde, cinnamic acid, icariside DC, dihydrocinnacasside	*Cinnamomum cassia*	Inhibit U46619-induced aggregation	Kim et al., 2010
Maltol 3-O-beta glucopyranoside	*Evodiopanax innovans*	Inhibit collagen, ADP and thrombin-induced aggregation	Tsuchiya et al., 2008
Quercetin 3,7,3',4'-tetrasulphate	*Flaveria bidentis*	Inhibit AA, collagen, ADP and epinephrine-induced aggregation	Guglielmone et al., 2005
Harmane, harmine	*Perganum harmala*	Inhibit collagen-induced aggregation	Im et al., 2009
Hydroxychavicol	*Piper betel*	Inhibit AA and collagen-induced aggregation; inhibit TXB_2 and COX-1 and COX-2	Chang et al., 2007

Sanguinarine	*Sanguinaria canadensis*	Inhibit collagen, U46619 and thrombin-induced aggregation	Jeng et al., 2007
Taxinine, taxinine A, taxinine B, 2-deacetoxytaxinine B, taxacin, taxchinin B, taxol	*Taxus cuspidata*	Inhibit AA and U46619-induced aggregation	Kim and Yun-Choi, 2010
Anticoagulation compounds			
7-hydroxycoumarin	*Aegle marmelos, Citrus aurantium*	Prolong PT, clotting time and bleeding time	Ramesh and Pugalendi, 2007
Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (3.5%), arabinose (21.1 %), xylose (0.8 %), mannose (0.9 %), glucose (21.0 %), galactose (22.8 %).	*Arnica motana*	Prolong PT and aPTT *in vitro*	Pawlaczyk et al., 2009
Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (4.2%), arabinose (28.6%), xylose (2.4%), mannose (0.6%), glucose (8.0%), galactose (23.5%).	*Echinacea purpurea*	Prolong PT and aPTT *in vitro*	Pawlaczyk et al., 2009

Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (5.5%), arabinose (13.6%), xylose (4.2%), mannose (0.9%), glucose (4.2%), galactose (15.7%).	*Filipendula ulmaria*	Prolong PT and aPTT *in vitro*	Pawlaczyk et al., 2009
Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (1.0%), arabinose (6.8%), xylose (1.4%), mannose (0.9%), glucose (8.0%), galactose (19.3%).	*Fragaria vesca*	Prolong PT and APTT *in vitro*	Pawlaczyk et al., 2009
Acidic glycoconjugate (MW ~12.500) consisting of carbohydrates (30%), phenolics (1 g contained 1.2mMof gallic acid equivalent) and proteins (0.8%). Carbohydrates predominated by uronic acids (~66%), galactose (~12%), rhamnose (~10%) and arabinose (~9%) residues	*Lythrum salicaria*	Prolong PT and aPTT *in vitro*. Procoagulation seen *in vivo*.	Pawlaczyk et al., 2010

Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (2.4%), arabinose (9.8%), xylose (1.1%), mannose (2.9%), glucose (25.8%), galactose (16.1%).	*Rubus plicatus*	Prolong PT and aPTT *in vitro*	Pawlaczyk et al., 2009
Polyphenolic-polysaccharide. Carbohydrate consists of rhamnose (6.0%), arabinose (17.2%), xylose (1.6%), mannose (1.6%), glucose (17.8%), galactose (30.8%).	*Solidago virgaurea*	Prolong PT and APTT *in vitro*	Pawlaczyk et al., 2009

AA- arachidonic acid; U46619-thromboxane A_2 mimic; ADP-adenosine diphosphate; TXB2-thromboxane $B_{2;}$ PT- prothrombin time; aPTT-activated partial thromboplastin time.

1.2.3 *Ardisia elliptica*

1.2.3.1 The genus *Ardisia*

Ardisia is a genus in the family of Myrsinaceae. Approximately 400 to 500 species of *Ardisia* exists in the tropical regions of East and Southeast Asia, Americas, Australia and the Pacific Islands (Chen and Pipoly, 1996; eFloras, 2010; Kobayashi and de Mejia, 2005). In this project, a traditional Malay herb, *Ardisia elliptica* Thunberg (*A. elliptica*), is chosen for the investigation of its antiplatelet and anticoagulant activities. Figure 1.3 shows the photograph of the trees, leaves, flowers and fruits of *A. elliptica*. There are many taxonomic confusions in this genus and correct identification of the plants are difficult (Kobayashi and de Mejia, 2005). For example, *A. crenata* is considered to be a synonym to *A. crispa* by some authorities (Duke and Ayensu, 1985). Perry (1980) and Burkill (1966) considered the two names to be synonymous, but according to eFloras (2010), they are separate species. In addition, taxonomical information on *A. elliptica* is confusing, leading to problems in plant identification. Burkill (1966) had written on *A. littoralis*, but this name had been recognised as a synonym of *A. elliptica* by different authors (Perry, 1980; HMRC and IMR, 2002). While Burkill (1966) stated that *A. humilis* is probably an older name of *A. elliptica*, Perry (1980) considered them separate but others agreed with Burkill (HMRC and IMR, 2002). In addition, a review by Kobayashi and de Mejia (2005) accepted *A. solanacea* and *A. squamulosa* as synonyms of *A. elliptica*. eFloras (2010) only accepts *A. squamulosa* as a synonym, but not *A. solanacea.*

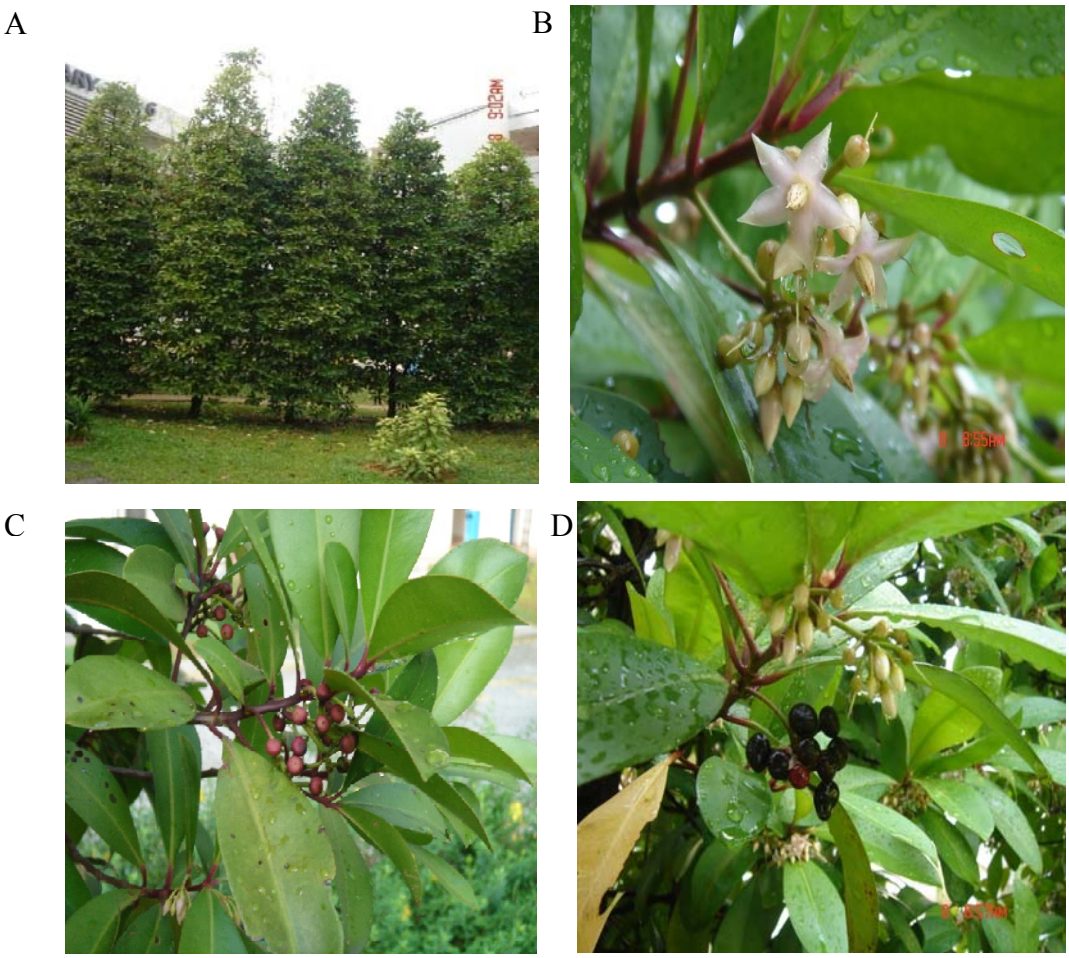


Figure 1.3 Photographs of A) trees B) flowers C) unripe fruits (Pink) D) ripe fruits (dark purple) of *A. elliptica*.

1.2.3.2 Description of *Ardisia elliptica*

A. elliptica is a tropical evergreen subshrub that is native to China, Malaysia, Singapore and Sri Lanka (Chong et al., 2009; Koop, 2004; NParks, 2006). Various common names include Shoebutton ardisia or Sea-shore ardisia in English and Cham in Thai (HMRC and IMR, 2002; NParks, 2006). In Malay, *A. elliptica* is known by different names such as mata pelanduk or mata ayam (HMRC and IMR, 2002), while in Chinese it is known as dong fang zi jin niu

(东方紫金牛) or chun bu lao (春不老) (Yen, 2005; eFloras, 2010). *A. elliptica* reaches maturity between two to four years. The trees are usually one to five meters in height with a single stem and perpendicular branches attached (Figure 1.3A) (Koop, 2004). *A. elliptica* belongs to the family of Myrsinaceae which consists of about 30 genera and 1000 species (Carr, 2005). Its leaves are typical of the Myrsinaceae family, being simple, glandular and alternate. Young leaves are often reddish while mature leaves are oval, fleshy, leathery in texture and can grow up to 20 cm in length (FLEPPC, 2010). The flowers of *A. elliptica* are auxillary and grow in inflorescences on the branches. The flowers are star shaped and about 13 mm in width, with the five petals forming a sympetalous corolla (Figure 1.3B) (Carr, 2005). Fruits of *A. elliptica* are drupes. They are round and not more than 2.5 cm in width, turning from red to black when ripe (Figure 1.3C and D) (Carr, 2005; FLEPPC, 2010). The seeds are spherical and approximately 5 mm in width. *A. elliptica* starts flowering during April and fruits by September. Fruiting continues until March (Koop, 2004).

According to eFloras (2010), *A. elliptica* can be differentiated from closely related species (*A. garrettii*, *A. solanacea* and *A. filiformis)* by its marginate petiole, flowers in umbels, rugose basal sepals and a revolute leaf blade margin.

1.2.3.3 Traditional uses of *Ardisia*

Several *Ardisia* species are used as ornamental plants, food or medicines. Out of the 400 to 500 species of *Ardisia* however, it is not known how many of the species were known to be medicinal due to the limited information available.

A review had been written on species of *Ardisia* known to be medicinal, including the phytochemical constituents isolated from the plants (Kobayashi and de Mejia, 2005). Plants in the *Ardisia* species are well known for some biological compounds such as bergenin and ardisin. Some examples of *Ardisia* species used in traditional medicine include *A. cornudetata*, *A. crenata*, *A. crispa*, *A. crassa*, *A. demissa* and *A. lanceolata*. *A. cornudetata* is used in folk medicine in the south eastern regions of China as an anti-inflammatory/analgesic medicine, antidote for snake bites and to improve general blood circulation (Tian et al., 1987).

The roots of *A. crenata* (朱砂根) is used in Traditional Chinese Medicine as an anodyne, detoxicant, febrifuge, for backaches, diphtheria, dog and snake bites, sore throat, toothache, traumatic injuries, pain in the thighs. The wine extract of the roots are used on broken bones, bruises or sprains (Duke and Ayensu, 1985). The roots of *A. crispa* are used by the Chinese for fever, sore throat, antidotal and diuretic. The plant is crushed and used for treatment of scurf. The juice is put in ears to treat ear aches. The plant is also used for broken bones, sprains, cough and other pulmonary diseases (Burkill, 1966; Duke and Ayensu, 1985). It was also stated that the sap of the plant is used in the Malay Peninsular for scurvy and an infusion of the root is used as an anti-pyretic, a bechic, antidysenteric and antidiarrheic (Perry, 1980).

A. crassa roots are used in central and southern parts of the Malay Peninsular for rheumatism (Burkill, 1966). *A. demissa* (synonym: *A. odontophylla*) is also used by the Malays for treatment of rheumatism and stomach ache (Burkill, 1966; HMRC and IMR, 2002). *A. lanceolata* is taken as an

entire plant and used during confinement and for wound healing (Burkill, 1966; HMRC and IMR, 2002).

Unlike *A. crenata*, a well known herb in TCM, there is comparatively very little information on the traditional uses of *A. elliptica* which is used by the Malays. According to Burkill (1966), the leaves and roots of *A. elliptica* is used traditionally in the Malay Peninsular as a herb. Usually, the leaves are boiled and the extract is drunk to treat pain in the region of the heart (chest pains), parturition complications, fever, diarrhoea and liver poisoning (Burkill, 1966; HMRC and IMR, 2002). The roots can be used to substitute the leaves. Young shoots of the plant are eaten as food (Burkill, 1966).

1.2.3.4 Scientific findings of *Ardisia elliptica*

A. elliptica has been studied scientifically for different biological activities which are summarised in Table 1.3.

Table 1.3 List of biological activities studied scientifically for *A. elliptica*.

Bioactivity	Reference
Antiproliferative	Moongkarndi et al., 2004
Antibacterial	Phadungkit and Luanratana, 2006
Antiplatelet	Ching et al., 2010; Jalil et al., 2004
Antiviral *	Kobayashi and de Mejia, 2005; Chiang et al., 2003
Induce apoptosis	Yen, 2005

* The exact identification of the plant specie described is unclear, as discussed in Section 1.2.3.1.

Ethanolic extracts of *A. elliptica* fruits were found to have antiproliferative activity on human breast adenocarcinoma (SKBR3) cell line with an IC_{50} of 103 μg ml^{-1} (Moongkarndi et al., 2004). *A. elliptica* was also reported to inhibit lung cancer cells (H661 cell line) by inducing apoptosis via up-regulation of TNFR, FADD, TRADD, Fas and caspase-3 gene expressions (Yen, 2005). In addition, the ethanolic extract of the fruits was found to be anti-salmonella. Three compounds, syringic acid, isorhamnetin and quercetin were isolated from the extract and were found to inhibit *S. enteritidis*, *S. weltevreden*, *S. typhimurium* and *S. blockley* with minimal inhibitory concentrations between 15.6 and 125.0 μg ml^{-1} (Phadungkit and Luanratana, 2006). The methanol extract of *A. elliptica* leaves was found to be a potent platelet-activating factor antagonist. The compound responsible for the activity was reported to be 5-(Z-Heptadec-4'-enyl)resorcinol, with an IC_{50} of 7.08 x 10^{-6} M (Jalil et al., 2004). A review by Kobayashi and de Mejia (2005) reported that *A.elliptica* has antibacterial and antiviral properties (Kobayashi and de Mejia, 2005). However, as mentioned before, original reports of the studies reported the plant as *A. solanacea* (Khan et al., 1991) and *A. sqamulosa* (Chiang et al., 2003) respectively. According to eFloras (2010), *Ardisia solanacea* is considered as a separate specie. Thus further identification of the plant is necessary.

1.2.3.5 Chemical constituents of *Ardisia elliptica*

Currently there are 10 phytochemical constituents that have been reported in *A. elliptica*. They are α-amyrin, β-amyrin, bauerenol, bergenin, isorhamnetin,

quercetin, rapanone, syringic acid, 5-(*Z*-heptadec-4'-enyl)resorcinol and 5-pentadecylresorcinol. These phytochemical constituents and the plant parts they are isolated from are listed in Table 1.4.

Table 1.4 Phytochemical constituents obtained from different parts of *A. elliptica*.

Phytochemical constituent	Plant part	Extract	Reference
α-amyrin	Leaves	Benzene	Ahmad et al., 1977
β-amyrin	Leaves, stems	Benzene, diethyl ether	Ahmad et al., 1977; Chow et al., 1991
bauerenol	Leaves	Benzene	Ahmad et al., 1977
bergenin	Whole plant	Methanol	Liu et al., 1993
isorhamnetin	Fruits	Ethanol	Phadungkit and Luanratana, 2006
quercetin	Fruits	Ethanol	Phadungkit and Luanratana, 2006
rapanone	Leaves, stems	Diethyl ether	Chow et al., 1991
syringic acid	Fruits	Ethanol	Phadungkit and Luanratana, 2006
5-(*Z*-heptadec-4'-enyl)resorcinol	Leaves	Methanol	Jalil et al., 2004
5-pentadecylresorcinol	Leaves	Methanol	Jalil et al., 2004

1.2.3.6 Biological activities of amyrins

The triterpenes, α- and β- amyrin are found in *A. elliptica*, and are of special interest in this work. The structures of α- and β- amyrin are shown in Figure 1.4. The compounds α- and β- amyrins are found naturally occurring in a

wide variety of plants. α- and β- amyrin belong to a class of compounds, the triterpenoids. Triterpenoids belong to the isoprenoid family and typically have a C_{30} backbone.

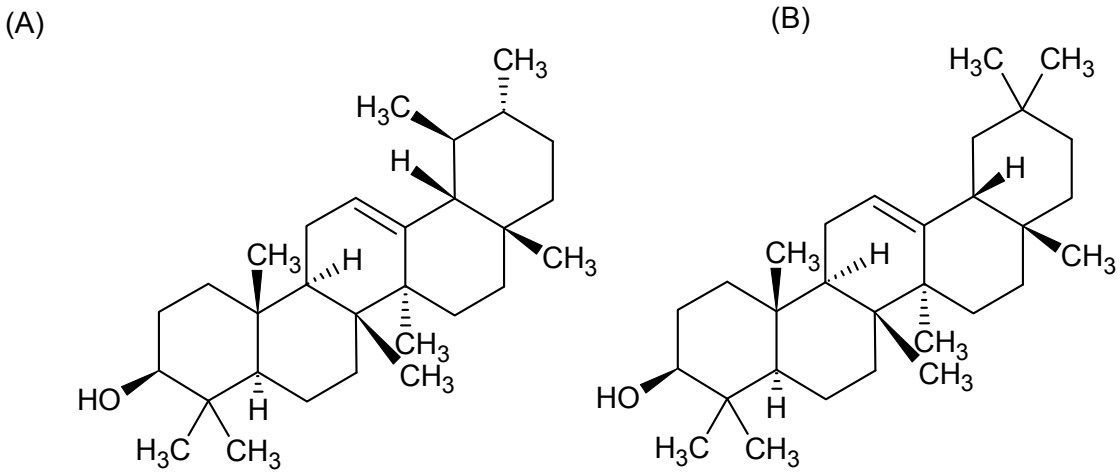


Figure 1.4 Chemical structures of (A) α-amyrin (B) β-amyrin.

There is much potential for α- and β- amyrins to be used as lead compounds. As seen in Table 1.2, α- and β- amyrins show a wide range of biological activities. In additional to that, the amyrins show weak cytotoxic activities. For example, α-amyrin showed anti-protozoal activity but is low in cytotoxicity against myoblast (L-6) cells (Mwangi et al., 2010). The amyrins are also only weakly cytotoxic against other cell lines such as A549, HL-60, SK-OV-3 and A2780 cell lines (Chaturvedula et al., 2004; Chung et al., 2009; Thao et al., 2010). The toxicities of a mixture of α- and β- amyrin had been studied (Oliveira, 2005). The α- and β-amyrin mixture showed low toxicity as a dose of up to 3 g kg^{-1} (p.o.) and 2 g kg^{-1} (i.p.) failed to cause any mortality in mice.

The numerous activities of α- and β- amyrin are summarised below in alphabetical order:

Acetylcholinesterase inhibition

An α- and β-amyrin mixture showed a weak acetylcholinesterase inhibition (13.4%) at 1 mM (Gurovic et al., 2010).

Analgesic / Antinociceptive

Writhing and formalin tests conducted in mice showed inhibition of writhing when a α- and β-amyrin mixture was dosed at 10 and 50 mg kg^{-1}, i.p. (Aragao et al., 2007). A triterpene mixture of α- -amyrin (43.7%), β-amyrin (24.9%) and baurenol (31.4%) administered i.p 100 mg kg^{-1} to mice, exhibited 51% analgesic activity when tested using the acetic acid-induced writhing test (Villasenor et al., 2004).

Rats pretreated with α- and β-amyrin mixture (10, 30 and 100 mg kg^{-1}, i.p.) significantly inhibited the face-rubbing response when the animals were treated with formalin or capsaicin. It was shown that the treatment reduced orofacial pain in part, through a peripheral opioid mechanism (Pinto et al., 2008).

Mice dosed with α- and β-amyrin mixture (30 and 100 mg kg^{-1}, p.o.) showed significant reduction in visceral pain-related behaviours and NK1 immunoreactivity. It was suggested that the mechanisms involve blocking either Substance P release or its receptor function, and also partly by opening K-ATP(+) channels (Lima-Junior et al., 2007).

Mice treated with α- and β-amyrin mixture (3 to 100 mg kg^{-1}, p.o.) significantly suppressed the nociceptive behaviours induced by either subplantar or intracolonic application of capsaicin. The treatment did not alter the pentobarbital sleeping time, nor the ambulation or motor coordination in open-field and rota-rod tests, respectively. This indicated the absence of sedative or motor abnormality that could account for its antinociception. It was suggested the analgesia inducing effect of the triterpene mixture, could involve the vanilloid receptor (TRPV1) and an opioid mechanism (Oliveira et al., 2005).

Intraperitoneal treatment with α- and β-amyrin mixture reduced the nociception caused by 8-bromo-cAMP (8-Br-cAMP) and by 12-O-tetradecanoylphorbol-13-acetate (TPA) or the hyperalgesia caused by glutamate. However unlike morphine, the treatment did not induce analgesia in thermal models of pain. The antinociception caused by the mixture of compounds seemed to involve mechanisms independent of opioid, alpha-adrenergic, serotoninergic and nitrergic system mediation. It was suggested that the mechanisms involved the inhibition of protein kinase A- and protein kinase C-sensitive pathways (Otuki et al., 2005).

Antiarthritic

α-amyrin was tested on models of destructive arthritic processes and was found to inhibit rat osteosarcoma cell growth with an IC_{50} of 14 μM (Kweifiookai et al., 1994).

Anti-bacterial

A α- and β-amyrin mixture was found to have Minimal Inhibitory Concentrations (MICs) of 80, 80 and 90 μg ml^{-1} against the gram positive bacteria, *Bacillus sphaericus, B. subtilis and Staphylococcus aureus* respectively. The mixture was also found to have MICs of 90 and 50 μg ml^{-1} against the gram negative bacteria, *Escherichia coli* and *Pseudomonas syringae* respectively (Mallavadhani et al., 2004).

α-amyrin (1 mg) showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacillus thuringiensis* with activity index (Inhibition area of test sample / Inhibition area of standard, 1 mg gentamycin) of 0.5, 0.44, 0.43 and 0.39 respectively (Singh and Singh, 2003).

β-amyrin exhibited strong antibacterial activity against *Salmonella typhimurium* with a minimum bactericidal concentration (MBC) value of 300 μg ml^{-1} and with a MIC value of 95 μg ml^{-1}. β-amyrin had moderate activities against *Escherichia coli* and *Pseudomonas aeruginosa* with MIC values of 97 μg ml^{-1} (Hichri et al., 2003).

β-amyrin (1 mg) showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae* with activity index (Inhibition area of test sample / Inhibition area of standard, 1mg gentamycin) of 0.64, 0.40 and 0.35 respectively (Jain et al., 2003).

Anti-colitis

A α- and β-amyrin mixture given intraperitoneally at 3 mg kg^{-1} improved tissue damage scores and abolished polymorphonuclear cell infiltration in mice

with trinitrobenzene sulphonic acid (TNBS)-induced colitis. The inhibition of TNBS-induced colitis was related to the local suppression of inflammatory cytokines and COX-2 levels. The mechanism was suggested to occur via inhibition of NF-kappa B and CREB-signalling pathways (Vitor et al., 2009).

Antidepressant

Mice dosed with 10 and 25 mg kg^{-1} of α- and β-amyrin mixture after intraperitoneal or oral administrations showed sedative effects. When dosed at 2.5 and 5.0 mg kg^{-1}, a decrease in the immobility time was observed in the forced swimming test (Aragao et al., 2006).

Anti-diarrhoeal

A triterpene mixture of α- -amyrin (43.7%), β-amyrin (24.9%) and baurenol (31.4%) given orally resulted in a 29 and 55 % antidiarrhoeal activity at dosages of 100 and 250 mg kg^{-1} (p.o.) of mouse, respectively in a charcoal tracing test (Villasenor et al., 2004).

Antifungal

A α- and β-amyrin mixture showed MICs of 30, 125, 125, 250 and 60 μg ml^{-1} against *Candida krusei*, *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis* respectively (Johann et al., 2007).

A triterpene mixture of α- -amyrin (43.7%), β-amyrin (24.9%) and baurenol (31.4%) showed an antimicrobial index *(*diameter of clearing zone − diameter of well*)/*diameter of well) of 0.3, 0.6 and 0.8 against *Staphylococcus*

aureus, *Candida albicans* and *Trichophyton mentagrophytes* the using the agar cup method (Villasenor et al., 2004).

α-amyrin (1 mg) showed antifungal activity against *Aspergillus niger*, *Rhizoctonia phaseoli* and *Penicillium chrysogenum* with activity index (Inhibition area of test sample / Inhibition area of standard, 1mg mycostatin) of 0.72, 0.5 and 0.83 respectively (Singh and Singh, 2003).

β-amyrin (1 mg) showed antifungal activity against *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum* with activity index (Inhibition area of test sample / Inhibition area of standard, 1mg mycostatin) of 0.9, 0.58 and 0.9 respectively (Jain et al., 2003).

Antihyperglycemic

α-amyrin, given orally, was found to lower the blood glucose levels by 13.9 and 8.12% at 5 and 24 h, respectively, in sucrose challenged streptozotocin induced diabetic rat (STZ-S) model at the dose of 100 mg kg^{-1} body weight. However the p-chlorobenzoic acid and nicotinic acid derivatives of α-amyrin showed more potent antihyperglycemic activity (Narender et al., 2009).

Anti-inflammatory

A α- and β-amyrin mixture (25 and 50 mg kg^{-1}, i.p.) was able to reverse the edema of the paw of mice induced by carrageenan. The mixture was also able to prevent dextran-induced paw edema (Aragao et al., 2007).

A single oral dose of α- and β-amyrin (10, 30 and 100 mg kg^{-1}) given to rats reduced the L-arginine-induced increase in pancreatic wet weight/body weight ratio, and decreased serum levels of amylase and lipase, and TNF-alpha

and IL-6. Pancreatic myeloperoxidase activity, lipid peroxidation and nitrate/nitrite were also lowered (Melo et al., 2010).

A triterpene mixture of α- -amyrin (43.7%), β-amyrin (24.9%) and baurenol (31.4%) administered i.p 100 mg kg^{-1} to mice, exhibited 20% anti-inflammatory activity when tested using the carrageenan-induced mouse paw edema test (Villasenor et al., 2004).

α-amyrin, applied topically on the ears of mice, was found to reduce 12-*O*-tetradecanoylphorbol-13-acetate- (TPA) induced inflammation in mice with an IC_{50} of 0.2 mg per ear (Akihisa et al., 1996).

Topical application of α-amyrin (0.1–1 mg/ear) in mice dose-dependently inhibited TPA -induced increase of prostaglandin E2 levels. It was found that α-amyrin dose-dependently inhibited TPA-induced COX-2 expression in the mouse skin. Results also showed that treatment with α-amyrin prevented IκBα degradation, p65/RelA phosphorylation and NF-κB activation, and inhibited the activation of extracellular signal-regulated protein kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC)α (Medeiros et al., 2007).

α-amyrin was shown to inhibit platelet activating factor (PAF) receptor binding to ^{3}H-PAF in rabbit platelets with IC_{50} values of 20.0 μM (Mazura et al., 2007).

α-amyrin and β-amyrin were individually found to inhibit TPA-induced inflammatory ear edema in mice with an IC_{50} of 0.50 and 0.83 μM per ear respectively (Yasukawa et al., 2010).

In another study, β-amyrin, applied topically on the ear of mice, was found to reduce TPA-induced inflammation with an IC_{50} of 0.4 mg per ear (Akihisa et al., 1996).

Antioxidant

β-amyrin (2.92 mM) showed 62.6% protection against *in vitro* low density lipoprotein (LDL) oxidation in a copper-induced LDL oxidation test (Andrikopoulos et al., 2003).

Antiplatelet

A α- and β-amyrin mixture was found to inhibit aggregation of human platelets induced by adenosine 5'- diphosphate (ADP), collagen and arachidonic acid *in vitro*. The IC_{50} values for the inhibitory effect were 90.0, 117.9 and 181.4 μM for collagen, ADP and arachidonic acid respectively (Aragao et al., 2007).

Antiprotozoal

α-amyrin was shown to exhibit anti-plasmodial activity against the parasitic protozoa, *Plasmodium falciparum* (K1 strain, chloroquine resistance) with an IC_{50} of 0.96 μg ml^{-1}. α-amyrin also showed low to moderate activity against *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi* and *Leishmania donovani* with IC_{50} value of 11.21 μg ml^{-1}, >30 μg ml^{-1} and 7.90 μg ml^{-1} respectively. At the same time, α-amyrin was shown to have low cytotoxicity against myoblast (L-6) cells (IC_{50} > 90 μg ml^{-1}), which makes it a good potential anti-protozoal drug (Mwangi et al., 2010).

In another study, α-amyrin and β -amyrin were shown to exhibit anti-plasmodial activity against a chloroquine-sensitive strain of *P. falciparum* (D10) with an IC_{50} value of 50.3 and 89.9 μM respectively (Chung et al., 2009).

Antipruritic

Mice fed orally with α- and β-amyrin (50, 100 and 200 mg kg^{-1}) showed significantly inhibited scratching induced by dextran T40 and compound 48/80. The results suggested that the antipruritic effect was related to a stabilizing action on mast cell membrane (Oliveira et al., 2004).

Antithrombin

α-amyrin (0.5 mg ml^{-1}) and β-amyrin (0.5 mg ml^{-1}) showed a 39% and 78% antithrombin activity in vitro in a chromogenic assay respectively (Medeiros et al., 2002).

Antitubercular

A mixture of lupeol, α- and β-amyrin showed a MIC of 31.5 μg ml^{-1} against *Mycobacterium tuberculosis*. Pure β-amyrin was shown to exhibit a MIC of 312.25 μg ml^{-1} against *M. tuberculosis* (Higuchi et al., 2008).

Anxiolytic

Mice dosed with 10, 25 and 50 mg kg^{-1} of α- and β-amyrin mixture after intraperitoneal or oral administrations showed decreased the number of crossings, grooming and rearing, indicating antianxiolytic effects (Aragao et al., 2006).

Cytostatic activity

α –amyrin showed cytostatic activity against cultured Hep-2 and Mc Coy cells with ED_{50} values of 4.63mg ml^{-1} and 4.59mg ml^{-1} respectively (Gomez et al., 2001).

Cytotoxic

α -amyrin exhibited weak cytotoxic activity against SK-OV-3 cancer cell lines with an IC_{50} value of 32.3 µM (Chung et al., 2009) and weak cytotoxic activity against A2780 ovarian cancer cell line with an IC_{50} value of 20.6 µg ml^{-1} (Chaturvedula et al., 2004).

β-amyrin exhibited weak cytotoxic activity against A549 and HL-60 cell lines with IC_{50} values of 46.2 and 38.6 µM, respectively (Thao et al., 2010).

β-amyrin exhibited cytotoxic activity against SK-OV-3 cancer cell lines with an IC_{50} value of 12.4 µM (Chung et al., 2009).

Gastroprotective

Mice pretreated orally with α- and β-amyrin (50 and 100 mg kg^{-1}) showed a lower intensity of ethanol-associated gastric mucosal damage (Oliveira et al., 2004).

Rats treated with α- and β-amyrin (100 mg kg^{-1}) orally showed 50% of gastroprotection in the animals with acute gastric ulcers induced by absolute ethanol (Navarrete et al., 2002).

Lipoxygenase inhibition

α –amyrin was found to inhibit soybean lipoxygenase with an IC_{50} of 15.0 μM (Gutierrez-Lugo et al., 2004).

β-amyrin (55.6 μg ml^{-1}) reduced the calcium-ionophore (A23187)-induced synthesis of 5-lipoxygenase products by human neutrophils, 5-HETE by 58%, 6-trans-leukotriene B_4 by 56% and 6-trans-12-epileukotriene B_4 by 58%. 5-HETE is proinflammatory and the results helped to account for the anti-inflammatory activities of β-amyrin (Kweifio-Okai and Macrides, 1992).

Liver-protective

Mice pretreatment with α- and β-amyrin (50 and 100 mg kg^{-1}, i.p before acetaminophen) reduced the acetaminophen-induced acute increase in serum alanine aminotransferase and aspartate aminotransferase activities, replenished the depleted hepatic glutathione and also reduced histopathological alterations (Oliveira et al., 2005).

Serine protease inhibitor

α –amyrin was found to be a competitive inhibitor of bovine trypsin and chymotrypsin with Ki values of 29 μM and18 μM respectively. The IC_{50} values are 41 μM and 23 μM for trypsin and chymotrypsin respectively (Rajic et al., 2000).

Inhibit binding to human endothelin 1 ET_A receptor

β-amyrin (100 μM) was found to inhibit 49% of [^{3}H]-BQ-123 from binding to the Endothelin receptor type A (ET_A) receptor. β-amyrin was found to

be specific for ET_A as the same concentration of compound could only inhibit 2% of [^{3}H]-angiotensin II from binding to the angiotensin II AT_1 receptor (Caballero-George et al., 2004).

Tables 1.5 to 1.7 list the activities of the amyrins from literature, together with the plants which the amyrins were found. The tables are categorised by the purity of the amyrins studied: mixture of α- and β-amyrin in Table 1.5; α-amyrin in Table 1.6; β-amyrin in Table 1.7.

Table 1.5 Biological activities reported for α- and β-amyrin mixture in alphabetical order.

Activity	Plant	Reference
Acetylcholinesterase inhibition	*Chuquiraga erinacea*	Gurovic et al., 2010
Analgesic / Antinociceptive	*Protium heptaphyllum* *Carmona retusa*	Villasenor et al., 2004; Aragao et al., 2007; Oliveira et al., 2005; Otuki et al., 2005; Lima-Junior et al., 2007; Pinto et al., 2008
Anti-bacterial	*Protium kleinii* *Diospyros melanoxylon*	Mallavadhani et al., 2004
Anti- colitis	*Protium kleinii*	Vitor et al., 2009
Antidepressant	*Protium heptaphyllum*	Aragao et al., 2006
Anti-diarrhoeal	*Carmona retusa*	Villasenor et al., 2004
Anti-inflammatory	*Protium heptaphyllum* *Carmona retusa*	Villasenor et al., 2004; Aragao et al., 2007; Melo et al., 2010
Antifungal	*Protium heptaphyllum* *Carmona retusa*	Villasenor et al., 2004; Johann et al., 2007

Antiplatelet	*Protium heptaphyllum*	Aragao et al., 2007
Antitubercular	*Byrsonima fagifolia*	Higuchi et al., 2008
Anxiolytic	*Protium heptaphyllum*	Aragao et al., 2006
Gastroprotective	*Protium heptaphyllum* *Hippocratea excels*	Navarrete et al., 2002; Oliveira et al., 2004
Liver-protective	*Protium heptaphyllum*	Oliveira et al., 2005
Antipruritic	*Protium heptaphyllum*	Oliveira et al., 2004

Table 1.6 Biological activities reported for α-amyrin in alphabetical order.

Activity	**Plant**	**Reference**
Antiarthritic	*N.A.*	Kweifiookai et al., 1994
Antibacterial	*Trichodesma amplexicaule*	Singh and Singh, 2003
Antifungal	*Trichodesma amplexicaule*	Singh and Singh, 2003
Antihyperglycemic	*N.A.*	Narender et al., 2009

Anti-inflammatory	*Chrysanthemum morifolium, Matricaria matricarioides, Calendula officinalis, Cosmos bipinnatus, Helianthus annuus, Helianthus. debilis, Arctium lappa, Carthamus tinctorius, Cirsium nipponicum, Cirsium tanakae, Cynara cardanculus, Silybum marianum, Taraxacumo fficinale, Taraxacumo platycarpum* *Melastoma malabathricum* *Cynara cardunculus*	Akihisa et al., 1996; Mazura et al., 2007; Medeiros et al., 2007; Yasukawa et al., 2010
Antiprotozoal	*Teclea trichocarpa*	Chung et al., 2009; Mwangi et al., 2010
	Dendropanax morbifera	
Antithrombin	*Hedera helix*	Medeiros et al., 2002
Cytostatic activity	*Achillea ageratum*	Gomez et al., 2001
Cytotoxic	*Dendropanax morbifera*	Chaturvedula et al., 2004; Chung et al., 2009
Lipoxygenase inhibition	*Vepris punctata* *Anadenanthera colubrina*	Gutierrez-Lugo et al., 2004
Serine protease inhibitor	*N.A.*	Rajic et al., 2000

Table 1.7 Biological activities reported for β-amyrin in alphabetical order.

Activity	Plant	Reference
Antibacterial	*Periploca laevigata*	Hichri et al., 2003; Jain et al., 2003
	Arnebia hispidissima	
Antifungal	*Arnebia hispidissima*	Jain et al., 2003
Anti-inflammatory	*Chrysanthemum morifolium, Matricaria matricarioides, Calendula officinalis, Cosmos bipinnatus, Helianthus annuus, Helianthus. debilis, Arctium lappa, Carthamus tinctorius, Cirsium nipponicum, Cirsium tanakae, Cynara cardanculus, Silybum marianum, Taraxacumo fficinale, Taraxacumo platycarpum*	Akihisa et al., 1996; Yasukawa et al., 2010
	Cynara cardunculus	
Antilipoxygenase activity	*Alstonia boonei*	Kweifio-Okai and Macrides, 1992
Antioxidant	*Pistacia lentiscus*	Andrikopoulos et al., 2003
Antiprotozoal	*Dendropanax morbifera*	Chung et al., 2009

Antithrombin	*Hedera helix*	Medeiros et al., 2002
Antitubercular	*Byrsonima crassa*	Higuchi et al., 2008
Cytotoxic	*Camellia japonica*	Chung et al., 2009; Thao et al., 2010
	Dendropanax morbifera	
Inhibit binding to human endothelin 1 ET_A receptor	N.A.	Caballero-George et al., 2004

1.3 Applications of metabolomics in drug discovery

Traditionally in drug discovery, a target is usually first chosen and the drug is then designed to interact with the specific target(s). This is a process of rational drug design. Advances in technology now allow for fast and automated processes such as High Throughput Screening (HTS), rapid DNA sequencing, cell assays and microarrays etc. These processes are critiqued as replacing rational scientific reasoning with numbers (Drews, 2000). It is difficult to determine whether the traditional or the newer rapid methods work better as the statistics are unclear. Some industries agreed that HTS has resulted in more drug hits, but on the other hand others showed that HTS did not contribute much to the new drug leads (Drews, 2000). In any case, it is generally agreed that there is a need for more haste in drug discovery to satisfy the demand of more potent drugs, changing diseases like influenza and cancer, as well as cheaper and improved drugs with lesser adverse effects. As discussed earlier, natural products form a large and important source for new drugs. In the case of natural product discovery, the process could be neither fast (using techniques like random screening, bioassay-guided fractionation etc.) nor rational (natural products discovered by serendipity, for example penicillin). Although HTS does help to increase the rate of screening, the process is still deemed to be non-rational. To solve the problem, metabolomic studies seemed a probable solution as it entails rapid screening and rational prediction of probable bioactive compounds from complex mixtures, thereby increasing the chances of a hit.

Metabolomics has been described as the analyses and interpretation of metabolite levels in a biological sample by target analysis, profiling, footprinting or fingerprinting (Fiehn, 2002). Metabolomics analysis is performed using techniques of chemoinformatics, bioinformatics, and multivariate data analysis (MVDA). Current applications of metabolomics include discovery of biomarkers of various diseases such as coronary heart disease (Brindle et al., 2002), Lesch-Nyhan syndrome (Ohdoi et al., 2003) and cancer (Odunsi et al., 2005), investigation of genotype-phenotype relationships, and optimising cell culture, metabolic engineering cell cultures etc (Oldiges et al., 2007).

In addition to the study of metabolites in animal and human models, metabolomics has also been developed for natural product research (Rochfort, 2005). As pointed out by Shyur and Yang, 2008, metabolomics is especially useful for studying the plant metabolome as metabolites are much more complex in plants than in the mammalian system. Also, the numerous compounds produced by plants are influenced by the external environmental factors such as temperature, physical stress, predators and drought as well as internal factors including different phases of their life cycle (Wang et al., 2005). Moreover, decomposition of phytoconstituents can occur after harvesting of the plant parts, during extraction and preparation of the drug. A research by Wang *et al.* (2005) showed how the concentrations of ginkgolides and bilobalides can differ when Ginkgo leaves were harvested at sunrise and sunset. As such, metabolomic techniques can aid in optimising the harvesting of the medicinal plants and the growth conditions so that the amount of bioactive components in the plant is

maximised. The metabolomic approach can also be used for quality control so that only medicinal plants with appropriate amounts or mixtures of active components are used. Examples of studies using the metabolomic approach to help identify bioactive components in medicinal plants will be elaborated later.

1.3.1 Metabolomics for quality control of medicinal plants

As mentioned above, metabolomics is often performed using multivariate data analysis (MVDA). As the name implies, MVDA is a technique that allows for the analysis of data sets with large number of variables. For example by using MVDA, Nuclear Magnetic Resonance (NMR) spectrums of crude plant extracts can be visualised, interpreted and compared with those of relatively similar extracts (Heinrich, 2008). NMR-spectroscopy and Principal component analysis (PCA) of *Tanacetum parthenium* was able to help distinguish between batches of 14 commercially obtained samples based on their metabolic profile (Bailey et al., 2002). Rasmussen et al. went a step further to use ^{1}H-NMR-spectroscopy to differentiate between various preparations according to their global composition, and also between batches from the same supplier (Rasmussen et al., 2006). Other than NMR, it was shown that by using Ultra-high performance liquid chromatography/time-of-flight mass spectrometry on *Panax notoginseng*, and analysing the metabolites by MVDA, the duration of steaming of the herb can be deduced (Toh et al., 2010). These examples show the potential of metabolomics for quality control. As traditional medicine becomes more popularised and prominent in the world, it is important to characterise the medicinal plants used

and to be able to check for adulteration of the products. However more work needs to be done in order to use metabolomic techniques for routine and large scale testing.

1.3.2 Metabolomics and analysis of pharmacological effects

A major aspect of drug discovery is concerned with the elucidation of the drug's pharmacological effects. In order to do so, extensive mechanistic studies need to be performed. It had been shown that by using metabolomics, the pharmacological effect of medicinal plant extracts can be linked to its chemical profile. Wang et al. (2010) attempted to elucidate the mechanism by which a medicinal plant, *Sophora flavescens* up-regulates CYP3A. *S. flavescens* is used traditionally to treat of viral hepatitis, cancer, viral myocarditis, gastrointestinal haemorrhage and skin diseases. In the study, Principal-component analysis (PCA) and Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were used to analyse the chemical constituents of the plant. The chemical constituents were further analysed for their effects on the pregnane X receptor (PXR) activation and CYP3A regulation. Analysis showed that it was a single chemical, *N*-methylcytisine that was responsible for activating PXR (Wang et al., 2010).

In the field of cancer therapy research, the mode of action of a plant extract obtained from *Anoectochilus formosanus* was compared to a single compound drug in MCF-7 cells (Yang et al., 2004). Metabolomic and transcriptomic analyses showed similar gene expression regulating profiles,

indicating a similar mode of action between the extract and the drug. By this technique, fewer possibilities with regards to the mechanism of action need to be considered, enabling more focus in research.

1.3.3 Using metabolomics for drug discovery from medicinal plants

Discovery of active compounds from plant extracts is difficult due to a number of reasons. First, plant extracts comprise of a complex mixture of compounds which can be difficult to isolate, especially if they are in small quantities. In many cases, herbal medicines are taken as a concoction of different plants, thus increasing the difficulty of pinpointing the active component. Conventional methods of discovering bioactive components from medicinal plants involve bioassay-guided fractionation which involves repeated fractionation and bioassays. For very complex mixtures, isolating a single compound can be tedious and not cost efficient. Second, there are many examples where two or more of the compounds in the extract act together in synergism in order for the activity to be exhibited. Individually, their activity is not as strong as that in a mixture. When this is the case, bioassay-guided fractionation would not be useful for identifying the active component. An example of synergism can be illustrated from an Ayurvedic formulation, 'Trikatu'. This preparation is used routinely for many diseases as a part of a multidrug formulation. Trikatu consists of a mixture of black pepper (*Piper nigrum*), long pepper (*Piper longum*) and ginger (*Zingiber officinalis*). It was found that the alkaloid, piperine found in pepper was a bioavailability enhancer. It was reported that blood levels of

rifampicin, phenytoin, pentobarbitone, theophylline and propanolol were increased when the drugs were taken together with piperine. When long pepper was taken together with vasaka leaves (*Adhatoda vesica)* for treatment of asthma, the bioavailability of vasicine (or peganine) from the leaves was enhanced (Johri and Zutshi, 1992). On top of synergism, there are other reasons that compounds should not be isolated from the plant extract. Plant extracts may contain unstable constituents, for example in *Valeriana* spp., *Allium sativum*, *Zingiber officinalis* and *Humulus lupulus*, which are "protected" from decomposition by antioxidants in the whole plant material (Williamson, 2001). Williamson further advised against isolation of the compounds when the active constituents are not completely identified, and also when the extracts have a range of activities (Williamson, 2001).

Another concern in natural product discovery is whether different plant parts can be used to replace parts used in existing remedies. The reason for the need to consider a replacement could be due to reasons such as ease of harvesting or for conservation purposes. For example, *Vismia guineensis* is an endangered shrub found in West Africa, and its roots are used in decoctions for various skin diseases such as dermatitis, leprosy, syphilis, herpes, scabies and eczemas. As harvesting of the root is destructive for the plant, a study was conducted to determine the feasibility of using the leaves of the plant instead. In the earlier days of metabolomics, the study involved the comparison of the chemical compositions of the root and leaves by HPLC-UV/PAD and HPLC-MS. The molecular weights and characteristic fragments of the chemicals were compared

with Electron Impact (EI) or HPLC-MS literature data, which allowed partial identification of the major peaks in the chromatograms. The results showed that only a minority of the constituents of the two organs were similar, which implied that the roots cannot be replaced by the leaves (Politi et al., 2004).

Successful reports on using metabolomics or MVDA for discovering bioactive compounds from medicinal plants are being reviewed. It has been hypothesised that by screening plant extracts with different composition, it would be "possible to calculate which compounds or group of compounds are associated with the highest bioactivity" using MVDA (Wang et al., 2005). Research on Quantitative composition-activity relationship (QCAR) has been conducted on herbal medicine using various algorithms and showed positive results (Cheng et al., 2006; Wang et al., 2006; Wang et al., 2008; Chau et al., 2009; Froufe et al., 2009). Using chemoinformatic techniques on QCAR, Cheng et al. demonstrated the suitability of the technique to discover active components from the mixed extracts of *Radix Salviae miltiorrhizae* and *Cortex Moutan* compared to bioassay-guided fractionation. Cryptotanshinone and dihydrotanshinone I were found using QCAR to be active for protecting cardiomyocytes against ischemia-induced injury (Cheng et al., 2006). Using a QCAR approach, Wang et al. managed to optimise a herbal medicine for decreasing plasma cholesterol levels, Qi-Xue-Bing-Zhi-Fang, by identifying and varying two active components in the herbal extract (Wang et al., 2006). In a separate report, Wang et al. also managed to identify a ginsenoside Rb_1 to be cytotoxic from *Panax ginseng* extracts using a data mining approach and a causal discovery algorithm (Wang et al., 2008). Chau et al. developed a

partial least-squares (PLS) model to reveal the chromatographic regions of a herbal medicine most strongly related to its activity. It was stated that by inputting whole chromatographic profiles and total bioactivity, a quantitative pattern-activity relationship approach can help to predict total activity from the chromatographic fingerprint and also to predict features in the fingerprint related to the activity (Chau et al., 2009). A QCAR model using the PLS method was also constructed by Froufe et al. to predict the antioxidant activities of wild mushrooms (Froufe et al., 2009).

From the examples listed, metabolomics and MVDA seemed to be a potential tool for aiding drug discovery from medicinal plants.

1.3.4 Techniques used in metabolomic studies

Figure 1.5 shows the general workflow of a metabolomic study. The workflow shows the relationship between analytical techniques such as LC-MS and GC-MS and using MVDA for the identification of marker metabolites. In the case of drug discovery from medicinal plants, the marker metabolites would be compounds that are bioactive.

Good reviews have been written to describe the techniques used in metabolomics (Wang et al., 2005; Shyur and Yang, 2008). Metabolomic studies on plant extracts can be performed using techniques such as gas chromatography-mass spectrometry (GC-MS) (Lisec et al., 2006; Ma et al., 2008), liquid chromatography-mass spectrometry (LC-MS) (Zhi et al., 2008), thin layer chromatography (TLC) (Roberts et al., 2008), Fourier transform infrared

spectroscopy (FT-IR) (Goodacre et al., 2007) and NMR (Lang et al., 2008; Mitova et al., 2008). After obtaining raw data from the above techniques, general data processing procedures include centroiding and deisotoping mass spectra to condense and reduce data, reduce variation in chromatograms, filtering off background signals, deciding on threshold windows of the mass (m/z) and retention times and normalisation of MS data (Chen et al., 2007).

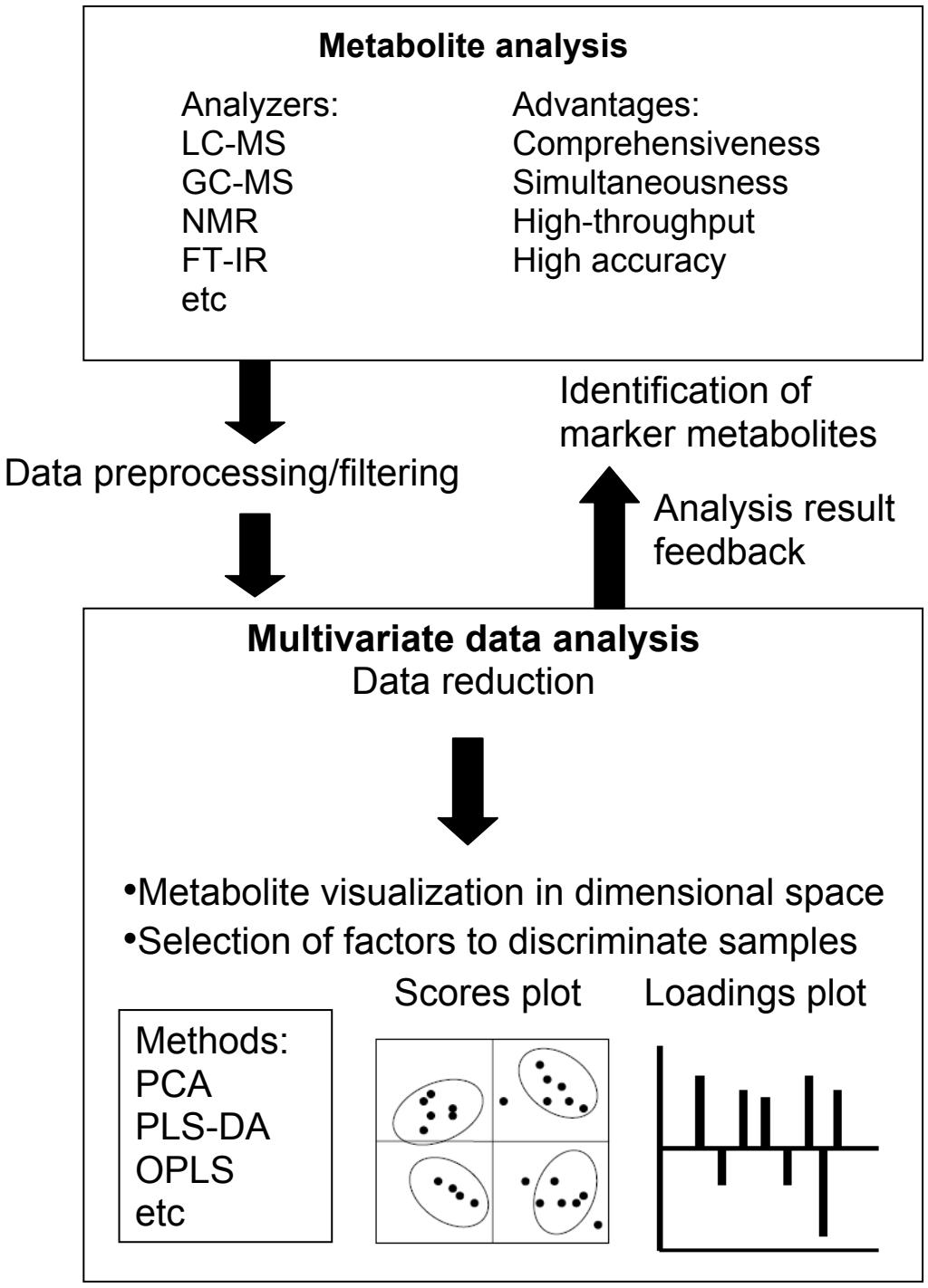


Figure 1.5 A general workflow of metabolomic study, adapted from Okada et al., 2010.

Thereafter, the data is being analysed by MVDA (Shyur and Yang, 2008). MVDA enables the analysis of data coupled with many variables (Sumner et al., 2003). There are two major classes in MVDA—supervised and unsupervised MVDA. In the unsupervised MVDA, sample classification is not revealed in the analysis. However in supervised MVDA, this information is made known for model construction (Chen et al., 2007). Popular unsupervised methods include PCA, hierarchical clustering, K-means clustering and self-organizing maps, while supervised methods include partial least squares, orthogonal partial least squares, soft-independent modelling of class analogy, and partial least squares-discriminant analysis (Sumner et al., 2003; Chen et al., 2007). One of the best known unsupervised methods of analysis is PCA (Ma et al., 2008). PCA is a well known technique for exploratory data analysis. As PCA can present data in low number of dimensions and also determine the least dimensions needed for replication of data (Andrews et al., 1996; Zhi et al., 2008), PCA allows data to be represented on a graph with different objectives. The representation on a graph enables data to be studied in a dimensional space for analysis of their proximity with other objects, hence facilitating classification and detection of unusual patterns and objects (Zhi et al., 2008).

CHAPTER 2

Hypothesis and Objectives

2.1 Hypothesis

The traditional uses of a medicinal plant may provide useful clues for drug discovery purposes. *A. elliptica* is traditionally used for treatment of chest pains (Burkill, 1966). Causes of chest pains include thromboembolism (Michota, 2005). Another reported traditional use of the herb is the treatment of parturition (childbirth) complications which is also related to problems in blood circulation. Hence it is hypothesised that *A. elliptica* possesses phytoconstituents with antiplatelet and/or anticoagulant activities that may be potential lead compounds for development of new drugs.

In this project, the traditional bioassay guided fractionation is employed for isolation and identification of bioactive constituents. However, bioassay guided fractionation is a slow process. Metabolomics and MVDA have been proven to be useful tools for the discovery of biomarkers in animal tissues for diseases (e.g. cancer, metabolic syndromes and coronary diseases) and in plants, MVDA had recently been used for the identification of compounds having anticancer, antioxidant activities etc. It is hypothesised that MVDA can also be developed as a drug discovery platform for rapid identification of bioactive constituents in plant extracts for antiplatelet activity.

2.2 Objectives

Adverse effects of current antiplatelet and anticoagulant drugs have been well documented. Currently used antiplatelet and anticoagulant drugs such as aspirin and warfarin have adverse effects including gastric mucosal erosions and excessive bleeding respectively. Given the limitations of the current treatments, the search for safer drugs is necessary.

The overall objectives of this work are to investigate the potential antiplatelet and anticoagulant effects of *A. elliptica* and to isolate and identify the active compounds(s) responsible for the activities. Hence to achieve these objectives, the specific objectives are to

1. Screen extracts of *A. elliptica* for antiplatelet and anticoagulant activities *in vitro* and *in vivo*.
2. Identify and isolate the active compound(s) responsible for the antiplatelet and/or anticoagulant activity of the plant.
3. Develop a platform involving MVDA to rapidly identify bioactive components from the plant extract without the need for bioassay-guided fractionation.
4. Study the pharmacokinetics of the isolated component(s) in rats.

The successful development of metabolomics in natural product discovery would help in rapid identification of active compounds from medicinal plant extracts. The bioactive component, after being isolated, will be studied for its *in*

vivo effects and pharmacokinetic profile in rats. The information obtained will be useful for future drug development.

CHAPTER 3

Chemical analysis, antiplatelet and anticoagulation studies of *A. elliptica* extract

3.1 Chemical analysis of *A. elliptica* extract

3.1.1 Introduction

It was previously discussed in Section 1.2.3.3 that *A. elliptica* is traditionally used for the treatment of various ailments such as parturition complications and chest pains which may be related to problems in blood circulation. Thus it is useful to know the chemical constituents of its extract. This chapter presents the work on the chemical analysis of *A. elliptica* extract and the screening for potential antiplatelet and anticoagulant activities. The information gained will be useful for the identification of bioactive components in the plant extract for the platelet aggregation and plasma coagulation assays to be performed.

The chemical constituents in the stem, leaves and fruits of *A. elliptica* had been previously reviewed and discussed in Section 1.2.3.5. Chemical constituents found in *A. elliptica* include α-amyrin, β-amyrin, bauerenol, bergenin, isorhamnetin, quercetin, rapanone, syringic acid, 5-(*Z*-heptadec-4'-enyl)resorcinol and 5-pentadecylresorcinol. It had been reported that a mixture of α-amyrin and β-amyrin were antiplatelet (Aragao et al., 2007). In this study, α-amyrin and β-amyrin were detected and found to be partly responsible for the antiplatelet

activity of the plant extract. Thus, it was of interest to develop a method to quantify, isolate and study the activities of both amyrins individually.

Since both α- and β-amyrin are not easily ionised, the detection of the amyrins using mass spectrometry has to be optimised. Literature has shown that detection of α- and β-amyrin using LC-MS with electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation mass spectrometry (APCI) gives a low sensitivity (Rhourri-Frih et al., 2009). LC-MS with atmospheric-pressure photoionisation (APPI) was more sensitive. Triterpenes are generally deemed to be non-volatile, so they are usually derivatised before analysing with GC-MS (Laurent et al., 2003; Regert et al., 2006; Coelho et al., 2007; Echard et al., 2007; Jacques et al., 2007; Lytovchenko et al., 2009; Rhourri-Frih et al., 2009; Scholz et al., 2009). However steps involving derivatisation are laborious when working with large number of samples, thus it would be useful to develop a sensitive analytical method without the need for derivatisation.

3.1.2 Objectives

In this study, the overall objectives are to develop chromatographic methods to analyse the components of the 70% v/v methanol *A. elliptica* leaf extract using HPLC and GC-MS. The specific objectives are to

1. Optimise a HPLC method for the isolation of amyrins from the plant extract.

2. Develop, optimise and validate a GC-MS method to quantify the amount of amyrins in *A. elliptica* leaf extract.

3.1.3 Materials and methods

3.1.3.1 Plant material

A. elliptica leaves were collected from Pasir Ris Park and identified by Mr Haji Samsuri bin Haji Ahmad from the Singapore Botanic Gardens. A voucher specimen of the plant (KHL0060/CJH/AE/29062006) is stored at the Department of Pharmacy, National University of Singapore.

3.1.3.2 Reagents and standards

Methanol was purchased from Tedia (Fairfield, OH). α-amyrin and β-amyrin were obtained from Extrasynthase (Genay, France). Aspirin was obtained from Sigma Aldrich (USA). Methyltestosterone was obtained from the United States Pharmacopeia (Rockville, MD).

3.1.3.3 Extraction and preparation of plant extracts

The leaves of *A. elliptica* were freshly collected, washed and air dried before blending and refluxed for six hours with the appropriate solvents. Soxhlet extraction with either 70% v/v methanol or water was carried out separately. A total of 860 g of freshly collected leaves were extracted with 5.0 l of 70% v/v methanol. The extracts were evaporated to dryness using a rotary evaporator.

3.1.3.4 Fractionation of *A. elliptica* 70% v/v methanol extract

Liquid-liquid fractionation was performed on 22.4 g of the *A. elliptica* 70% v/v methanol extract by first suspending the extract in a total of 600 ml of water, and subsequently fractionating it sequentially with hexane, chloroform and butanol four to six times.

In addition, 27.0 g of the dried 70% v/v methanol extract was fractionated using a preparative HPLC system. The dried extract was dissolved in 100 ml of HPLC grade methanol. After centrifuging at 14,000 rpm, the supernatant was removed using a pipette and fractionated using a preparative HPLC system (Agilent 1100 series, CA, USA) and a preparative Zorbax Eclipse XDB-C18 column (250 mm x 21.2 mm I.D., particle size: 7 μm; Agilent, CA, USA) using methanol as the mobile phase. The injection volume was 2 ml, flow rate 20 ml min^{-1} and run time was 40 min. In total, 13 fractions were collected. All fractions were dried using a rotary evaporator.

3.1.3.5 Analysis of the 70% v/v methanol extract using HPLC

An analytical HPLC system (Agilent 1100 series, CA, USA) and a Zorbax Eclipse XDB-C18 column (250 mm x 4.6 mm I.D., particle size: 5 μm; Agilent, CA, USA) was used. Separation of compounds was achieved using a binary gradient elution system consisting of water (A) and acetonitrile (B) at the following gradient: 0-30 min, 70-95% B; 30-40 min, 95-100% B; 40-100 min, 100% B. The injection volume was 5 μl, flow rate 1.5 ml min^{-1} and run time was 100 min. The UV detection was set at 210 nm, while the DAD was scanned from 190 to 400 nm.

3.1.3.6 Analysis of phytoconstituents in the 70% v/v methanol extract using GC-MS

Analyses by GC-MS were carried out with a Shimadzu, gc2010 gas chromatograph coupled to a Shimadzu gp2010 mass spectrometer, a Shimadzu AOC-20i auto-injector and an AOC-20s auto-sampler. A DB-5 MS column of internal diameter 0.25 mm, length 30.0 m and film thickness 0.25 μm was used. The oven temperature was set at 60 °C and held for 5 min, increased to 200 °C at a rate of 7 °C/ min and held for 10 min. The temperature was finally increased to 280 °C at a rate of 5 °C/ min and held for 30 min. The ion source and interface temperatures were kept at 200 °C and 180 °C respectively. Samples were injected under a splitless mode with split ratio -1.0. Each injection volume was 1 μl and an injection port dwell time of 0.3 s was set. The pressure of the Helium carrier gas was set at 100 kPa with a total flow of 50.0 ml min^{-1}. The column flow was 1.61 ml min^{-1} and the linear velocity was 46.3 cm s^{-1}. Data recording was set from 5 to 81 min for 50 to 900 m/z.

Phytoconstituents were preliminarily identified by the Wiley Mass Spectral Library Registry 7 (NJ, USA). Identities of the α-amyrin and β-amyrin detected were confirmed by comparing the retention times and mass spectra of the signals with those of the standards.

3.1.3.7 Isolation of β-amyrin using preparative and semi-preparative HPLC

The 70% v/v methanol *A. elliptica* leaf extract (13.3 g) was dissolved in a total of 50 ml of HPLC grade methanol and fractionated by preparative HPLC as

described in Section 3.1.3.4. The fraction containing amyrins was collected and further purified by chromatography using a semi-preparative Zorbax SB-C18 column (250 mm x 9.4 mm I.D., particle size: 5 μm; Agilent, CA, USA) using methanol as the mobile phase. The injection volume was 2 ml, flow rate 8 ml min^{-1} and run time was 35 min. The UV detection for both preparative and semi-preparative HPLC was set at 210 nm. Fractions containing β-amyrin was dried *in vacuo*. Melting points of the compound are uncorrected values and determined using a melting point apparatus (A Krüss Optronic, Germany).

3.1.3.8 Sample preparation for amyrin quantification

The 70% v/v methanol *A. elliptica* leaf extract was reconstituted in methanol to a concentration of 100 μg ml^{-1} for the GC-MS analysis. A 1 mg ml^{-1} standard stock solution in methanol was prepared for both amyrins, and diluted to concentrations ranging from 0.5 ng ml^{-1} to 2 μg ml^{-1} for the GC-MS analysis. An internal standard, methyltestosterone, was spiked into all samples at a concentration of 1 μg ml^{-1} before analysis. All samples are centrifuged at 10, 000 *g* for 10 min before the supernatants were used for analysis.

3.1.3.9 GC-MS assay for amyrin quantification

A Shimadzu (Kyoto, Japan) GC-MS system (gc2010 and qp2010 MS) was used for quantifying both α- and β-amyrins in *A. elliptica* 70% v/v methanol extract. Chromatographic separation was achieved with a DB5 column of film

thickness 0.25 μm, length 30.0 m and diameter 0.25 mm (Agilent Technologies, Santa Clara, CA). The initial oven temperature was 180 ^{o}C, and increased to 280 ^{o}C at a rate of 50 ^{o}C min^{-1}. This temperature was held for another 19 min. The helium flow rate was 1.6 ml min^{-1} and the interface and ion source temperature was 250 ^{o}C and 200 ^{o}C respectively. At the beginning of the assay development, the mass spectrometer was operated in the scan mode to obtain the mass spectra of α-amyrin, β-amyrin and methyltestosterone. After the identification of the major ions, the selected-ion monitoring (SIM) mode was applied, detecting m/z 203, 218 and 426 for both α- and β-amyrins and m/z 43, 124 and 302 for the internal standard methyltestosterone. Quantification was done using the total ion chromatogram (TIC) of the quantifier ions, m/z 218 and 302 for the amyrins and methyltestosterone respectively.

3.1.3.10 Method validation for GC-MS assay

To assess linearity, the calibration curves were analysed using the linear least-squares regression. The sensitivity of the GC-MS assay was represented by the LOD and LOQ, The LOD and LOQ were obtained by diluting the α- and β-amyrin samples until the signal to noise ratio reached 3:1 and 10:1 respectively. The method was also validated for its specificity, precision and accuracy (recovery).

For precision, repeatability and intermediate precision were determined. For repeatability, 8 concentrations of α- and β-amyrin standards were each assayed five times within a day. Intermediate precision assessed by determining

the RSD of the concentrations on different days. For inter-day variation, triplicate analyses of 8 concentrations of α- and β-amyrin standards were performed on three different days.

Recoveries of α- and β-amyrin from the plant matrix were determined by spiking α- and β-amyrin standards at 3 different levels (0.5, 1.0, 1.5 μg ml^{-1}) into the *A. elliptica* samples before extraction and subsequently determining the added amounts. 600 mg of *A. elliptica* leaves were extracted using Soxhlet extraction with 100 ml of 70% v/v methanol. Extraction was first carried out in triplicates on *A. elliptica* leaves with no added amyrins. The extraction was repeated by spiking the leaves with either 0.25 mg, 0.5 mg or 0.75 mg of both α- and β-amyrin standards. After extraction, the solvents were evaporated to dryness and the dried residues were reconstituted with 5 ml of HPLC grade methanol. Each of the samples was diluted 100 times with HPLC grade methanol and filtered before GC-MS analyses.

3.1.4 Results and discussion

3.1.4.1 Extraction and fractionation of *A. elliptica* 70% v/v methanol extract

69.7 g of dried crude extract (8.1% w/w yield) was obtained from soxhlet extraction of 860 g of fresh *A. elliptica* leaves using 70% v/v methanol while 7.0 g of dried crude extract (8.5% w/w yield) was obtained from 82 g of fresh *A. elliptica* leaves when water was used as the extraction solvent. Of the 69.7 g of crude 70% v/v methanol extract, 22.4 g was used for liquid-liquid partitioning. The hexane, chloroform, butanol and water fractions obtained were 3.0 g, 1.5 g, 2.6 g and 11.4 g respectively. From the 27.0g of 70% v/v methanol extract used for fractionation using preparative HPLC, a fraction weighing 127.8 mg containing α- and β-amyrin was obtained.

3.1.4.2 Analysis *A. elliptica* crude extract using HPLC

Analysis of the crude 70% v/v methanol extract was also performed using HPLC. Figure 3.1 shows the chromatograms of the crude 70% v/v methanol extract (0.5 mg ml^{-1}) and those of the standards of α- and β- amyrin (0.1mg ml^{-1}).

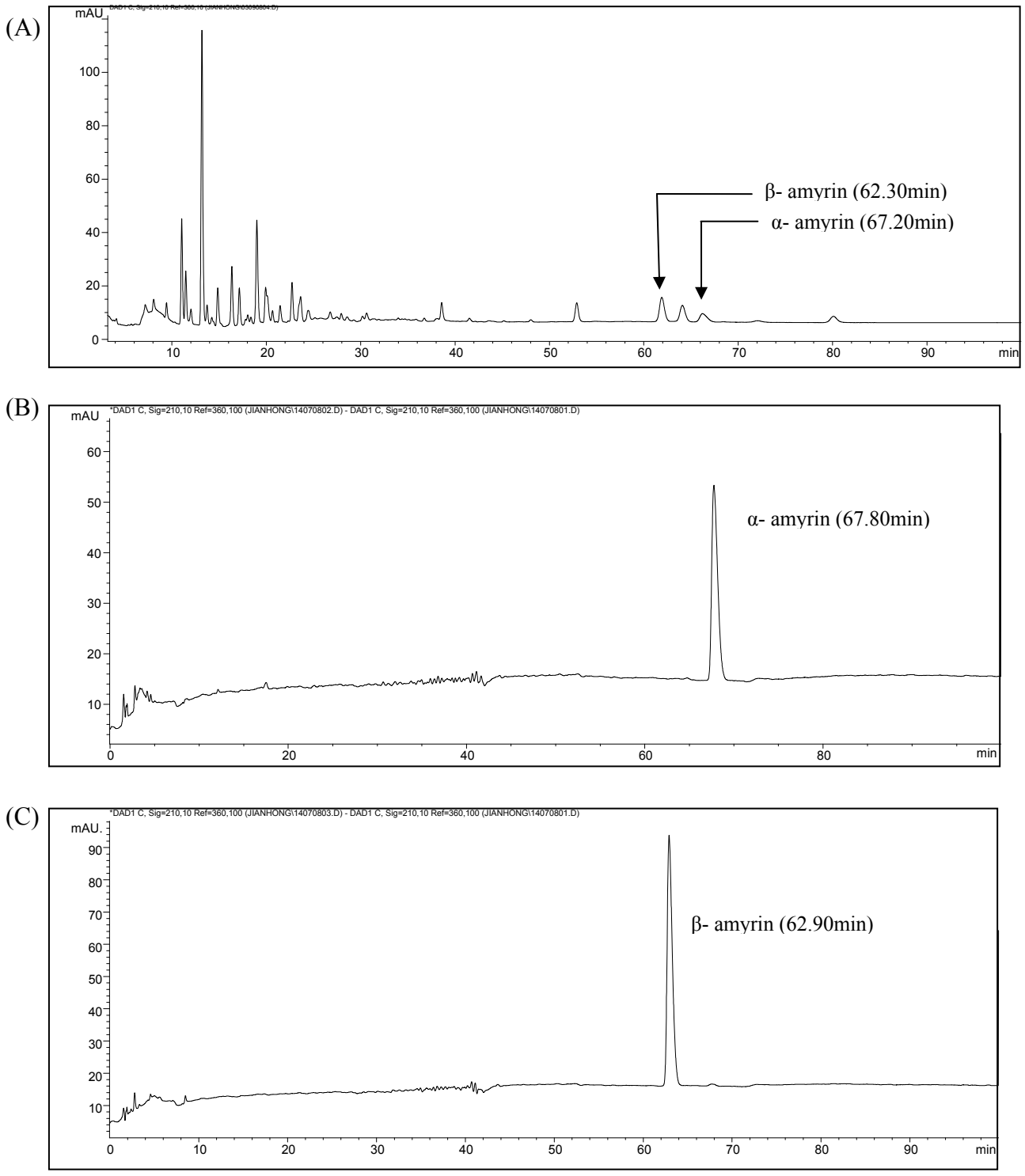


Figure 3.1 HPLC chromatograms of (A) 70% v/v methanol extract (0.5 mg ml^{-1}), (B) α- amyrin standard (0.1 mg ml^{-1}) and (C) β- amyrin standard (0.1 mg ml^{-1}).

3.1.4.3 Identification of phytoconstituents in *A. elliptica* using GC-MS

A GC-MS method was developed to analyse the *A. elliptica* extracts and fractions. A temperature of 280 ^{o}C was required to allow elution of all phytoconstituents in the extracts and fractions. As the phytoconstituents were co-eluting at a steep temperature gradient, a gentle gradient of 5 to 7 ^{o}C/ min was used for the analysis.

The gas chromatograms of the 70% v/v methanol extract are shown in Figure 3.2. An autolibrary search using the Wiley mass spectral library reveal that the compounds found in the 70% v/v methanol extract consisted mainly of fatty acids (palmitic acid, stearic acid), esters of fatty acids (methyl palmitate, methyl heptadecanoate, methyl linolenate), triterpenes (α- and β- amyrin), α-tocopherol and a resorcinol (5-pentadecylresorcinol).

The gas chromatograms of the crude 70% v/v methanol extract, the hexane fraction, and those of α- and β- amyrin are shown in Figure 3.2. The retention times of β- amyrin and α- amyrin were 67.8 min and 79.0 min respectively, similar to those of the authentic standards. α- and β- amyrin were also differentiated by their MS (Figure 3.3). The intensity of the peak with m/z 203 in β- amyrin is approximately twice that of the intensity of the peak with m/z 203 in α- amyrin (Morita et al., 2000; Martelanc et al., 2007).

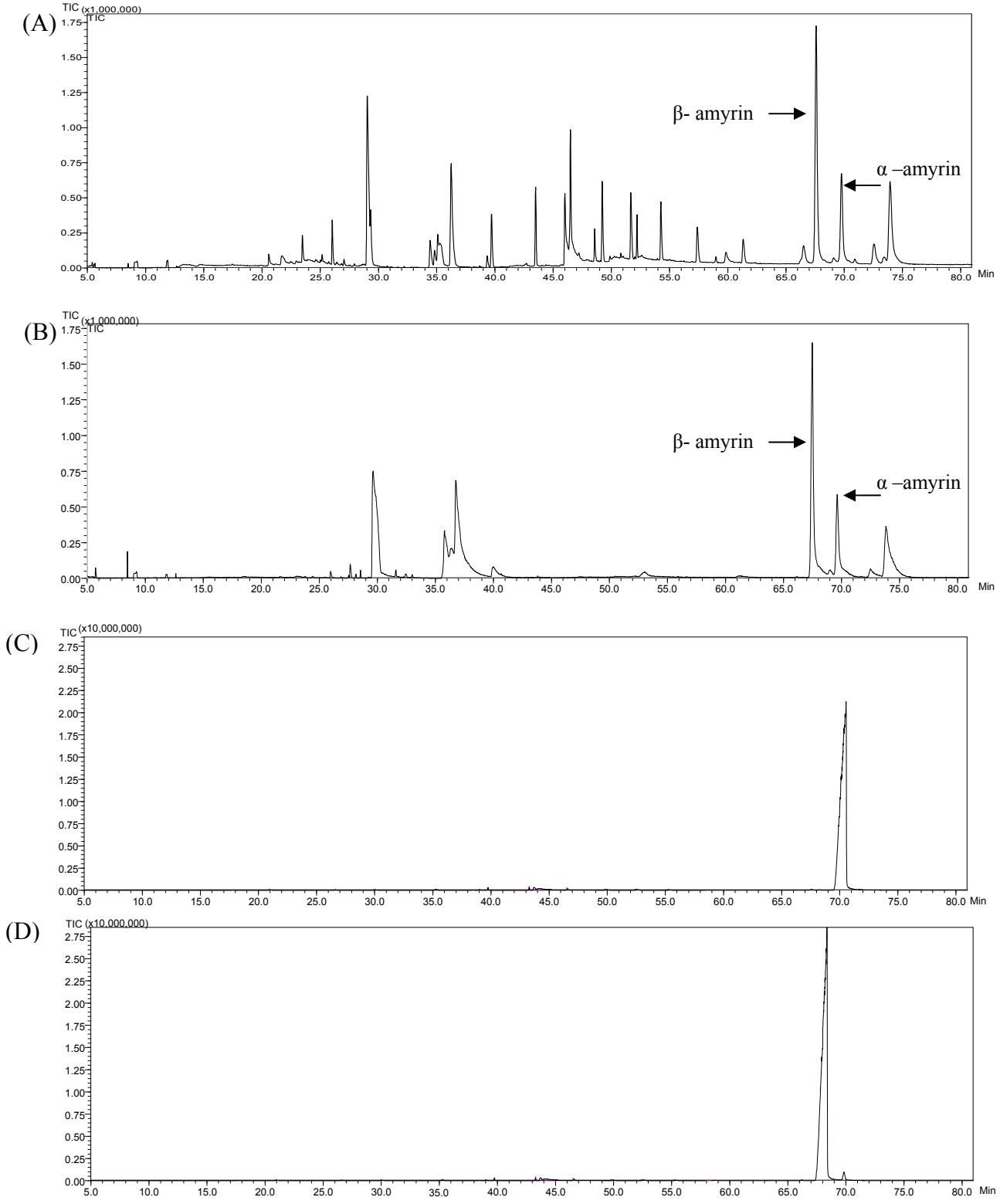


Figure 3.2 Gas chromatograms of (A) 70% methanol extract of *A. elliptica*, (B) hexane fraction, (C) α-amyrin standard and (D) β-amyrin standard.

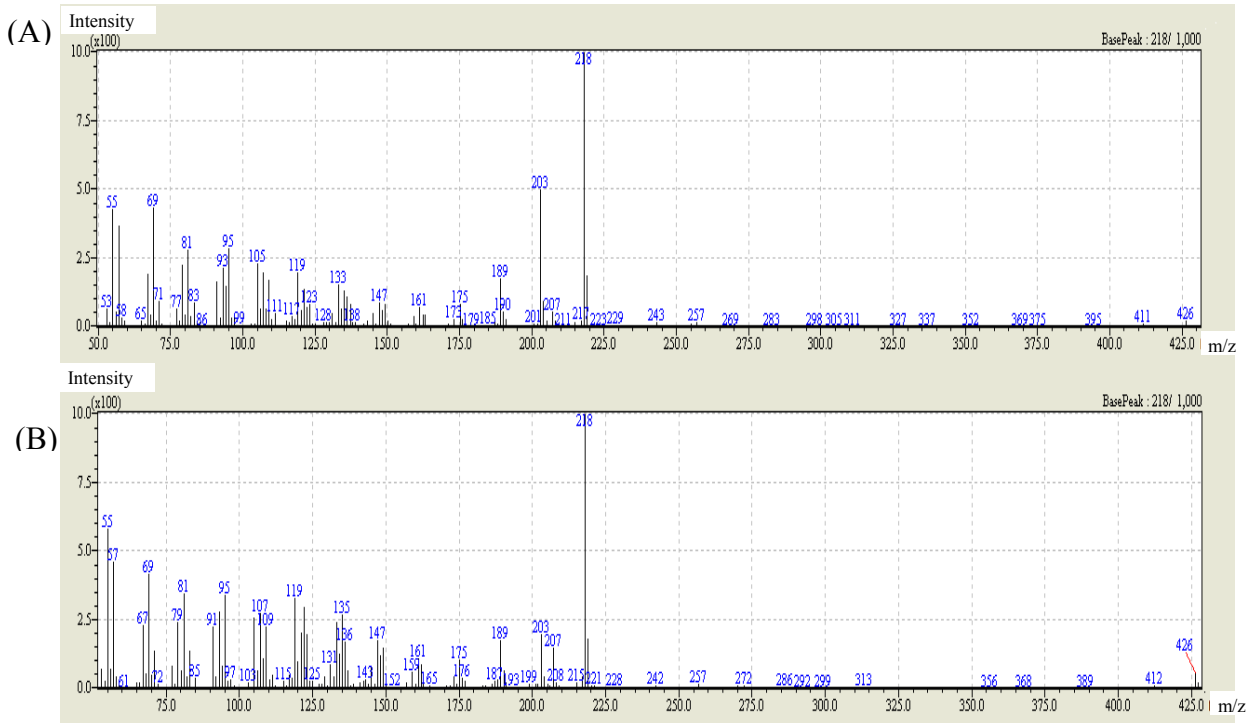


Figure 3.3 Mass spectra of the standards (A) β- amyrin and (B) α- amyrin.

3.1.4.4 Isolation of β- amyrin from *A. elliptica*

β- amyrin was isolated first by fractionating the 70% v/v methanol extract through a preparative column. The HPLC chromatogram from preparative HPLC isolation is shown in Figure 3.4. As observed, α-amyrin is not well resolved from a neighbouring peak using this method of isolation, and thus not isolated. β-amyrin was found to elute at 15.5 min. The fraction containing β-amyrin was concentrated and further purified using a semi-preparative column. The chromatogram of the β-amyrin fractionated by semi-preparative HPLC is shown in Figure 3.5.

Identification of the isolated β- amyrin from *A. elliptica* leaves was done by HPLC (Figure 3.6) as well as GC-MS (Figure 3.7). In total, 20.4 mg of β-amyrin was isolated from 13.3 g of dried 70% v/v methanol extract in the form of white crystals. The purity of the β- amyrin isolated was determined to be 96.1% by HPLC and 99.8% by GC-MS. Melting point of the crystals was determined to be 189.2 ^{o}C to 191.2 ^{o}C, comparable with those of the β-amyrin standards (187.0 ^{o}C to 188.2 ^{o}C). The melting point of β-amyrin reported by Sowemimo et al. (1973) was 197 ^{o}C to 199 ^{o}C but agreed well with those with material and safety data sheet of Extrasynthese (189 ^{o}C to 196 ^{o}C) (Extrasynthese, 2009).

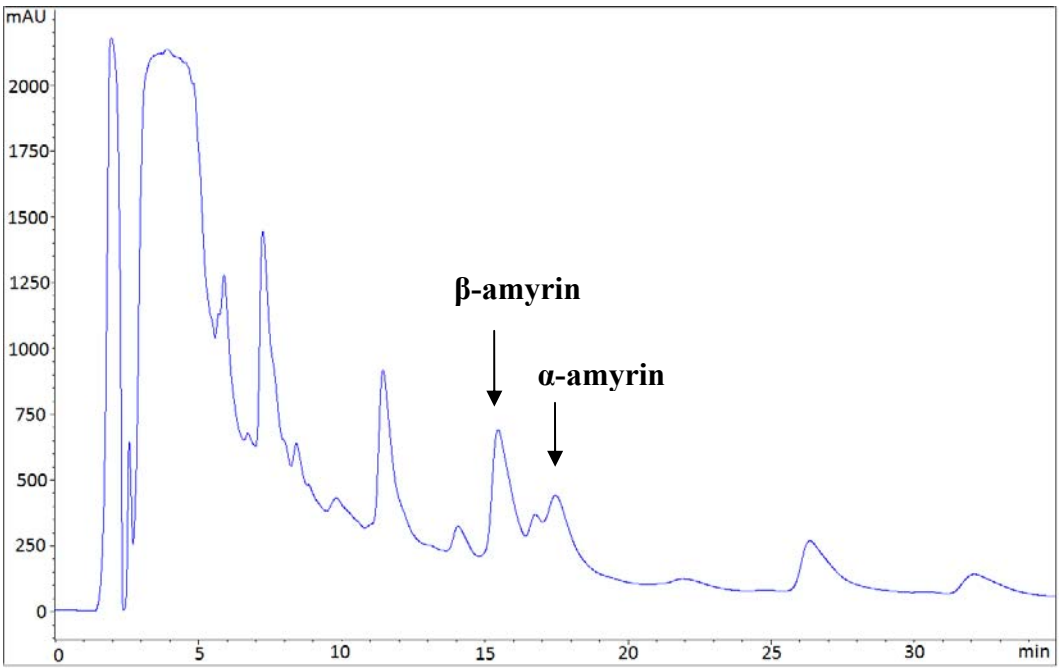


Figure 3.4 HPLC chromatogram of 70% v/v methanol leaf extract from preparative HPLC isolation.

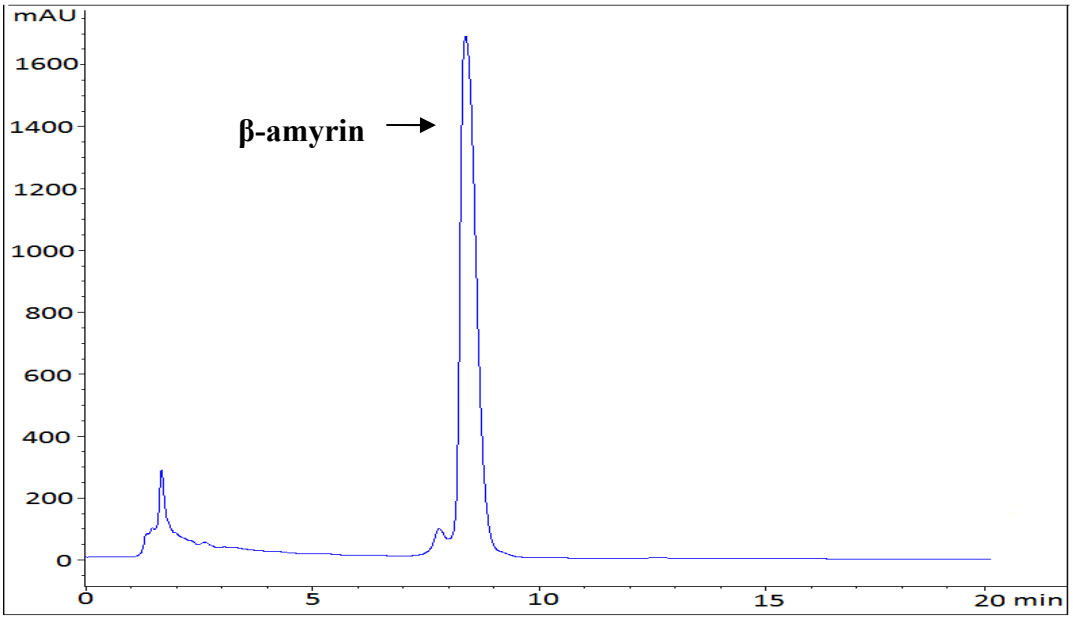


Figure 3.5 HPLC chromatogram of 70% v/v methanol leaf extract from semi-preparative HPLC isolation.

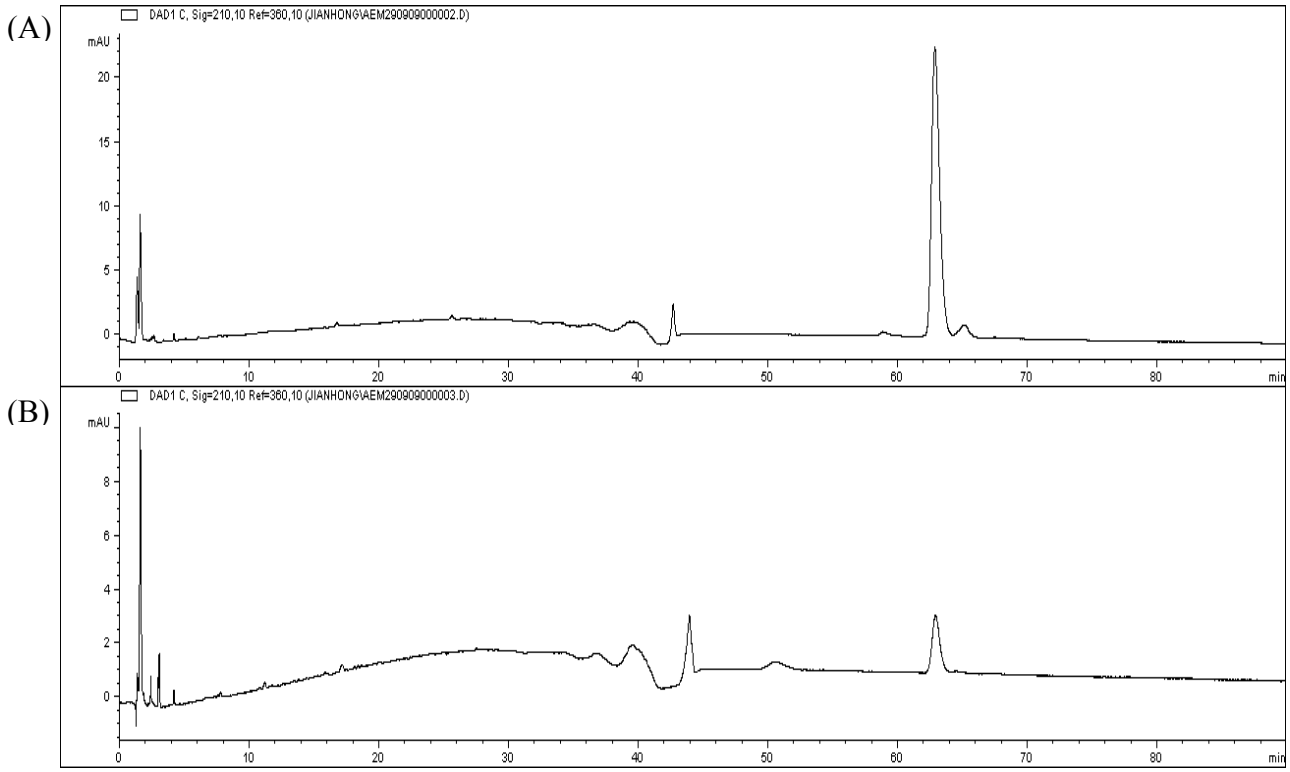


Figure 3.6 HPLC chromatograms of (A) isolated and purified β-amyrin and (B) β-amyrin standard.

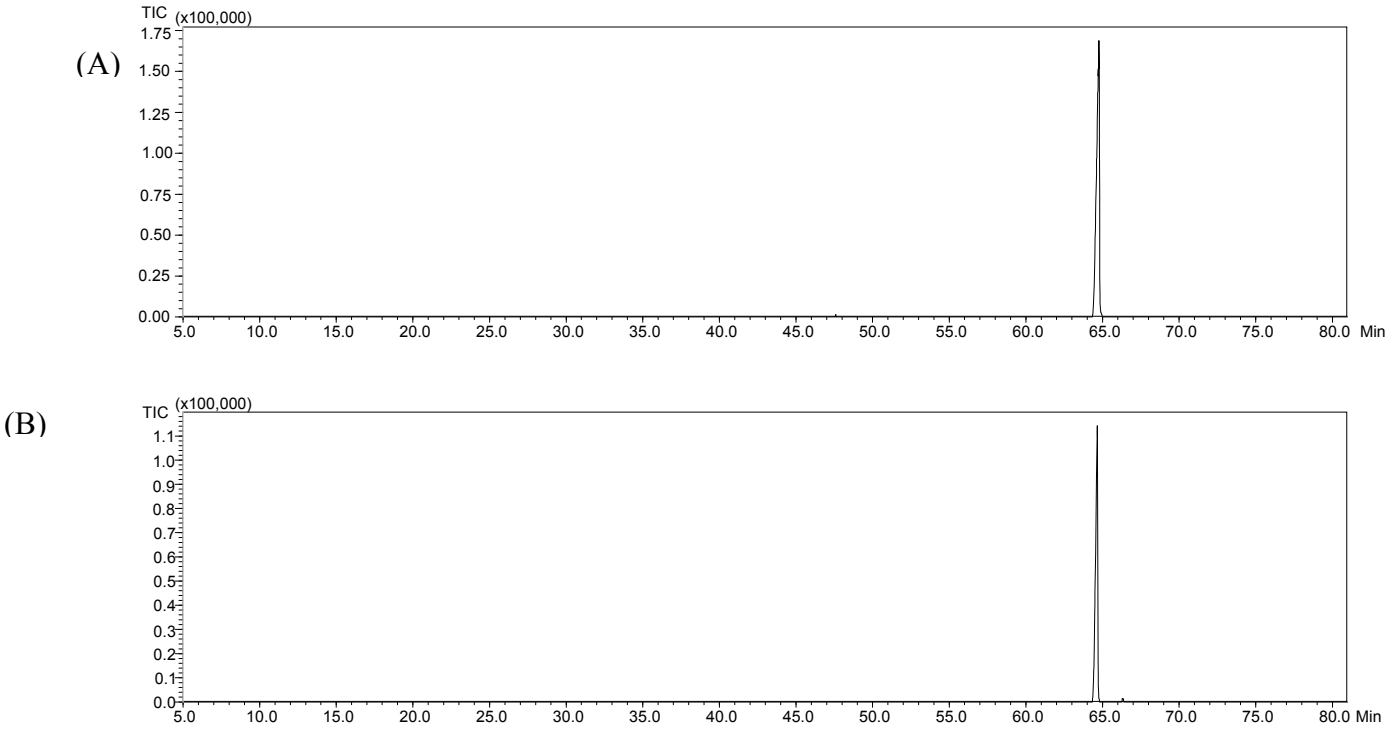


Figure 3.7 Gas chromatograms of (A) isolated and purified β-amyrin and (B) β-amyrin standard.

3.1.4.5 GC-MS method for analysis of amyrins

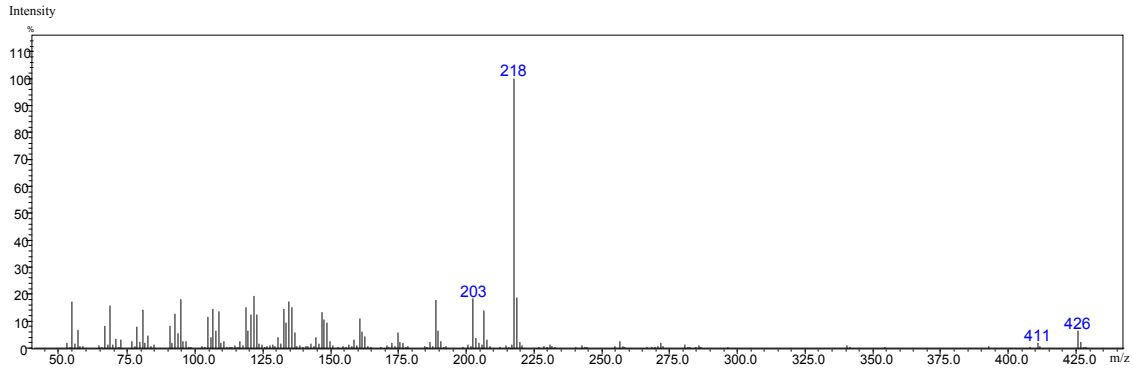
Various methods using HPLC, LC-MS and GC-MS had been developed for the detection or quantification of amyrins. The use of HPLC for the quantification of α-amyrin had been investigated. It was found that the LOD and LOQ were 0.4 μg ml^{-1} and 12 μg ml^{-1} respectively (Kumar et al., 2010). Although the method was determined to be precise, accurate and selective, the method is not sensitive enough at an LOD of 0.4 μg ml^{-1}. Another method that is used for the analysis of amyrins involves the use of LC coupled with mass spectrometry. However, LC-ESI/MS is not preferred for the detection of neutral triterpenes with only one hydroxyl group in the structure as they are not easily ionised by ESI

(Rhourri-Frih et al., 2009). It was found that LC-APPI-MS was more sensitive than LC-ACPI-MS for the analysis of α- and β-amyrin. APPI is a relatively new ionisation method for LC-MS. Samples are evaporated by heating and a nebulising gas. The samples are then passed into the photoionisation region. An ionisable dopant in the region is vaporised, which then collides with the solvents and samples, producing analyte ions $[M + H]^+$ or $[M]^{\bullet+}$ in the positive ion mode and $[M - H]^+$ in the negative ion mode (Robb et al., 2000). The LOQ for α- and β-amyrin in the SIM mode using LC-APCI-MS was 0.55 μg ml^{-1} and 0.56 μg ml^{-1} respectively (Rhourri-Frih et al., 2009). LC-APPI-MS was 36 to 46 times more sensitive than LC-APCI-MS, with the LOQ for α- and β-amyrin in the SIM mode being 0.015 μg ml^{-1} and 0.012 μg ml^{-1} respectively (Rhourri-Frih et al., 2009). Gas chromatography-electron impact mass spectrometry (GC-EI/MS) has been popularly used for the analysis of triterpenes but silylation or derivatisation is required in the method to make the solutes volatile enough for analysis (Laurent et al., 2003; Regert et al., 2006; Coelho et al., 2007; Echard et al., 2007; Jacques et al., 2007; Lytovchenko et al., 2009; Rhourri-Frih et al., 2009; Scholz et al., 2009). However steps involving derivatisation is laborious for large number of samples. A GC-MS method without the need for derivatisation of samples was reported for the quantification of β-amyrin (Kirby et al., 2008). The reported method required the GC oven temperature to be ramped up to 300 ^oC and the quantification was performed in SIM mode, detecting ions of m/z 203, 218 and 426.

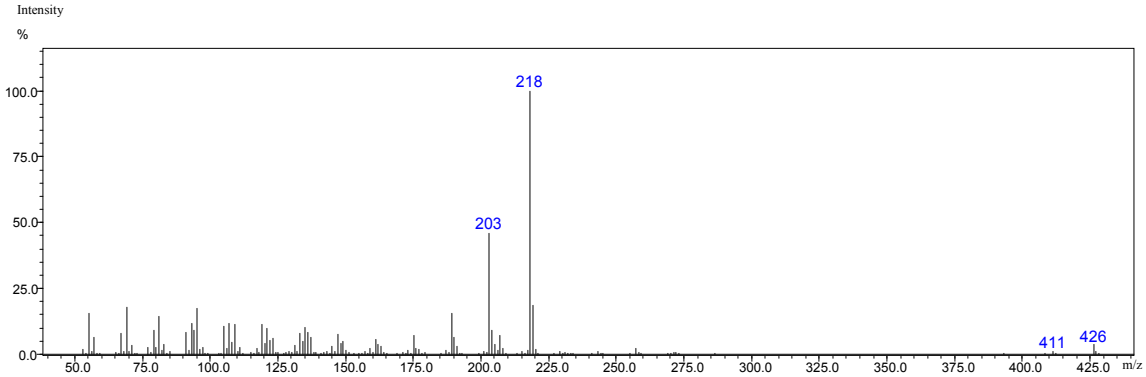
Initially, liquid chromatography-mass spectrometry (LC-MS) was attempted for assay development. However the sensitivity of the assay was low when APCI was used for analysis. The LOQ of β-amyrin was found to be about 400 ng ml^{-1}. This was agreeable to that reported in literature (Rhourri-Frih et al., 2009). As the sensitivity of LC-MS was low, GC-MS was subsequently employed for the assay development.

In order to select the ions for quantification, full scans of the mass spectra of α-amyrin, β-amyrin and methyltestosterone were first obtained. The mass spectra were shown in Figure 3.8. Ions with relatively high intensities were selected to monitor amyrins and methyltestosterone under SIM mode. Subsequently, ions with m/z 203, 218 and 426 were used for detecting both α- and β-amyrin while those with 43, 124 and 302 were used for detecting methyltestosterone. The mass spectra of both α- and β-amyrin were in agreement with those reported in literature (Gawronska-Grzywacz and Krzaczek, 2007; Boszormenyi et al., 2009). Ions with m/z 218 and 302 were chosen for quantification of the amyrins and methyltestosterone respectively.

(A)



(B)



(C)

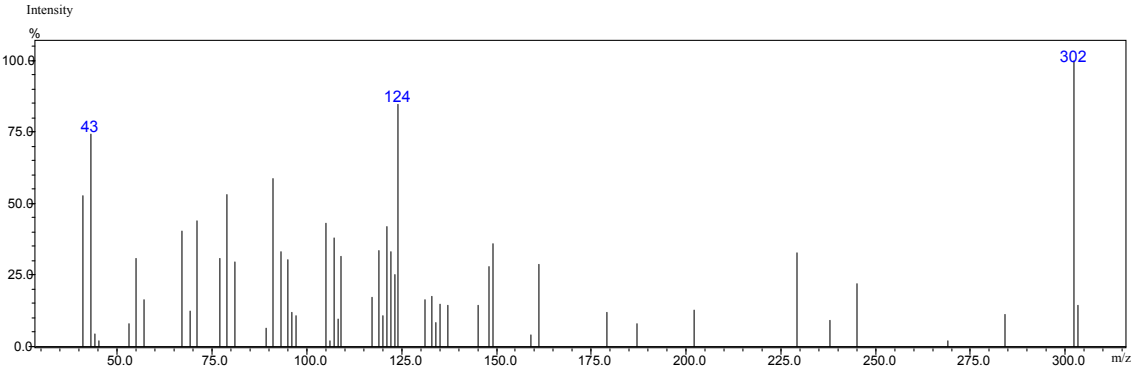


Figure 3.8 Scanning mode mass spectra of (A) α-amyrin, (B) β-amyrin and (C) methyltestosterone.

3.1.4.6 GC-MS method validation

The LOD (S/N = 3) and LOQ (S/N = 10) for α-amyrin were found to be 0.1 ng ml^{-1} and 1 ng ml^{-1} respectively. The LOD and LOQ for β-amyrin were found to be 0.1 ng ml^{-1} and 0.4 ng ml^{-1} respectively. The calibration curves for both α –amyrin and β-amyrin show good linearity over the concentration range 0.5 ng ml^{-1} to 2 μg ml^{-1} with a correlation coefficient $R^2 > 0.995$. The intra-day retention time variations (RSD) for α –amyrin and β-amyrin were 0.17 ± 0.04% (n = 5) and 0.23± 0.09% (n = 5) respectively. The inter-day retention time variations (RSD) for α –amyrin and β-amyrin were 0.28 ± 0.03% (n = 3) and 0.26 ± 0.05% (n = 3) respectively. The intra-day assay variations (RSD) for α –amyrin and β-amyrin was 10.4 ± 0.17% (n = 5) and 9.6 ± 0.23% (n = 5) respectively. The inter-day assay variations (RSD) for α –amyrin and β-amyrin were 12.1 ± 0.28% (n = 3) and 9.1 ± 0.26% (n = 3) respectively.

Specificity of the method is demonstrated. Specificity is defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. In this method, the GC-MS assay is validated for its ability to discriminate between compounds of closely related structures (α- and β-amyrin), and to ensure no interferences between the compounds studied. The GC-MS assay developed is able to discriminate between compounds of closely related structures (α- and β-amyrin), indicating good specificity (Figure 3.9). There are no interferences between the compounds studied.

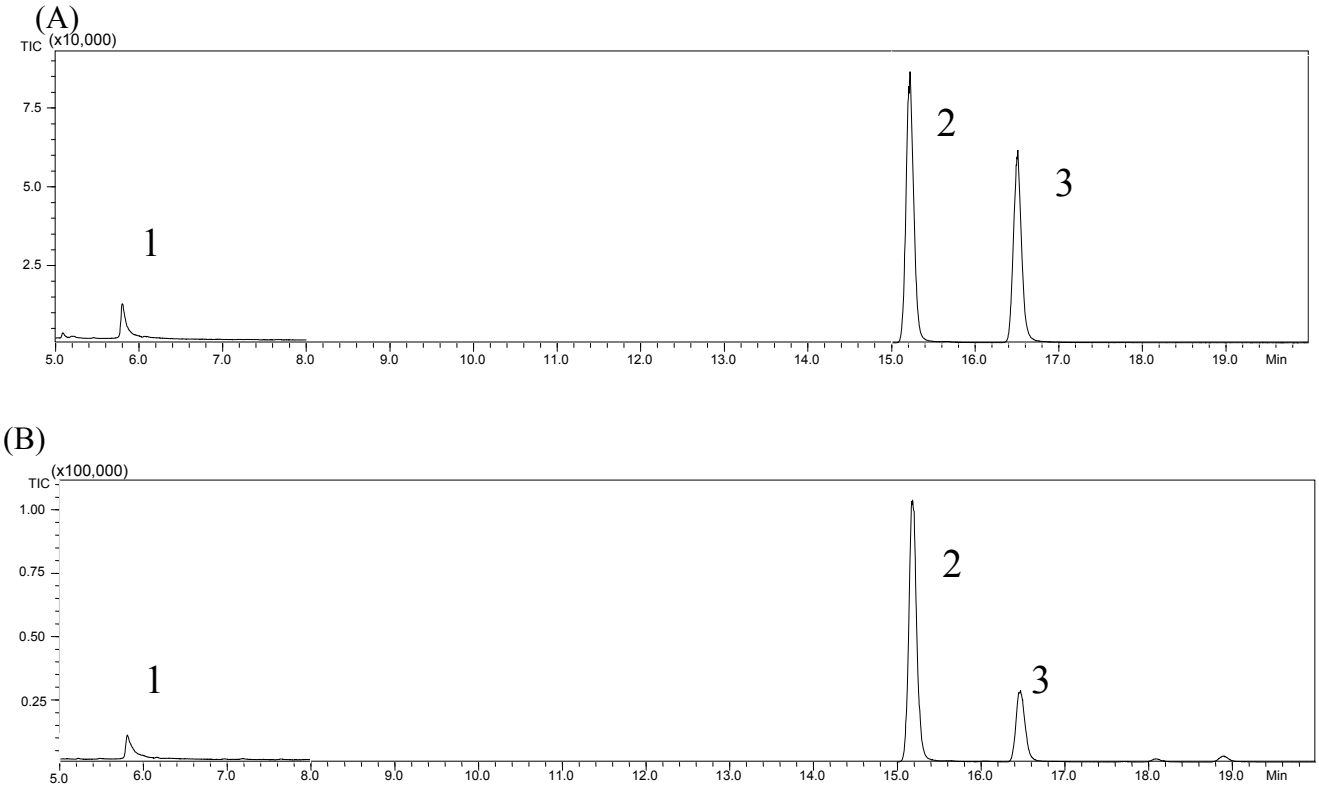


Figure 3.9 Gas chromatograms of (A) mixture of α-amyrin (peak 3; 2 ppm) and β-amyrin standards (peak 2; 2 ppm) and the internal standard, methyltestosterone (peak 1; 1ppm) spiked into HPLC grade methanol (B) *A. elliptica* 70 % methanol extract (100 ppm). Chromatograms are total ion chromatograms of selective ion monitoring (SIM) of α- and β-amyrin (m/z 203, 218, 428) and methyltestosterone (m/z 43, 124, 302).

Accuracy of the method was assessed by spiking known amyrin standards into the 70% v/v methanol *A. elliptica* leaf extract. To investigate the accuracy, recovery studies were done by spiking three different amounts of amyrin into the extract. The recoveries of the three concentration levels (0.5, 1.0, 1.5 μg ml^{-1}) of spiked α-amyrin were 102.3 ± 7.7% (n=3), 100.2 ± 7.8% (n=3) and 106.9 ± 13.0% (n=3) respectively. The recoveries of the three concentration levels (0.5, 1.0, 1.5 μg ml^{-1}) of spiked β -amyrin were 100.4 (n=2), 95.1 ± 17.5% (n=3) and 117.8 ± 2.6% (n=3) respectively.

While Kirby et al. (2008) also developed a GC-MS method without requiring the β-amyrin samples to be derivatised, the method had not been validated. The method developed here is validated for both α- and β-amyrin. The time required for analysis is also shorter for this method (retention time of β-amyrin = 15.2 min) compared to that of Kirby et al. (2008) (retention time of β-amyrin = 18 min). Another advantage is that the GC oven temperature used in this assay (280 ^{o}C) is lower than that used by Kirby et al. (2008) (300 ^{o}C). The lower temperature used could be beneficial for preserving the integrity of GC columns.

3.1.4.7 Quantification of α- and β-amyrins in the *A. elliptica* leaf extract and the fresh leaves

To quantify the amount of amyrins in the extract and leaves, three separate batches of leaves were individually extracted and analysed in triplicates. Calibration curves of α- and β-amyrin were generated. It was found that the concentrations of α- and β-amyrin were 0.6 ± 0.01% (w/w) and 1.0 ± 0.01% (w/w) of the dried *A. elliptica* leaf extract respectively. This translated to a concentration of 0.05 ± 0.001% (w/w) and 0.08 ± 0.001% (w/w) for α- and β-amyrins in the fresh leaves. Thus it can be seen that α- and β-amyrin exist naturally in *A. elliptica* leaves in a 1 : 1.6 ratio.

3.2 Antiplatelet and anticoagulation studies of *A. elliptica* extract

3.2.1 Introduction

Adverse effects of current antiplatelet and anticoagulant drugs have been well documented. Currently used antiplatelet and anticoagulant drugs such as aspirin and warfarin have adverse effects including gastric mucosal erosions and excessive bleeding. Given the limitations of the current treatments, the objective of part of the work is to discover lead compounds or novel antiplatelet and anticoagulant drugs from *A. elliptica*.

In the previous section, *A. elliptica* extracts were chemically analysed and β-amyrin was isolated and quantified. In this section, the *A. elliptica* extract and isolated β-amyrin are studied for potential antiplatelet and anticoagulant activities. Currently, information on the traditional uses of the plant is very limited. While records are available for the disease symptoms the plant can treat, there is no information on the doses of the plant. It is understood that traditional records indicated that the leaves were boiled and drunk (Burkill, 1966), presumably with water. However in certain cases, whole leaves were eaten. In this study an organic solvent 70% v/v methanol was used, in the aim of extracting more compounds, compared to using either a non-polar or polar solvent.

3.2.2 Objectives

The overall objectives of this section are to investigate the antiplatelet and anticoagulant activities of the *A. elliptica* extract, as well as its bioactive components *in vitro*. The specific objectives are to

1. Investigate the antiplatelet activities of *A. elliptica* extracts and their bioactive components *in vitro* in rabbit blood by means of the impedance method using a whole blood aggregometer.
2. Investigate the anticoagulant activities of *A. elliptica* extracts and their bioactive components *in vitro* in human blood by determining their effects on the prothrombin time (PT) and the activated partial thromboplastin time (aPTT).

3.2.3 Materials and methods

3.2.3.1 Plant material

A. elliptica leaves were collected and stored as described in Section 3.1.3.1.

3.2.3.2 Reagents and standards

Methanol was purchased from Tedia (Fairfield, OH). Dimethyl sulfoxide (DMSO) was obtained from MP Biomedicals (Illkrich, France) while phosphate buffer saline (PBS, 8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4, 0.24 g l^{-1} KH_2PO_4) bought from 1st Base (Singapore). Collagen (1 mg ml^{-1}) was purchased from Chronolog (Havertown, PA). α-amyrin and β-amyrin were obtained from Extrasynthase (Genay, France). Aspirin was obtained from Sigma Aldrich (USA).

3.2.3.3 Extraction and preparation of plant extracts

The preparation of the 70% v/v methanol leaf extract of *A. elliptica* was as in Section 3.1.3.3. In addition, 82 g of leaves were extracted with 500 ml of distilled water. The extracts were evaporated to dryness using a rotary evaporator.

3.2.3.4 Fractionation of *A. elliptica* crude extract

Liquid-liquid fractionation was performed as described in Section 3.1.3.4.

3.2.3.5 Measurement of platelet aggregation

Whole blood aggregation assay was carried out by the measurement of impedance using a whole blood aggregometer (Chronolog Corporation, Havertown, PA) as described by (Cardinal and Flower, 1980). Rabbit blood (Male New Zealand Whites, 2.5 ± 0.5 kg, anaesthetised with ketamine/xylazine mixture at 0.2 ml kg^{-1} body weight of each) from the central ear artery of the rabbits was collected into a citrated tube (Tapval, 4 ml blood collection tubes, 0.106 M citrate, Deltalab S.A., Barcelona, Spain). The animals were kept in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). 450 µl of the blood prewarmed to 37 ^{o}C was then diluted with pre-warmed PBS (1:1) and allowed to equilibrate for two minutes. For the assay on plant extracts, 4.6 µl of plant extracts in DMSO was added and the solution was allowed to equilibrate for two minutes. As for assays using the standards, 4.6 µl of either α- or β-amyrin or a mixture of both in DMSO was used. 2 µl of collagen was then added to initiate platelet aggregation. The test was allowed to run for 5 min. The final concentration of DMSO in the test solutions were kept constant at 0.5% v/v. All tests were carried out at least three times. Aspirin was used as a positive control to determine the extent of anti-platelet activity of the plant extracts and fractions. Inhibition of platelet aggregation (Z) is calculated as follows:

Percentage inhibition of platelet aggregation (%), $Z = \frac{X - Y}{X} \times 100$

where X is the impedance value for the control and Y is the impedance value of the sample. IC_{50} values were calculated using Graph Pad Prism version 3.03 **(**GraphPad Software, Inc., CA, USA).

3.2.3.6 Plasma coagulation assays

The procedure for the plasma coagulation assays was modified from Lau (2006). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) times were determined with a Sysmex CA-530 blood coagulation analyser (Sysmex Corporation, Kobe, Japan). The commercial lyophilised pooled human plasma (Ci-Trol level 1; Dade Behring, DE, USA) was reconstituted with 1 ml of distilled water and left to stand for at least 15 min. It was used within 8 h after reconstitution. To prepare the sample mixture, 2 µl of plant extracts or standards dissolved in DMSO was added to 133 µl of plasma for the measurement of the PT and aPTT. For the control, 2 µl of DMSO was used instead.

For measurement of PT, 50 µl of the sample mixture was incubated for 180 s. 100 µL of PT reagent (Thromborel S; Dade Behring, DE, USA) was then added to the sample mixture. Time course of the test protocol was 120 s. If the blood coagulation was greatly inhibited, resulting in a PT time that was more than 120 s, no coagulation would be detected.

For measurement of aPTT, 50 µl of sample mixture was incubated for 60 s. 50 µl of aPTT reagent (Actin FSL; Dade Behring, DE, USA) was then added to the sample mixture and further incubated for 180 s. Finally, 50 µl of aqueous

$CaCl_2$ was added. Time course of the test protocol was 190 s. If the blood coagulation was greatly inhibited, resulting in an aPTT that was more than 190 s, no coagulation would be detected.

3.2.3.7 Statistical analysis

All results are expressed as mean ± standard deviation. Statistical analyses of the groups and IC_{50} calculation were performed using ANOVA and the nonlinear regression function respectively on Graph Pad Prism version 3.03. Significant differences were concluded for $p < 0.05$.

3.2.4 Results and discussion

3.2.4.1 Antiplatelet effects of *A. elliptica* extracts and fractions

The antiplatelet activities of the *A. elliptica* water extract and 70% v/v methanol extract, as well as the fractions obtained by liquid-liquid partition are presented in Figure 3.10. At a concentration of 0.2 mg ml^{-1}, all extracts and fractions showed between 18% to 35% inhibition of platelet aggregation induced by collagen, which were not significantly different from each other ($p > 0.05$). Chemical analyses showed that the hexane fraction contained high concentrations of α- and β-amyrin. This implied that the amyrins could have contributed to the antiplatelet activity observed in the hexane fraction and 70% v/v methanol extract. On the other hand, the water fraction (obtained from liquid-liquid partitioning of the 70% v/v methanol extract) showed pro-aggregation activities. Further analysis of the components in the water fraction is required to understand why a pro-aggregating effect was observed.

Furthermore, the IC_{50} values for the inhibition of platelet aggregation by the 70% methanol extract of *A. elliptica*, the water extract of *A. elliptica*, and aspirin (positive control) were determined and presented in Table 3.1. While the 70% v/v methanol extract showed a dose dependent increase in antiplatelet activity, the antiplatelet activity of the water extract decreased for concentrations above 40 μg ml^{-1}. The 70% v/v methanol extract was about 10 times less active than aspirin, however the IC_{50} of the water extract was almost similar to that of aspirin.

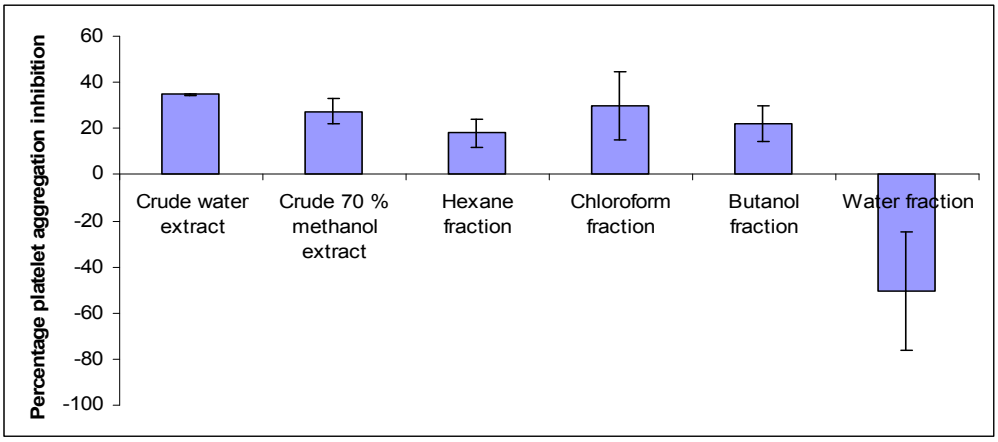


Figure 3.10 Platelet aggregation inhibition by different *A. elliptica* extracts and fractions derived from the 70% v/v methanol extract at 0.2 mg ml^{-1}. ($n \geq 3$)

Table 3.1 IC_{50} values of *A. elliptica* extracts for inhibition of collagen-induced platelet aggregation.

Sample	IC_{50} values (µg ml^{-1})
70% v/v methanol extract of *A. elliptica*	166.9 ± 46.7
Water extract of *A. elliptica*	18.4 ± 1.1
Aspirin	16.5 ± 4.4

n = 3. Values are reported as mean ± standard deviation.

For concentrations of above 40 µg ml^{-1}, the antiplatelet activity of the water extract decreased with increasing concentrations. This implied either the presence of both antiplatelet and proaggregating components in the water extract, or presence of a component with dual activities. Similar observations had been reported previously for a flavonoid, arcapillin, where antiplatelet activity was seen

at low concentrations but proaggregation activity was observed at high concentrations (Wu et al., 2001). According to published literature (Wee, 1992; HMRC and IMR, 2002; Jalil et al., 2004), the leaves are boiled and drunk by Malays for treatment of pain in the region of the heart. However, the dosage was not indicated and the exact liquid to prepare the decoction was not clearly specified. In general, the usual liquid used for decocting medicinal herbs is water.

Jalil *et al.* (2004) previously reported a platelet activating factor inhibitor, 5-(*Z*-heptadec-4'-enyl)resorcinol from the leaf extract of *A. elliptica* with an IC_{50} value of 7.08 μM for the inhibition of PAF receptor binding on rabbit platelet using ^{3}H-PAF as a ligand. Besides 5-(*Z*-heptadec-4′-enyl)resorcinol, other phytoconstituents identified in *A. elliptica* include 5-pentadecylresorcinol (Jalil et al., 2004), rapanone, β- amyrin (Chow et al., 1991), syringic acid, isorhamnetin and quercetin (Phadungkit and Luanratana, 2006). Bauerenol, α-amyrin and bergenin had also been reported in *A. elliptica* (Ahmad et al., 1977; Chow et al., 1991; Kobayashi and de Mejia, 2005). Syringic acid was reported to have very weak antiplatelet activities on washed rabbit platelets induced by arachidonic acid and collagen (Yang et al., 2002). Numerous studies had been done on the antiplatelet activities of quercetin. Quercetin was found to inhibit PAF receptor binding on rabbit platelets with an IC_{50} value of 33.0 μM (Mazura et al., 2007). In addition, it was reported to inhibit platelet activation by ADP (Tzeng et al., 1991; Kobzar et al., 2005), collagen and arachidonic acid (Tzeng et al., 1991). Calcium mobilization in human platelets was inhibited by quercetin as well (Xiao et al., 1995; Guerrero et al., 2007). In addition to collagen, ADP was also used to induce

platelet aggregation in the investigations here. However the results were inconclusive as very weak aggregation was observed with the control.

To investigate if the amyrins detected were giving rise to the antiplatelet activity, the 70% v/v methanol was further fractionated by preparative HPLC into 13 fractions as described in Section 3.1.3.4. Of the 13 fractions collected, the fraction containing both α- and β-amyrin showed the highest antiplatelet activity among the different fractions. A 35.6% platelet aggregation inhibition was observed when that highly active fraction was tested at a low concentration of 10 μg ml^{-1}. On the other hand, only 27.4% platelet aggregation inhibition was observed when 0.2 mg ml^{-1} of the 70% v/v methanol extract was tested (Figure 3.10). This implied that the amyrins were responsible for the antiplatelet activity observed.

3.2.4.2 Antiplatelet effects of α- and β- amyrin

Following the identification of the two active components detected, α- and β- amyrin, their IC_{50} values were determined and compared to aspirin (positive control) in Table 3.2.

Table 3.2 IC_{50} values of *A. elliptica* extract and bioactive components for inhibition of collagen-induced platelet aggregation.

Sample	IC_{50} values (μg ml^{-1})	IC_{50} values (μM)
70 % methanol extract of *A. elliptica*	166.9 ± 46.7	-
α-amyrin	9.1 ± 1.0	21.3 ± 2.3
β-amyrin	4.5 ± 0.6	10.5 ± 1.0
Aspirin	11.3 ± 2.0	62.7 ± 11.1

n = 3. Values are reported as mean ± standard deviation.

The pure components α-amyrin and β-amyrin gave IC_{50} values of 9.1 μg ml^{-1} (21.3 μM) and 4.5 μg ml^{-1} (10.5 μM) respectively (Ching et al., 2010). They are at least 6 folds more active compared to aspirin (IC_{50} value 62.7 μM) and 3 times more active than the water extract of *A. elliptica*. In order to study any potential synergistic effect of the amyrins on inhibition of platelet aggregation, a mixture of amyrins (5 μg ml^{-1}) in different ratios of the 2 amyrins were also used. The results showed no significant differences ($p > 0.05$) in platelet aggregation inhibition between the mixtures of amyrins (Table 3.3).

Table 3.3 Percentage inhibition of platelet aggregation of amyrin standards

Standards at 5 μg ml^{-1}	Percentage inhibition of platelet aggregation (%)
α-amyrin	20.6 ± 6.21
β-amyrin	49.0 ± 15.4
7 parts of α-amyrin : 3 parts of β-amyrin	32.3 ± 10.0
1 part of α-amyrin : 1 part of β-amyrin	29.8 ± 6.5
3 parts of α-amyrin : 7 parts of β-amyrin	35.4 ± 14.5

Values are reported as mean ± standard deviation. Activities of amyrin mixtures are not significantly different from each other ($p > 0.05$). n = 4-9.

Both α- amyrin and β- amyrin have been reported to have diverse interesting biological activities, including anxiolytic, antidepressant (Aragao et al., 2006), antinociceptive (Otuki et al., 2005), liver-protective (Oliveira et al., 2005) and gastroprotective (Oliveira et al., 2004) effects. Both the amyrins were

also separately found to exhibit anti-inflammatory activity (Akihisa et al., 1996). As both α- and β- amyrin are poorly soluble in water, this implied the possible presence of other bioactive components in the crude water extract with anti-platelet activity.

A mixture of α- and β- amyrin isolated from *Protium heptaphyllum* in a ratio of 2:1 respectively was shown to inhibit collagen induced platelet aggregation with an IC_{50} value of 90.0 μM (Aragao et al., 2007). The mixture was also found to inhibit platelet aggregation induced by ADP and arachidonic acid with IC_{50} values of 117.9 μM and 181.4 μM respectively. The current IC_{50} values for inhibition of collagen induced platelet aggregation at 5.3 μg ml^{-1} (12.4 μM) for α- amyrin and 3.6 μg ml^{-1} (8.4 μM) for β- amyrin are at least 7 fold smaller. α-amyrin was also reported to inhibit PAF receptor with an IC_{50} value of 20.0 μM (Mazura et al., 2007). This is equivalent to an IC_{50} of 8.5 μg ml^{-1} using the molecular mass of α-amyrin of 426.72 g mol^{-1}.

3.2.4.3 Anticoagulant effects of *A. elliptica* extracts and fractions

The effects of the *A. elliptica* extracts and fractions on the PT and aPTT of plasma coagulation are shown in Figure 3.11. The hexane fraction showed a slight but significant prolongation of the PT ($p < 0.05$), while the 70% v/v methanol extract ($p < 0.01$), the butanol fraction ($p < 0.05$), water extract and water fraction ($p < 0.001$) showed significant shortening of the PT. Both the crude water extract and the chloroform fraction showed no effects on the PT. Strong anticoagulant activities on aPTT were exhibited by both the crude extracts and the hexane and

chloroform fractions ($p < 0.001$). The butanol fraction and water fraction showed weaker effects in prolonging the aPTT ($p < 0.01$).

At a concentration of 0.2 mg ml^{-1}, the water extract showed only prolongation of the aPTT. However, the 70% v/v methanol extract exhibited both anticoagulant and procoagulant effects. It was generally observed that the fractions extracted from more non-polar solvents (hexane and chloroform) exhibited anticoagulant effects on the aPTT. Plasma coagulation activities on the PT were shown on more polar fractions, the butanol and water fractions. This could imply that components with anticoagulant found in *A. elliptica* were of a more non-polar nature while those that caused increased coagulation were more polar.

The phenomenon of plant extracts having both opposing activities on plasma coagulation had also been previously reported. Plasma coagulant activity was detected at high concentrations of the *Jatropha curcas* latex while anticoagulant activity was detected at low concentrations of the latex (Osoniyi and Onajobi, 2003). Two possible explanations were proposed to explain the phenomenon. The first was that the two different activities were induced by two separate components which may have acted optimally at different concentrations. The second possible explanation was that the same component had differing activities under different conditions (Osoniyi and Onajobi, 2003). For example, thrombin exhibits procoagulant activities when it cleaves fibrinogen to induce a fibrin clot. However it can also act as an anticoagulant when it activates protein C in the presence of the cofactor, thrombomodulin (Cantwell and Di Cera, 2000). In

this study, the anticoagulant effects on the aPTT (prolonging aPT) were seen in non-polar fractions while plasma coagulation activities on the PT (shortening PT) were obtained with the more polar fractions. The opposing activities on different pathways of plasma coagulation might imply the presence of different compounds acting separately. Further work should be carried out to investigate this phenomenon.

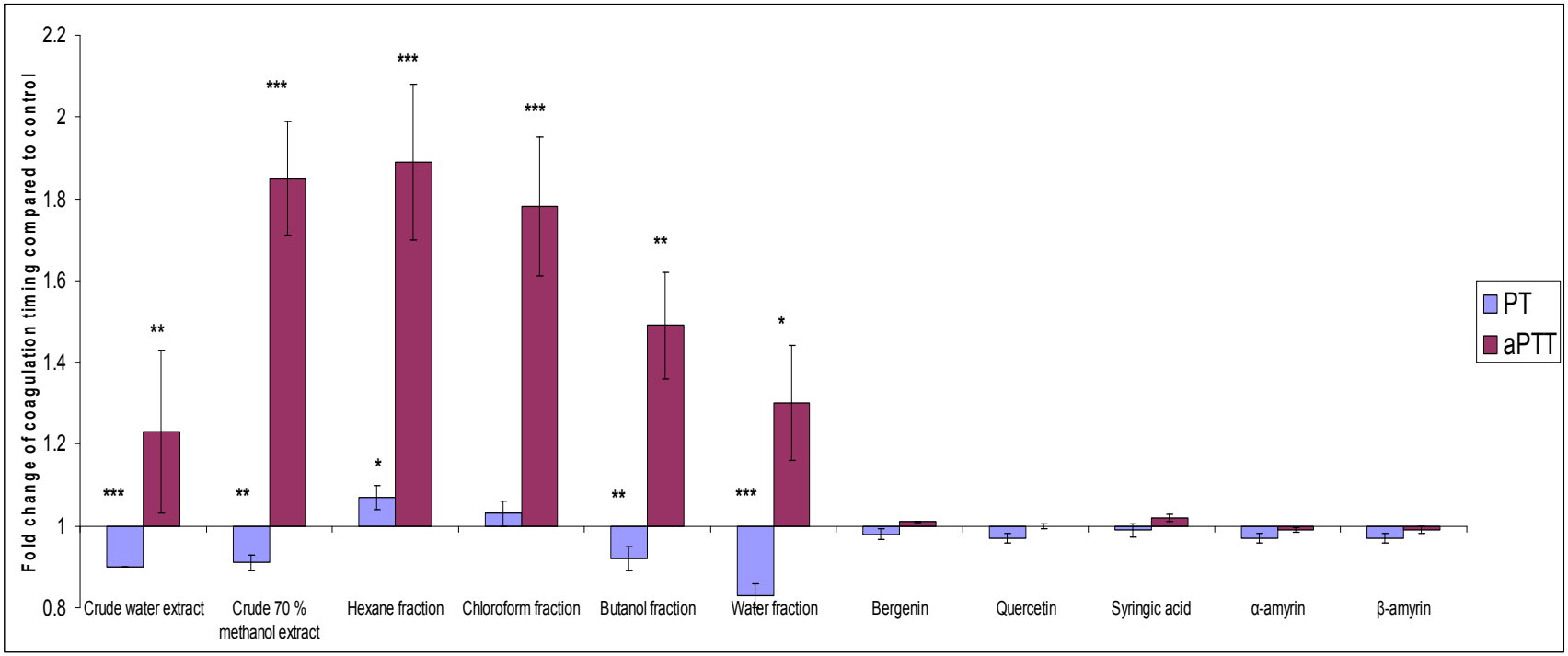


Figure 3.11 Plasma coagulation effects by different *A. elliptica* extracts and fractions at 0.2 mg ml^{-1}; bergenin, quercetin, syringic acid at 0.1 mg ml^{-1}; α- and β- amyrin at 0.01 mg ml^{-1}. ($n \geq 3$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

3.2.4.4 Anticoagulant effects of phytoconstituents found in *A. elliptica*

In addition to α- and β- amyrin, phytoconstituents reported in *A. elliptica* in the literature include bergenin, quercetin and syringic acid (Chow et al., 1991; Phadungkit and Luanratana, 2006). Bergenin, quercetin and syringic acid standards were tested for effects on plasma coagulation at a concentration of 0.1 mg ml^{-1} while the two amyrins were tested at 0.01 mg ml^{-1} due to their lower solubilities. All the compounds tested were found to have no significant effects on plasma coagulation ($p > 0.05$) (Figure 3.11). Although α- and β- amyrin were reported to exhibit antithrombin activity *in vitro* (Medeiros et al., 2002), no anticoagulant activity was exhibited by the compounds at 0.01 mg ml^{-1} in this study. The discrepancies may be due to the difference in concentration and the bioassay method used. The study by Mederios et al. showed that α- and β- amyrin at a concentration of 0.5 mg ml^{-1}, were found to exhibit 39% and 78% antithrombin activity respectively. However this high concentration was not achieved in this study due to solubility problems. Moreover, Mederios et al. investigated only the antithrombin activity using a different experimental setup from that used in this study. Mederios et al. used a chromogenic reagent for detection of the antithrombin activity, while a light scattering method was used to determine the PT and aPTT in human plasma in this study.

3.3 Conclusion

In the first section of this chapter, HPLC and GC-MS methods have been successfully developed for the analysis of the 70% v/v methanol extract of *A.*

elliptica leaves. It was shown that α- and β-amyrin are major components in the plant extract. Short preparative and semi-preparative HPLC methods had also been developed to isolate β-amyrin from the leaf extract. α- amyrin co-eluted with other components and attempts to isolate it was not successful. A GC-MS method had been successfully developed for the analysis of α- and β-amyrins in the *A. elliptica* leaf extract. The method had been validated for its sensitivity, specificity, precision and accuracy. Unlike previous methods which require derivatisation (Laurent et al., 2003; Coelho et al., 2007; Echard et al., 2007), this method has been streamlined to exclude the derivatisation steps so that laborious steps are removed for future assays requiring large samples. This is also the first report of the quantification of both α- and β-amyrin in the leaves of *A. elliptica*.

In the second section of this chapter, the antiplatelet and anticoagulant activities of *A. elliptica* and its isolated component had been investigated *in vitro*. The 70% v/v methanol extract of *A. elliptica* was shown to have both antiplatelet and anticoagulant activities. β-amyrin was found to be one of the components responsible for the antiplatelet activity. This compound was isolated and purified**.** β-amyrin was found to be six times as active as aspirin in inhibiting platelet aggregation. A naturally occurring isomer of β-amyrin in the plant extract, α-amyrin was also tested and found to have strong antiplatelet activities. While the 70% v/v methanol extract of *A. elliptica* was shown to have anticoagulant activities, α- and β-amyrin were not found to be active. This showed that the anticoagulant properties of *A. elliptica* were attributed by other components and further studies have to be performed to identify them.

CHAPTER 4

Multivariate data analysis method for discovery of bioactive components from *A. elliptica*

4.1 Introduction

The conventional method of identifying bioactive compounds from plant extracts usually involves bioassay-guided fractionation. The process of bioassay-guided fractionation is tedious, involving repeated steps of fractionation and bioassays. Although the process may eventually yield bioactive components, much time is required. An added drawback is its difficulty to detect compounds which are in low concentrations. Chemoinformatics and Multivariate data analysis (MVDA) are used extensively in metabolomics which has applications in studying pathways of cardiovascular disease (Wheelock et al., 2009), discovery of biomarkers of various diseases such as diabetes mellitus (Li et al., 2009) and cancer (Kim et al., 2009), investigation of genotype-phenotype relationships, and optimising cell culture, metabolic engineering cell cultures (Oldiges et al., 2007), quality control of herbal medicines (Xie et al., 2008) etc. Metabolomics has also been developed for natural product research (Rochfort, 2005; Cheng et al., 2006; Wang et al., 2006; Wang et al., 2008; Chau et al., 2009; Froufe et al., 2009; Kim et al., 2010). It has been discussed in Section 1.3 about the different examples metabolomics can help in natural product drug discovery. In one example, the pharmacological action of a herb can be elucidated by comparing the metabolites of treated cells with a known drug. Metabolomics also enabled studies of

synergistic effects of complex herbal concoctions, and can help to predict active compounds from plant extracts. An additional advantage of metabolomics is the application of simple, sensitive and well established techniques for analysing samples. Rochfort (2005) had reviewed on the application of different analytical techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy, mass spectrometry, gas and liquid chromatography, as well as a combination of the techniques in studying plant metabolites using metabolomics.

As discussed in Section 1.3.3, there had been reports of successful application of different algorithms for the prediction of active components from natural products with different activities, such as cholesterol level reduction, cytotoxicity and antioxidant effects. This is the first report of using an MVDA method to investigate the antiplatelet and/or anticoagulant components of a plant extract. *A. elliptica* is used as the model plant in this case. In this chapter, the *in vitro* effects of plant extracts on platelet aggregation were correlated to the chemical constituents as determined by gas chromatography-mass spectrometry (GC-MS). The modeling used in this work is performed using easily available commercial software which can be applied simply to different extracts or bioactivities.

In Chapter 3, an evaluation had been performed on the platelet aggregating and plasma coagulating activities of *A. elliptica in vitro* in rabbit blood and human plasma respectively. The results of the activity studies were correlated to the individual compounds in the extracts and fractions, and analysed using MVDA for prediction of potential active compounds in this chapter. The MVDA method

was further refined by increasing the number of different extracts studied, and also performing the platelet aggregating assay in human blood. In addition, a more comprehensive MVDA method was designed using more statistical tests for the prediction of active compounds.

4.2 Objectives

The overall objective of this work is to develop a platform method to rapidly identify bioactive compounds from crude plant extracts and their partially purified fractions using MVDA. The specific objectives are to

1. Use existing data of biological activities (from Chapter 3) to explore the design of a platform MVDA method for rapid identification of compounds in plant extracts that are active, with minimal fractionation steps.
2. Further development of the MVDA method using biological activities obtained from testing human blood. The MVDA method used will include additional modelling by Orthogonal Partial Least Squares (OPLS), Partial Least Squares projection of latent structures-Discriminant Analysis (PLS-DA), Chi-Squared weighting, InfoGain weighting and also correlating the constituents of the plant extracts with bioactivity.
3. Identify the antiplatelet and/or anticoagulation compounds present in the *A. elliptica* extract with the MVDA method developed.

4.3 Methods and Materials

4.3.1 Plant material and chemicals

A. elliptica leaves (716 g) were collected from Pasir Ris Park, Singapore, with permission from the National Parks Singapore.

All extraction solvents used were of analytical grade. Methanol and 1-butanol used were from Merck (Singapore). Ethanol and hexane were obtained from Fisher Scientific (UK). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) reagent with 1% trimethylchlorosilane and methoxyamine hydrochloride (MOX reagent) were purchased from Thermo Scientific (USA).

4.3.2 Extraction and preparation of plant extracts

Extraction and preparation of plant extracts for the preliminary studies were described in Section 3.1.3.3.

For further development of the MVDA method, freshly collected *A. elliptica* leaves were again washed with distilled water, air dried and blended. Soxhlet extractions of the leaves were performed by refluxing with the appropriate solvents for six hours to obtain three batches each of 70% v/v methanol extract, ethanol extract and water extract. In total, 179.7 g of leaves were extracted with 2100 ml of 70% v/v methanol, 101.9 g of leaves were extracted with 450 ml of 100% ethanol and 107.4 g of leaves were extracted with 750 ml of distilled water. The quantity of leaves extracted for each batch is the same for the same extraction solvent used. For the purpose of compound isolation,

327 g of leaves were extracted with 70% v/v methanol. The extracts were dried using a rotary evaporator. The three batches of fresh leaves were collected from the same day. The extracts were evaporated to dryness *in vacuo* and reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 40 mg ml^{-1} for testing in the platelet aggregation assay.

4.3.3 Fractionation of *A. elliptica* extract

For preliminary studies, fractionation of the *A. elliptica* 70% v/v methanol extract was described in Section 3.1.3.4. For further development of the MVDA method, liquid-liquid fractionation was carried out on three separate dried 70% v/v methanol extracts of *A. elliptica* leaves. The extracts were reconstituted in 150 ml of distilled water and fractionated with 100 ml of hexane, butanol and distilled water sequentially to obtain three batches each of hexane fraction, butanol fraction and water fraction. All fractions were dried *in vacuo* and subsequently reconstituted in DMSO at a concentration of 40 mg ml^{-1}.

4.3.4 Derivatisation and development of GC-MS analysis of samples

For the preliminary studies, 0.11 mg each of the *A. elliptica* 70% v/v methanol extracts, its hexane, chloroform, butanol and water fractions, were aliquoted into glass centrifuge tubes. The aliquoted extracts were dried using a nitrogen evaporator at 50 ^{o}C and derivatised. Derivatisation of the extracts was performed as described by Lisec et al., 2006. After drying, the extracts were

incubated with 40 µl of MOX reagent and shaken for 2 hours at 37 ^{o}C. Subsequently, 70 µl of MSTFA reagent was added to the mixture and shaken for 30 min. The final solution was centrifuged at 2000 g for 20 min to obtain a clear solution and the supernatant was analyzed by GC-MS (Shimadzu, gc2010 and qp2010 MS, Japan). A DB-5MS column of film thickness 0.25 µm, length 30.0 m and diameter 0.25 mm was used. The oven temperature of the GC was held at 60 ^{o}C for 5 min and increased to 200 ^{o}C in 7 min. The temperature was held for 10 min and increased to 280 ^{o}C within 5 min. This temperature was held for another 30 min.

For further studies, fresh leaves were extracted in triplicate again. All 3 batches of *A. elliptica* 70% v/v methanol extracts, its hexane, butanol and water fractions, the ethanol and water extracts, 0.11 mg each, were aliquoted into glass centrifuge tubes. The aliquotted extracts were dried using a nitrogen evaporator at 50 ^{o}C and derivatised as above.

The phytoconstituents were preliminarily identified using the Wiley Mass Spectral Library Registry 7 (NJ, USA). Analysis of all extracts or fractions was repeated twice, to obtain six gas chromatograms for each extract, fraction and blank (MOX and MSTFA only).

4.3.5 GC-MS validation for MVDA

The GC-MS method was validated for repeatability and intermediate precision. The intra-day and inter-day variations were assessed. To assess intra-day variation, the first 70% v/v methanol extract was analysed three times within

a day, and the three different 70% v/v methanol extracts were analysed once each within a day. To assess inter-day variation, the first 70% v/v methanol extract was analysed once a day over three days. The relative standard deviations (RSDs) were calculated for the retention time and peak areas of 5 common compounds present in the samples. The compounds were chosen based on various retention times over the whole chromatogram.

4.3.6 Measurement of platelet aggregation

For preliminary studies, the platelet aggregation study of *A. elliptica* extracts on rabbit's blood from Chapter 3 (Section 3.2.4.1) was employed for analysis using MVDA.

For further development of the MVDA method, tests were performed on human blood using a fresh batch of extracts. Blood from three (one male and two female) normal healthy volunteers was drawn by a clinician according to an Institutional Review Board (IRB)-approved protocol by a clinician. Blood from the cubital vein was collected into citrated tubes (Tapval, 4 mL blood collection tubes, 0.106 M citrate, Deltalab S.A., Barcelona, Spain), and warmed to 37 ºC before usage. The whole blood aggregation assay was carried out with similar steps as described in Section 3.2.3.5.

4.3.7 Plasma coagulation assay

For preliminary studies, results of the plasma coagulation study of *A. elliptica* extracts reported in the previous chapter (Section 3.2.4.3) were employed for MVDA in this Chapter. For further studies, details of the plasma coagulation assays are as described in Section 3.2.3.6.

4.3.8 Preliminary data processing

As introduced earlier, a preliminary study of the data processing methods was first performed before the method was further developed.

For the analysis of the chromatograms obtained, the Automated Mass Spectral Deconvolution and Identification System (AMDIS) version 2.65 (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used to convert the files into a format readable by GeneSpring MS version 1.2 (Agilent, CA, USA). The data was normalised and the experiment parameter (the effect of the extracts on platelet aggregation or plasma coagulation) was associated with the different extract and fractions. The activities of the extract and fractions were determined by the platelet aggregation assay and plasma coagulation assay in Chapter 3. For the multivariate data analysis of the data, principle component analysis (PCA) was performed to check if the different extracts and fractions were distinguishable from each other, and also to observe the clustering trend of active and inactive extracts. To evaluate which are the compounds associated with the particular activity studied, GeneSpring MS was

used to filter compounds from the extracts with the desired activity with intensities at least 100 folds bigger than those without the activity.

4.3.9 Data processing

After the preliminary study, the GC-MS and bioassay data were analysed by different MVDA methods to identify potential antiplatelet compounds.

4.3.9.1 Analysis using Mass Profiler Professional

The chromatograms and data from all the extracts and fractions were first deconvoluted using AMDIS as described previously, then analysed using Mass Profiler Professional (Agilent, CA, USA). PCA and PLS-DA was used in the programme to observe the clustering of the different extracts and fractions.

4.3.9.2 Analysis using OPLS, PLS-DA, Chi-squared weighting and InfoGain weighting

SIMCA-P+ Version 12.0.1 (Umetrics, Sweden) was used to perform Orthogonal Partial Least Squares (OPLS) and Partial Least Squares projection of latent structures—Discriminant Analysis (PLS-DA) tests (Ma et al., 2008). The minimum number of non median values was set at 4, i.e. a particular compound must appear in a minimum of 4 out of 6 extract/fractions before it is considered in the analysis. This is to ensure that any potential contaminants that are present in only some of the extracts are excluded from the analysis. The spectrum was scaled using Pareto Variance (Ma et al., 2008). RapidMiner Version 4.5 (Rapid-I,

Germany) (Ibáñez et al., 2010) was used to perform Chi-Squared weighting and InfoGain weighting. For each test, contribution scores for biological activity were assigned to each compound. The top ten compounds with the highest contribution scores were listed as potential bioactive compounds. Based on the lists generated by the four tests, a consensus list that consisted of compounds identified as the top ten hits in at least three out of the four tests was created. This method is termed the consensus method in this study.

4.3.9.3 Analysis by correlating compounds with bioactivity

The second MVDA method was based on the assumption that the higher the concentration of the bioactive compound, the more biologically active is the extract or fraction. To identify bioactive compounds, a correlation was performed using Microsoft Excel on the concentrations and biological activities in the various extracts and fractions for each compound. The top ten compounds with the highest correlation coefficients were identified as potential bioactive compounds. This method is termed the correlation method in this study.

For both methods stated above, noise or column bleeds, simple sugars, compounds that are not present in at least four out of the six samples, and compounds in inactive extracts and fractions were removed. The compounds were removed by deleting the data for these compounds and other information (concentration of the compound) from the combined compound list manually. This list was then used for analysis by the different methods

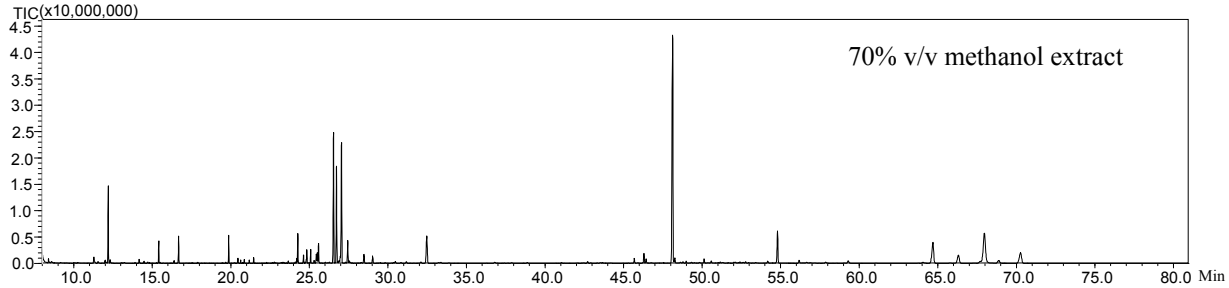
4.4 Results and discussion

4.4.1 Preliminary development of the MVDA method

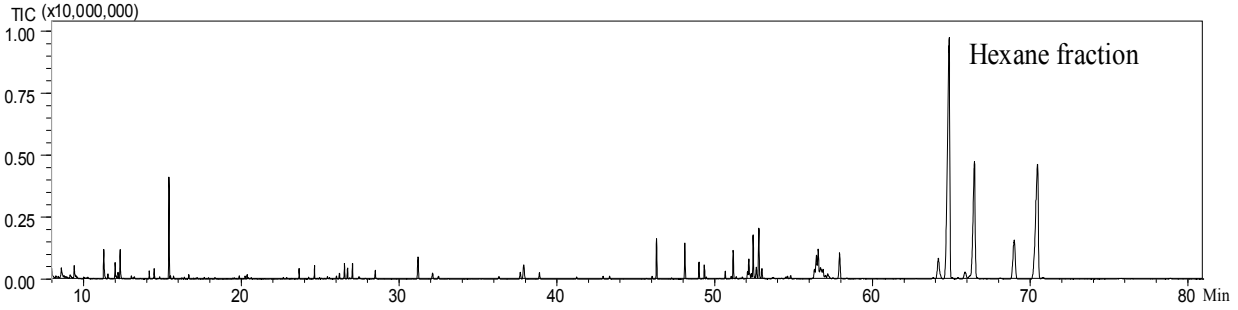
The GC-MS data of the derivatised crude extract and fractions obtained (Figure 4.1) were directly analysed by GeneSpring MS. No prior identification of the metabolites was done.

The results were as expected from a non-polar GC column. Non-polar compounds were eluted at longer retention times as can be seen from the non-polar fractions (Figure 4.1A to C), while more polar compounds were eluted earlier (Figure 4.1D and E). It can be observed that similar compounds of the 70% v/v methanol extract from nonderivatised samples (Figure 3.2A) were eluted earlier in the derivatised samples (Figure 4.1A). This is due to the effect of the derivatisation, which increases the volatility of the compounds, causing them to be eluted earlier.

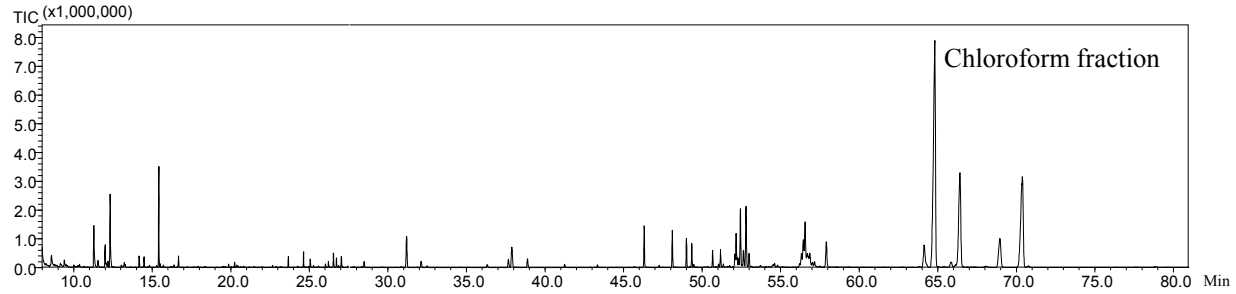
(A)


TIC (x10,000,000)
70% v/v methanol extract
Min

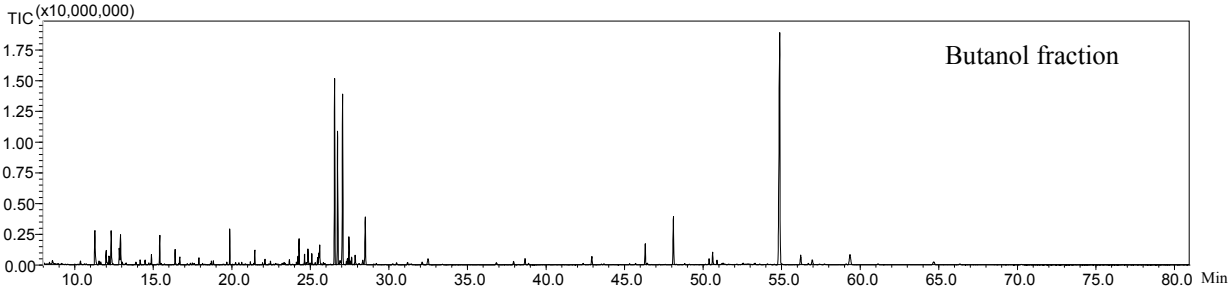
(B)


TIC (x10,000,000)
Hexane fraction
Min

(C)


TIC (x1,000,000)
Chloroform fraction
Min

(D)


TIC (x10,000,000)
Butanol fraction
Min

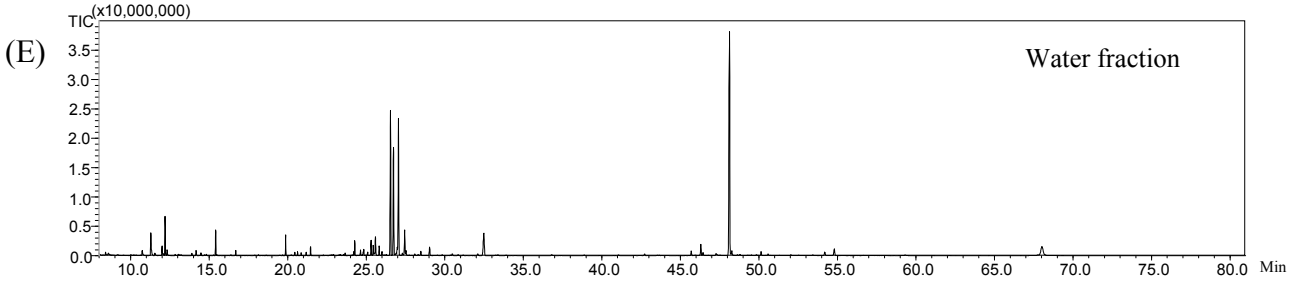


Figure 4.1 Typical gas chromatograms of derivatised (A) 70% v/v methanol extract, (B) hexane fraction, (C) chloroform fraction, (D) butanol fraction, (E) water fraction of *A. elliptica* 70% v/v methanol extract.

4.4.1.1 PCA analysis of all extracts and fractions

In this work, the chromatograms were first classified according to their identity, namely the 70% v/v methanol extract and the four different fractions, hexane, chloroform, butanol and water fraction. Figure 4.2 shows a PCA plot of the chromatograms of the different extracts. The clustering of the extract or fractions was based on the similarity of compounds present in the individual extract or fractions. Extract or fractions with closely similar compounds will cluster closer to each other. It can be seen that there was good separation of the different fractions, except the hexane (dark blue spots) and chloroform (yellow spots) fractions. This implied presence of similar compounds in the hexane and chloroform fractions. The water fraction (orange spots) and the 70% v/v methanol extract (purple spots) are clustered in separate groups, yet closely together. This shows that the majority of the compounds detected in the 70% v/v methanol extract by GC-MS consisted of polar compounds, as can also be seen from Figure 4.1a and 4.1e.

A PCA plot of the extract and fractions labelled by their different platelet aggregating activity is shown in Figure 4.3. The extract and fractions with antiplatelet activity was clearly separated from that with pro-aggregating activity. All extracts and fractions except the water fraction showed antiplatelet activity. However as the water fraction is closely clustered with the 70% v/v methanol extract which is antiplatelet, this implies the presence of strongly active non-polar compounds in the 70% v/v methanol extract which confer it its activity. Other non-polar fractions, i.e. the hexane, chloroform and butanol fractions showed antiplatelet activity (Figure 3.10).

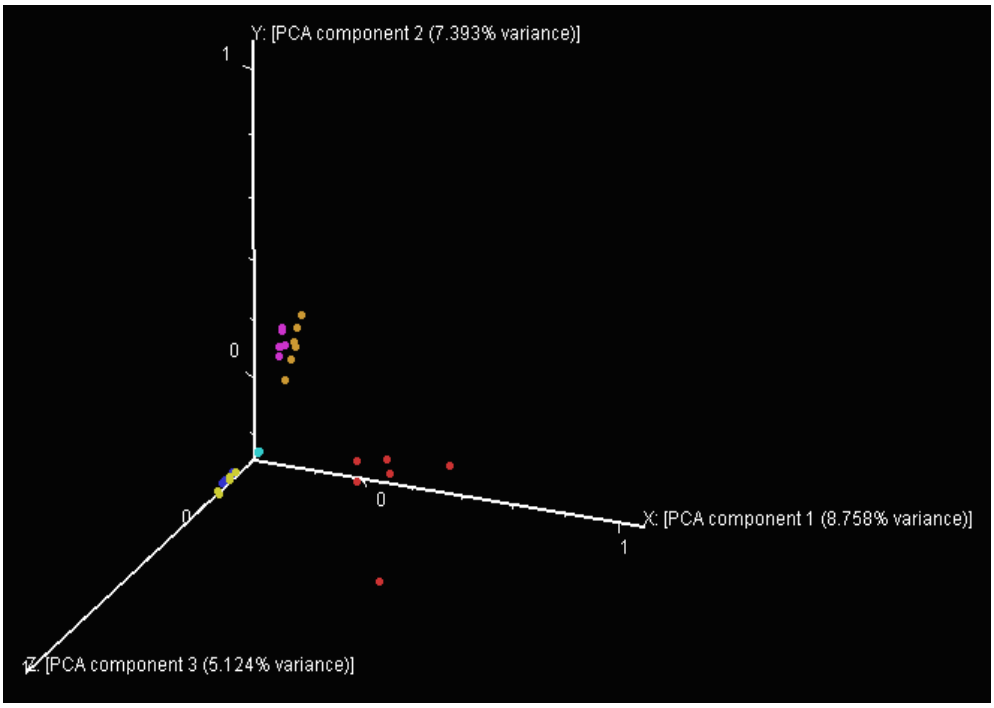


Figure 4.2 PCA analysis of chromatograms of the crude extracts and its four fractions. The PCA plot shows good separation of the 70% v/v methanol extract (●), the hexane fraction (●), chloroform fraction (●), butanol fraction (●), water fraction (●) and control (●) respectively. (n=6)

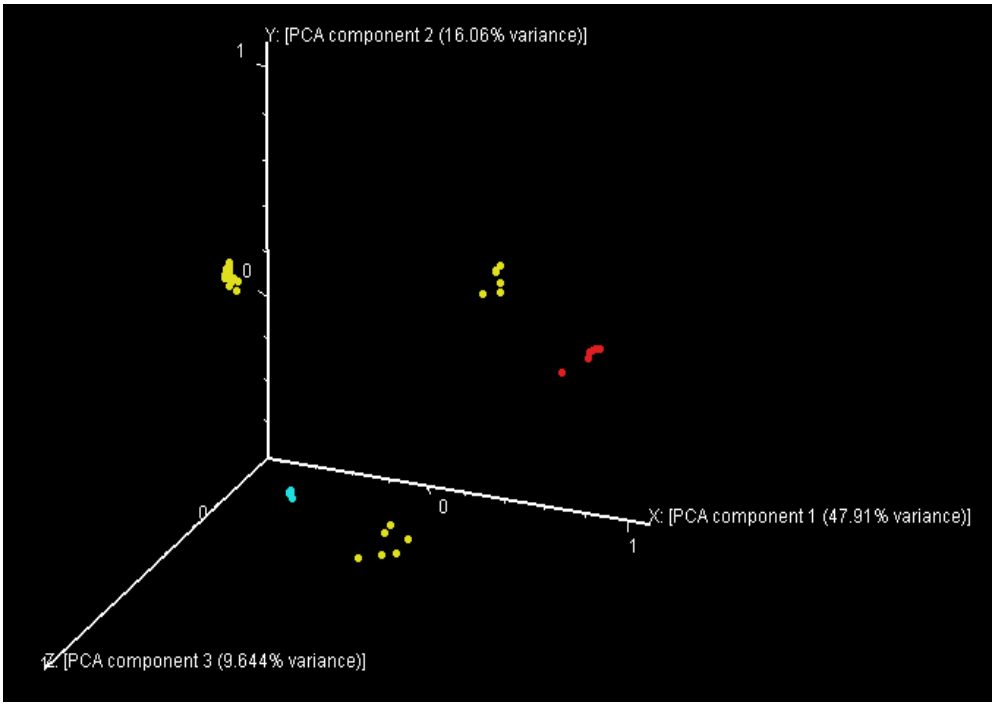


Figure 4.3 PCA analysis of chromatograms based on the extracts' platelet aggregating activity. Yellow spots (●) represent antiplatelet activity and red spots (●) represent and pro-aggregating activity. Light blue spots (●) represent controls.

A PCA plot of the extracts labelled by their plasma coagulation activity in affecting PT is shown in Figure 4.4. Procoagulation was seen for the crude extract, butanol and water fractions. The chloroform fraction showed no activity while the hexane fraction showed anticoagulant activity. This implied the presence of compounds which were able to prolong PT only in the non-polar fractions.

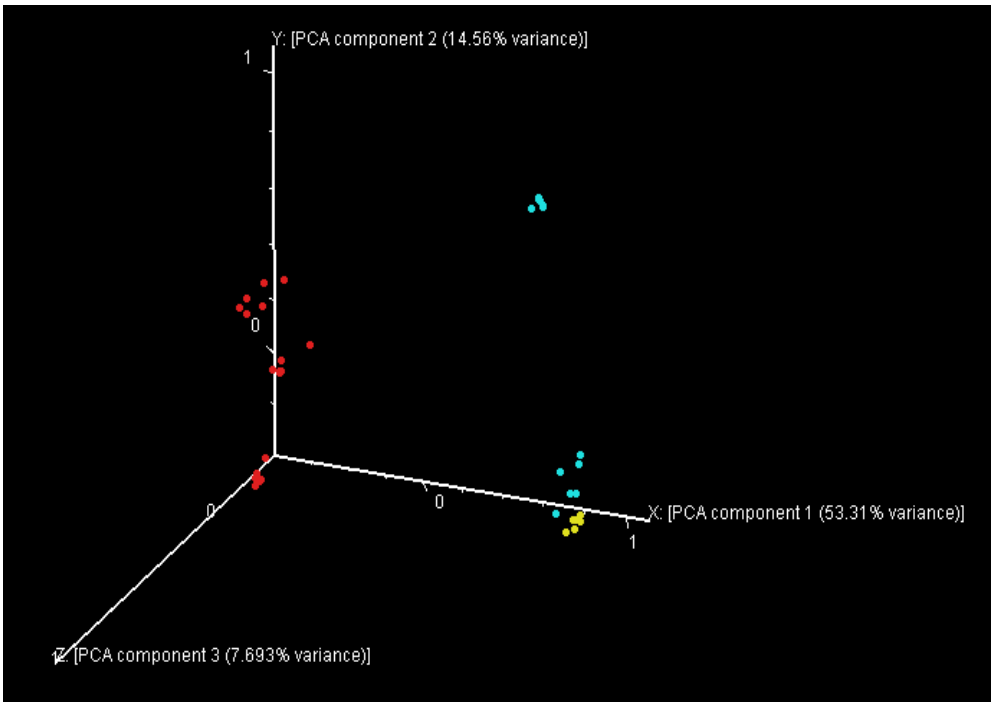


Figure 4.4 PCA analysis of chromatograms based on the extract's activity in affecting PT. Yellow (●) and red (●) spots represent anticoagulation and procoagulation respectively. Light blue spots (●) represent controls and extracts with no effect on PT.

A PCA plot of the extract and fractions labelled by their anticoagulant activity in prolonging aPTT is shown in Figure 4.5. Strong anticoagulant activity was seen in the 70% v/v methanol extract, hexane fraction and chloroform fraction, while weak activity was observed in the butanol and water fractions. As before, the extract and fractions showing strong activity was clustered distinctly from those exhibiting weak activity. As the water fraction is closely clustered with the 70% v/v methanol extract, some compounds in the 70% v/v methanol extract existing in low concentrations could have contributed to the stronger activity. However because the 70% v/v methanol extract contained a wider variety of constituents, there may be synergistic effects by the different constituents,

leading to stronger activity. Further work should be done to investigate this phenomenon.

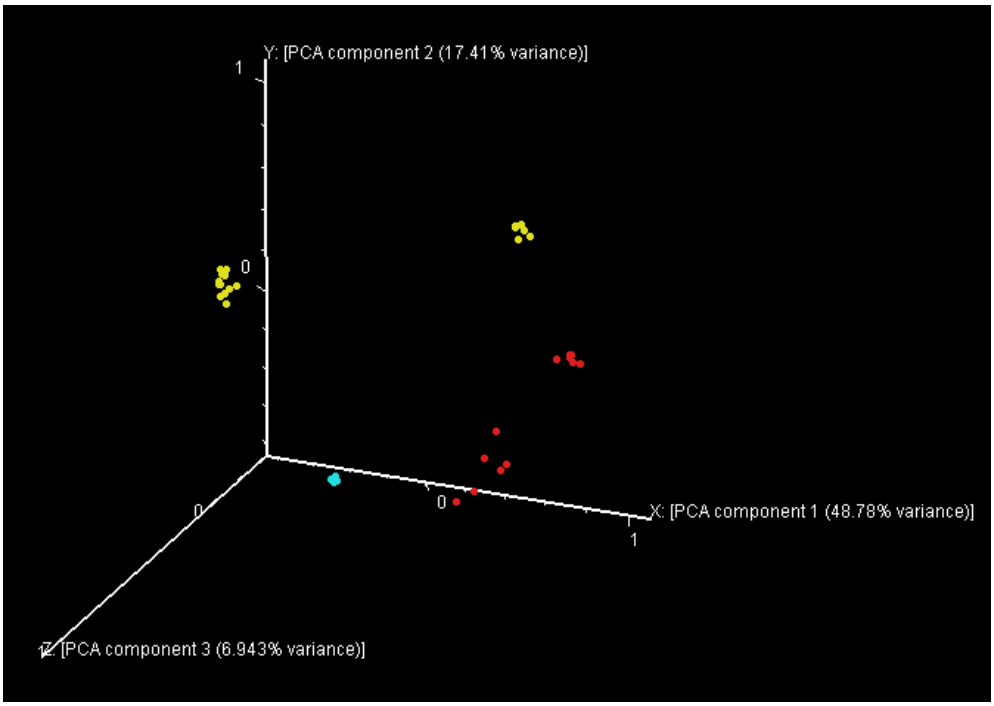


Figure 4.5 PCA analysis of chromatograms based on the extracts' anticoagulant activity in prolonging aPTT. Yellow (●) and red (●) spots represent strong ($p < 0.01$ and $p < 0.001$) and weak ($p < 0.05$) activity respectively. Light blue spots (●) represent controls.

4.4.1.2 Prediction of compounds with effects on platelet aggregation

In total, 8636 masses (or referred to as a particular compound in the extract) were identified by GeneSpring MS. The activities studied and tagged with the chromatograms are "antiplatelet", "proaggregating", "anticoagulant" (against PT or aPTT) and "procoagulant" (against PT). By comparing the biggest fold changes of a certain mass from chromatograms with the activity to those that are inactive, the compounds with the highest concentrations in the chromatograms are

suspected to possess the particular activity. This allows a reduction in the number of compounds to be studied, but excludes those compounds that are highly active but in very low concentrations. Due to the large number of hits given by GeneSpring, only the top five hits with the largest fold changes and good MS spectral library matches (80% and above using the Wiley Mass Spectral Library) will be discussed. The putative compound identities discussed below were given only by the Wiley Mass Spectral Library, hence further confirmation studies would be needed.

The top five hits for compounds predicted with antiplatelet activities are listed in Table 4.1. Of the compounds identified to have antiplatelet activity, α-amyrin and β-amyrin were among the first five hits with biggest fold changes when comparing antiplatelet extracts against proaggregating extracts in the compound list after PCA analysis (Table 4.1). This output by the software is consistent with previous reports of the antiplatelet effects of α- and β -amyrin (Aragao et al., 2007; Ching et al., 2010; Medeiros et al., 2002).

Table 4.1 List of putative compounds predicted with antiplatelet and anticoagulation (prolong aPTT) activities.

Top 5 putative compounds	Reported platelet aggregation effects	Reported plasma coagulation effects	References
α-amyrin	Antiplatelet	Antithrombin	Aragao et al., 2007; Ching et al., 2010; Medeiros et al., 2002
β-amyrin	Antiplatelet	Antithrombin	Aragao et al., 2007; Ching et al., 2010; Medeiros et al., 2002
tetradecanoic acid	No effect	Procoagulation	Temme et al., 1998; Tholstrup et al., 1994
linoleic acid	No effect	No reports	Li et al., 2006
linolenic acid	Proaggregation	Procoagulation/No effect	Hoak et al., 1967; Kelley et al., 1993

Other filtered compounds with high match factors identified by the Wiley Mass Spectral Library include fatty acids such as tetradecanoic acid (myristic acid), 9,12-octadecadienoic acid (linoleic acid) and α-linolenic acid. Different forms of linoleic acids were reported to have differing effects on platelet aggregation. Li et al., 2006 reported that 10-*trans*, 12-*cis*-conjugated linoleic acid, 9-*trans*, 11*trans*-conjugated linoleic acid and conjugated nonadecadienoic acid are antiplatelet. 9-*cis*, 11-*trans*-conjugated linoleic acid has moderate antiplatelet activity and linoleic acid and 9-*cis*, 11-*cis*-conjugated linoleic acid have no effect on platelet aggregation. Tetradecanoic acid was also reported to have no effects on whole blood aggregation (Temme et al., 1998). On the other hand, linolenic acid was reported to cause increased platelet aggregation as well as plasma coagulation (Hoak et al., 1967). While some compounds which are known to cause platelet aggregation were detected, it was possible that the presence of highly active compounds, α-amyrin and β-amyrin, could have contributed to the overall antiplatelet activity of the extracts.

Compared to the list of predicted compounds with antiplatelet activity, the list of compounds with proaggregant activity are mostly carbohydrates such as galactonic acid, sucrose, α-D-galactoside and α-D-galactopyranose. Carbohydrates like sucrose is very polar and are found in the water fraction. As sugars are not heat stable, the numerous hits indicating presence of simple sugars could imply that they were fragmented from oligosaccharides or other compounds. Polysaccharides are well studied for their anticoagulant activities (Bray et al., 1989; Mourao, 2004; Fonseca et al., 2008). Heparin itself is a sulfated

glycosaminoglycan. Heparin-mimetics being synthesised are also oligosaccharides (Petitou et al., 1999; Herault et al., 2002). However, it had been reported that sugar moieties of pennogenin glycosides are important for inducing platelet aggregation (Fu et al., 2008). Another compound listed was nonadecanoic acid which did not have any reported effects on platelet aggregation.

4.4.1.3 Prediction of compounds with effects on plasma coagulation

Prediction of compounds with anticoagulant activity (active in prolonging PT) was performed by filtering the compounds with the first five hits with biggest fold changes when comparing extracts/fractions with anticoagulant activity against those with procoagulant activity. Compounds predicted to prolong PT are listed in Table 4.2.

β- amyrin is among the compounds predicted to prolong PT. Although both α- and β- amyrin have been reported to exhibit antithrombin activity *in vitro* (Medeiros et al., 2002), there are no reports on the amyrins affecting plasma coagulation. However there are no reported effects of hexanedioic acid, methylethylketone and tetracosanoic acid on plasma coagulation.

Table 4.2 List of putative compounds with anticoagulation (prolong PT) activity.

Top 5 putative compounds	Reported platelet aggregation effects	Reported plasma coagulation effects	References
β-amyrin	Antiplatelet	No reports	Ching et al., 2010
hexanedioic acid	No reports	No reports	NIL
methylethylketone	No reports	No reports	NIL
tetracosanoic acid	No reports	No reports	NIL
linolenic acid	Proaggregation	Procoagulation/No effect	Hoak et al., 1967; Kelley et al., 1993

Compounds predicted by the software to shorten PT (procoagulation) consisted of maltose, α-D-galactoside, 2(3H)-furanone, citric acid and D-ribonic acid. As discussed in the previous section, polysaccharides are usually known to be anticoagulant. Such metabolites identified by the software could have been fragmented from a larger moiety, or exist in high concentrations, leading to masking of the presence of other active components. There are no reported activities related to plasma coagulation for 2(3H)-furanone, citric acid and D-ribonic acid.

Prediction of compounds with anticoagulant activity (active in prolonging aPTT) was performed by filtering the compounds with first five hits with biggest fold changes when comparing extracts/fractions with strong activity against those with weak activity (Table 4.1). As most of the extracts and fractions with anticoagulant activity overlap with those with antiplatelet activity, most of the

compounds identified that can prolong aPTT are similar to those detected with predicted antiplatelet effects (Table 4.1), including α- and β- amyrin.

Other compounds listed were 9,12-octadecadienoic acid, α-linolenic acid and tetradecanoic acid. Studies of dietary α-linolenic acid on humans showed no effect on the bleeding time, prothrombin time and partial prothrombin time for the subjects involved (Kelley et al., 1993). However, studies have not been conclusive so far. While Chan et al., 1993 also showed that the ratio of dietary α -linolenic acid to linoleic acid had no effect on bleeding time, Tohgi, 2004 reported a reduction in plasmin α2-plasmin inhibitor complex level, plasminogen activator inhibitor-1 activity, and thrombin antithrombin III complex level in type 2 diabetic patients when the ratio of dietary α -linolenic acid to linoleic acid is reduced. Tetradecanoic acid was reported to increase Factor VII coagulant activity (Tholstrup et al., 1994).

The preliminary results show that metabolomics is a promising method to predict bioactive components with antiplatelet and/or anticoagulant activities. The antiplatelet activities of α- and β –amyrin that were predicted by GeneSpring MS agreed with experimental results as well as reports from literature. However the disadvantage of the current method of filtering for bioactive compounds is that too many hits are obtained. It is preferable to have not more than 10 hits per activity in order for more targeted screening. More investigations have to be done to optimise the filtering of compounds by GeneSpring MS or by other softwares. In addition, the compounds with smaller fold changes should be explored.

With the results obtained from the optimization assay, some parameters were changed in the subsequent assay. Instead of only analyzing the *A. elliptica* 70% v/v methanol extract, its hexane, chloroform, butanol and water fraction, additionally ethanol and water extracts were obtained and incorporated in the assay. The chloroform fraction however was removed. This was because the compounds in the chloroform and hexane fractions were largely similar as can be seen from their chromatograms (Figure 4.1 b and c). As the gas chromatograms of the fractions were complex and difficult to compare by visual inspection, MVDA was employed to analyse the chromatograms. The two fractions were found to be clustered with each other (Figure 4.2), implying that an extra fractionation step using chloroform was not essential. Moreover, the antiplatelet activities of the hexane and chloroform fractions were not significantly different from each other (Figure 3.10). As a result, it was decided that the chloroform fraction was to be removed from the subsequent assay. The water and ethanol extracts were added as these two solvents were commonly used in the preparation of traditional herbal concoctions. Thus it would be interesting to investigate the bioactive compounds in these two extracts as well.

4.4.2 Further development of the MVDA method

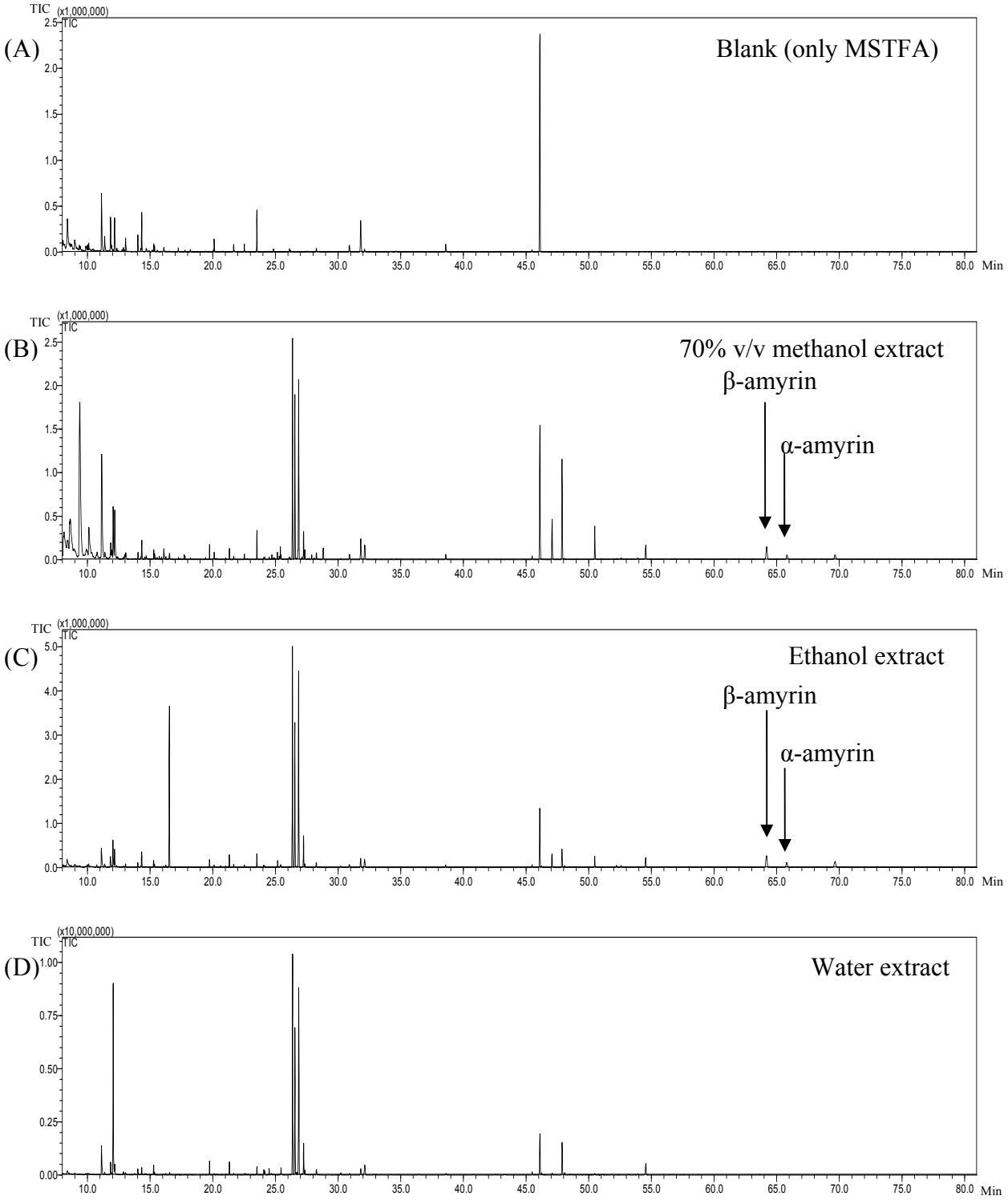
4.4.2.1 Validation of GC-MS method for MVDA study

The GC-MS method for performing the MVDA was validated for its intermediate precision. The intra-day variation was assessed by determining the RSDs of the retention times and peak areas for five compounds. They were 0.01%

to 0.05% and 5.3% to 14.7% respectively. The inter-day variation was also assessed. The RSDs of the retention times and peak areas were 0.01% to 0.03% and 10.7% to 14.5% respectively. The method was deemed precise as the RSDs of retention times and peak areas were lower than 15% for five compounds (Pasikanti et al., 2008).

4.4.2.2 GC-MS analysis of all extracts and fractions

The gas chromatogram of the crude extract and fractions obtained are shown in Figure 4.6. Preliminary identification of the compounds present was carried out using the autolibrary search function of the Wiley Mass Spectral Library. From the chromatograms, a combined compound list, which included all the different compounds and their peak areas in all extracts, fractions and controls, were compiled. 855 different compounds were obtained. This compound list was later used for MVDA. The hexane fraction contained more compounds with longer retention times, followed by the butanol fraction and lastly, the water fraction. These compounds were the non-polar compounds that partitioned into the more non-polar solvents.


TIC (x1,000,000)
TIC
(A)
Blank (only MSTFA)
Min
TIC (x1,000,000)
TIC
(B)
70% v/v methanol extract
β-amyrin
α-amyrin
Min
TIC (x1,000,000)
TIC
(C)
Ethanol extract
β-amyrin
α-amyrin
Min
TIC (x10,000,000)
TIC
(D)
Water extract
Min

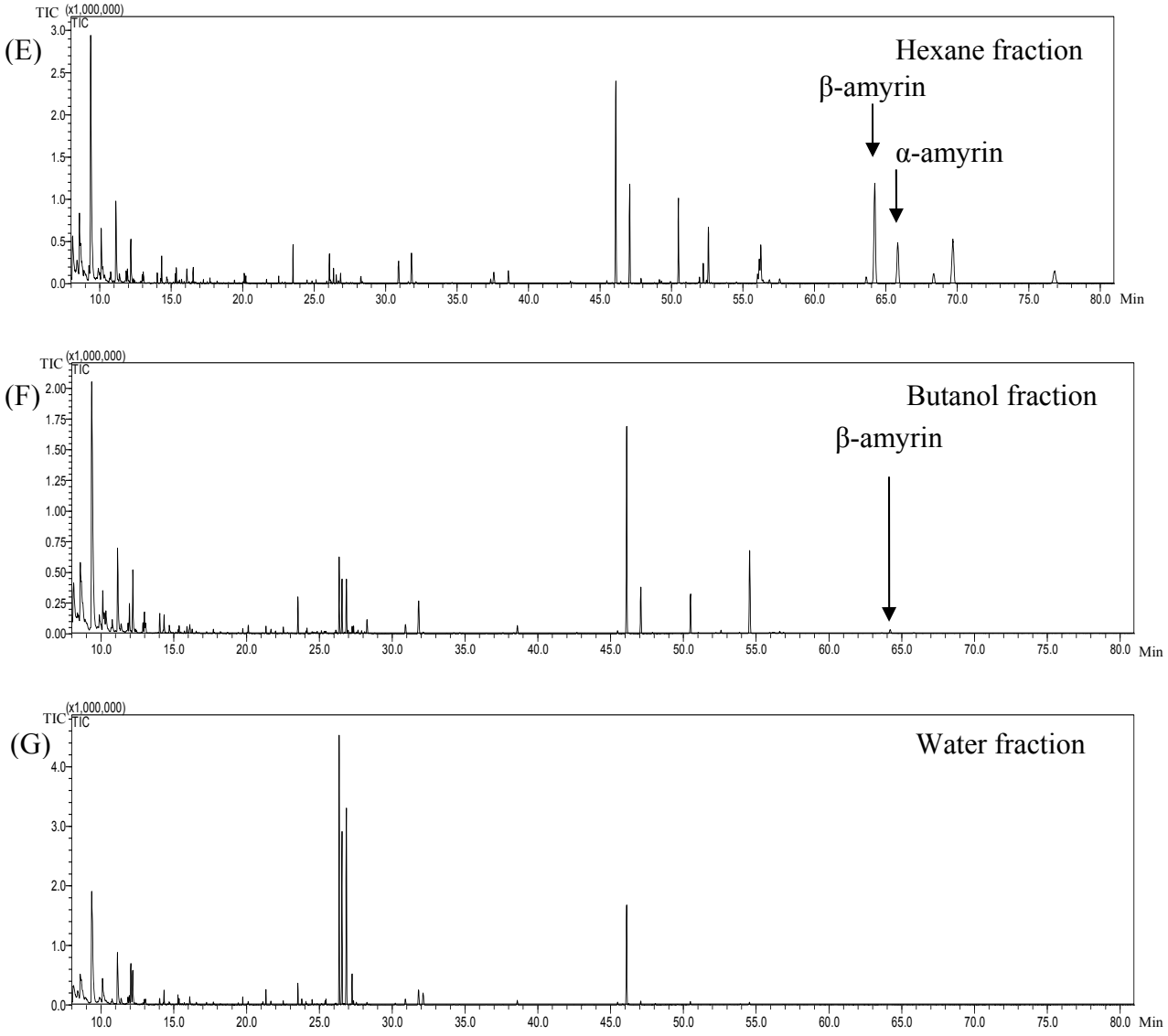


Figure 4.6 Typical gas chromatograms of (A) blank, (B) 70% v/v methanol extract, (C) ethanol extract, (D) water extract, (E) hexane fraction, (F) butanol fraction and (G) water fraction.

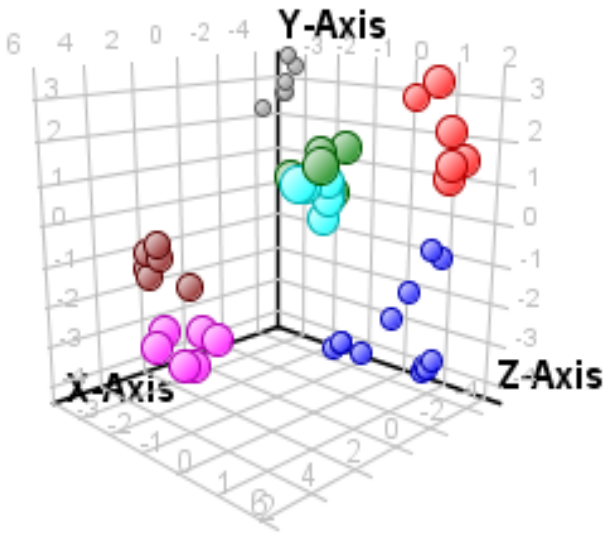
4.4.2.3 PCA and PLS-DA analysis of the extracts and fractions

Analysis of the chromatograms was first performed using PCA and PLS-DA on MPP. MPP is an upgraded version of GeneSpring MS, both programmes from Agilent Technologies.

It can be seen from Figure 4.7 that the different extracts and fractions showed clear clustering from each other in both the PCA and PLS-DA plots. Only the 70% v/v methanol extract and its water fraction were clustered closely together. The close clustering of the 70% v/v methanol extract and its water fraction is also seen in the preliminary study in Figure 4.2. This implied that the majority of the compounds present in the 70% v/v methanol extract consisted of polar compounds which are also present in the water fraction. While the 70% v/v methanol extract and water fraction show close similarity in compounds, they show opposing platelet aggregating activities. At a concentration of 0.2 mg ml^{-1}, the 70% v/v methanol extract showed antiplatelet activity. At the same concentration, the water fraction enhanced platelet aggregation. This implied that the antiplatelet activity is attributed to the non-polar compounds found in the 70% v/v methanol extract but not its water fraction.

From Figure 4.7, it can also be seen that the 70% v/v methanol extract, the water extract, and the ethanol extract shows distinct clustering from each other. This implied that there are compounds that are unique to each of the extracts, thus giving rise to the spatial differences as seen in the PCA and PLS-DA plots.

(A)



(B)

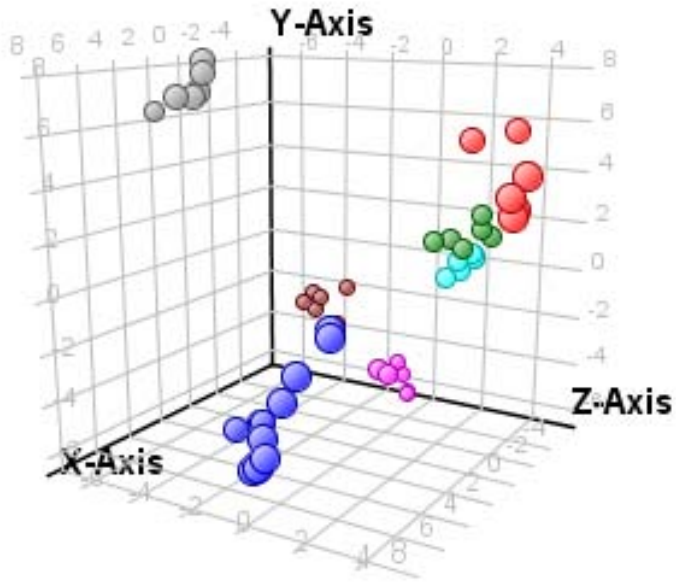


Figure 4.7 (A) PCA scatter plot (B) PLS-DA scatter plot of the chromatograms showing distinct clustering of the different extracts and fractions. ●—blank (MSTFA); ●--70% v/v methanol extract; ●-- ethanol extract; ●--water extract; ●-- hexane fraction; ●--butanol fraction; ●-- water fraction.

4.4.2.4 Antiplatelet activities of *A. elliptica* crude extract and its fractions

The percentage inhibition of platelet aggregation due to *A. elliptica* extracts and fractions as compared to the control are shown in Figure 4.8.

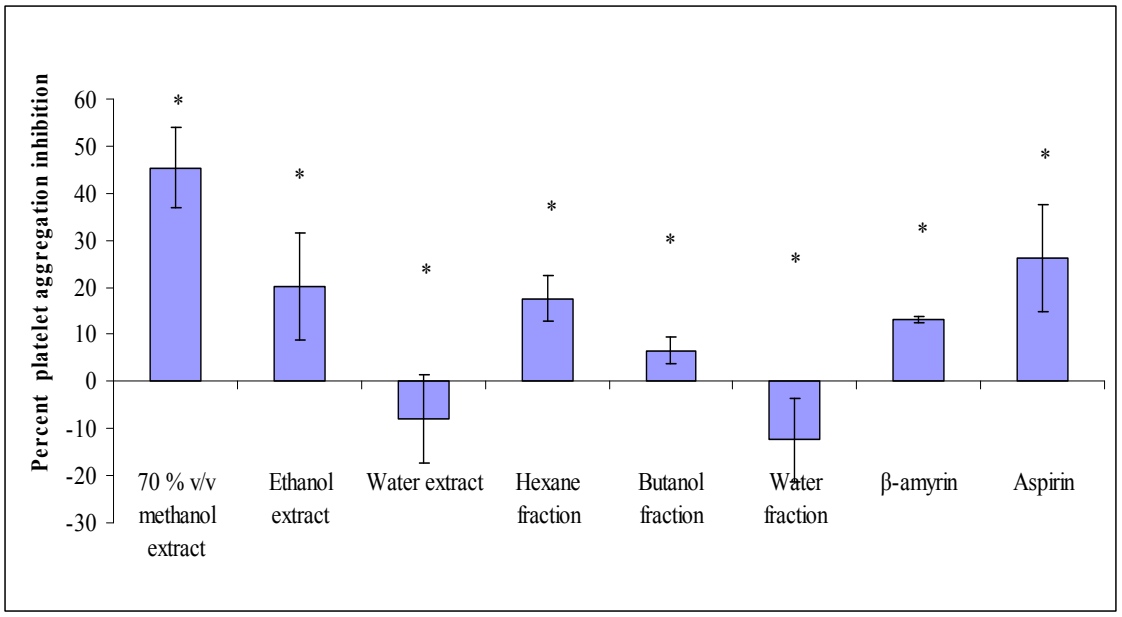


Figure 4.8 Percent inhibition of platelet aggregation by different *A. elliptica* extracts (0.2 mg ml^{-1}) and fractions (0.2 mg ml^{-1}), β-amyrin (10 µg ml^{-1}) and aspirin (10 µg ml^{-1}) compared to control; n = 6 except for aspirin where n = 3; * $p < 0.001$

At 0.2 mg ml^{-1}, the 70% v/v methanol extract, ethanol extract, hexane fraction and butanol fraction inhibited platelet aggregation significantly as compared to the control ($p < 0.001$). The percentage inhibitions ranged from 6% to 45%, but they did not differ significantly from one another ($p > 0.05$). Aspirin, a positive control, produced a significant platelet aggregation inhibition of 26% at 10 µg ml^{-1} as compared to the control ($p < 0.001$).

The water extract and water fraction showed opposing activities as compared to the other extracts and fractions. Platelet aggregation was

significantly enhanced at 0.2 mg ml^{-1} as compared to the control ($p < 0.001$). The proaggregating activity of the water fraction was consistent with that reported in Section 3.2.4.3. But the water extract showed antiplatelet activity instead in Section 3.2.4.3 at the same concentration. However, it was observed earlier that the water extract showed different activities at different concentrations. Antiplatelet activity was observed to decrease at increasing concentrations, and the compounds with antiplatelet activities in this batch of extract could be different as compared to previously.

The various extracts and fractions had different effects on collagen-induced platelet aggregation as they contained different compounds. The 70% v/v methanol extract, ethanol extract, hexane fraction and butanol fraction might contain antiplatelet compounds which were absent in the water extract and water fraction. This implied that the compounds responsible for the antiplatelet effects could be non-polar in nature. These non-polar compounds would correspond to peaks with longer retention times in the gas chromatograms (Figure 4.6).

4.4.2.5 Effects of *A. elliptica* crude extract and its fractions on plasma coagulation

The plasma coagulation assay provided information on the anticoagulant effects *A. elliptica* extracts, fractions and potential bioactive compounds.

4.4.2.5.1 Effects of extracts and fractions on PT

The effects of *A. elliptica* extracts and fractions on PT as compared to the control are shown in Figure 4.9.

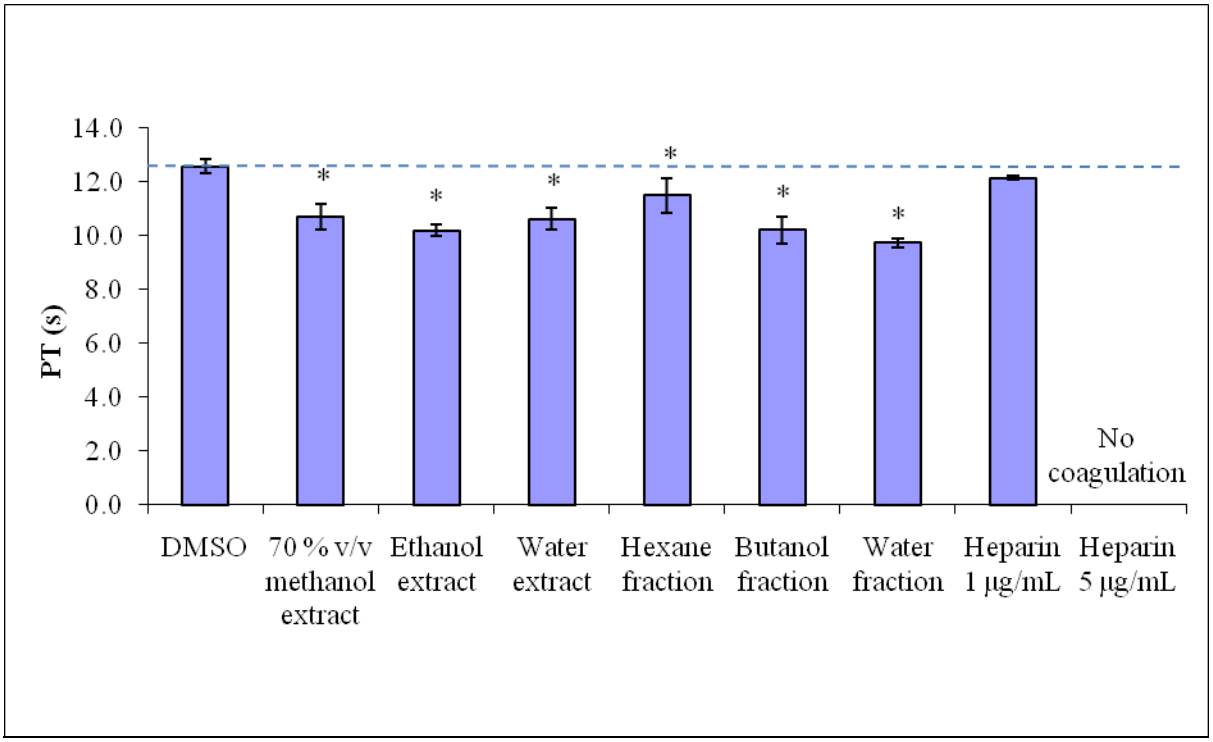


Figure 4.9 Effects of *A. elliptica* extracts (0.2 mg ml^{-1}), fractions (0.2 mg ml^{-1}) and heparin (1 µg ml^{-1} and 5 µg ml^{-1}) on PT compared to control; n = 6 except heparin where n = 3; * $p < 0.001$

All extracts and fractions shortened PT significantly at 0.2 mg ml^{-1} as compared to the control ($p < 0.001$) and they are comparable to that reported in Section 3.4.2.3. The PT of the extracts and fractions did not differ significantly from one another ($p > 0.05$). Heparin at 1 µg ml^{-1} did not affect PT significantly as compared to the control ($p > 0.05$). However, at a higher concentration of 5µg ml^{-1}, heparin prolonged PT significantly and no coagulation was detected within 120 s.

As the extracts and fractions shortened PT, compounds that are able to enhance the extrinsic pathway of plasma coagulation might be present. Since their effects did not differ significantly from one other, these procoagulant compounds might be present at comparable concentrations in all the extracts and fractions. Heparin did not prolong PT at 1 μg ml^{-1}, but this is not unexpected as heparin prolongs aPTT rather than PT (Hirsh et al., 2001). However, it prolonged PT at 5 μg ml^{-1} as high concentrations of heparin may affect PT (Fenyvesi et al., 2002).

4.4.2.5.2 Effects of extracts and fractions on aPTT

The effects of *A. elliptica* extracts and fractions on aPTT as compared to the control are shown in Figure 4.10.

Other than the water fraction, all extracts and fractions were found to prolong aPTT significantly at 0.2 mg ml^{-1} as compared to the control ($p < 0.001$). These results are also comparable to those reported in Section 3.2.4.3. The aPTT ranged from 34.0 s to 41.8 s, and they did not differ significantly from one another ($p > 0.05$). Only the water fraction had no significant activity at 0.2 mg ml^{-1} as compared to the control ($p > 0.05$). Heparin, a positive control, prolonged aPTT significantly to 37.8 s at 1 μg ml^{-1} ($p < 0.001$). It resulted in no coagulation detected within 190 s at 5 μg ml^{-1}.

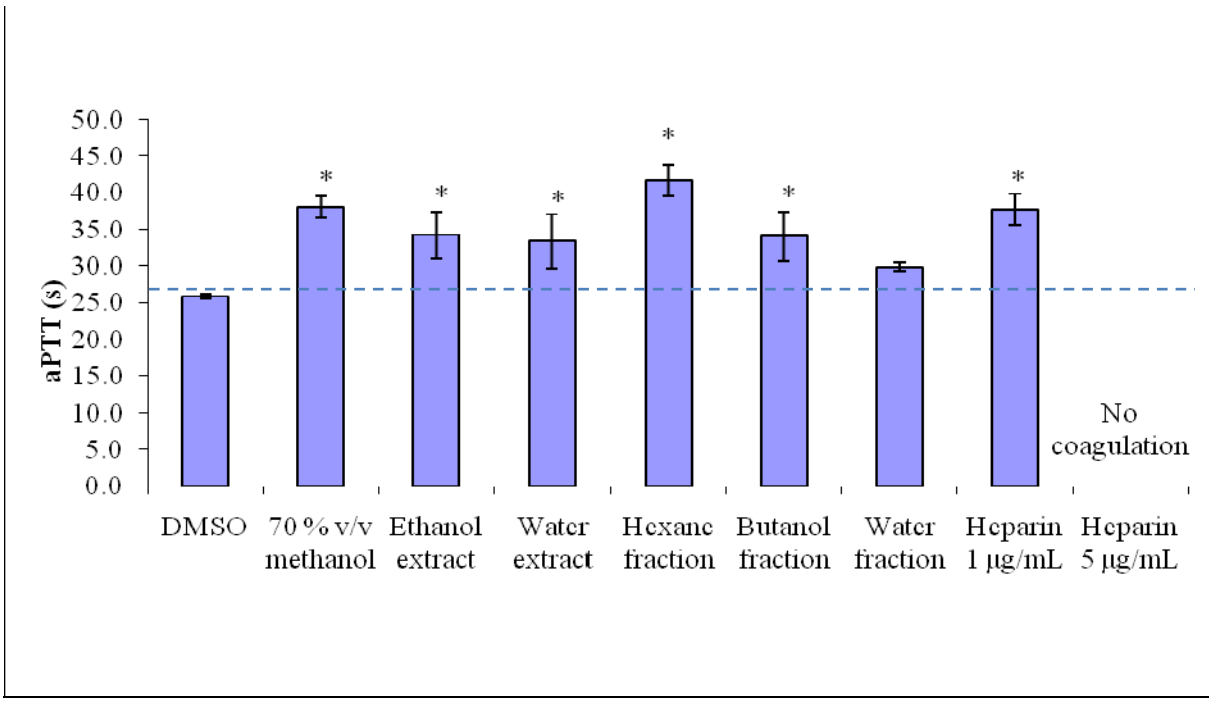


Figure 4.10 Effects of *A. elliptica* extracts (0.2 mg ml^{-1}), fractions derived from the 70% v/v methanol extract (0.2 mg ml^{-1}) and heparin (1 µg ml^{-1} and 5 µg ml^{-1}) on aPTT compared to control; n = 6 except heparin where n = 3; * $p < 0.001$

The extracts and fractions exhibited anticoagulant effects via the intrinsic pathway, implying that they contain anticoagulant compounds that prolonged aPTT. These anticoagulant compounds were likely to be in higher concentrations in all the extracts and fractions except the water fraction, which did not show significant prolongation of the aPTT.

4.4.2.6 Prediction of potential antiplatelet compounds by MVDA

The platelet aggregation assay showed that the 70% v/v methanol extract, ethanol extract, hexane fraction and butanol fraction were antiplatelet, while the water extract and water fraction enhanced platelet aggregation (Figure 4.8). Assuming that the compounds in the water extract and water fraction did not have

antiplatelet activity, the compounds from the water extract and water fraction were removed manually during data pre-processing. Noise, sugars and compounds that were not present in at least four out of the six samples were also removed. A consensus list shown in Table 4.3 was produced after performing the four tests using the two software: OPLS and PLS-DA using SIMCA-P+ and Chi-Squared weighting and InfoGain weighting using RapidMiner. Seven compounds were identified in at least three out of the four tests. All the compound names were exact quotes from the Wiley Mass Spectral Library Registry 7.

Table 4.3 Consensus list of potential antiplatelet compounds (compounds identified as the top ten hits in at least three of the four tests)

No.	Potential antiplatelet compound	Extract/fraction compound is present in*	No. of tests the compound appeared as top 10 hits	Match factor with Wiley MS Library
1	β-amyrin trimethylsilyl ether	BF, EE, HF, ME	4	98
2	α-amyrin	EE, HF, ME	4	98
3	Silane, [[(3.beta.)-lanosta-8,24-dien-3-yl]oxy]trimethyl- (CAS) lanosterol trimethylsilyl ether	HF	4	99
4	9,14-Bis(4-cyanophenyl)benzo[b]triphenylene	BF, EE, HF	4	97
5	Phosphine, tris[4-(trimethylsilyl)phenyl]- (CAS)	HF	4	99
6	4'-tert-butyl-3,5-bis(4-tert-butylphenyl)biphenyl-2,2'-diol	BF, HF	3	84
7	Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone	BF	3	98

*BF, butanol fraction; EE, ethanol extract; HF, hexane fraction; ME, 70% v/v methanol extract

Table 4.4 shows a list of top ten compounds with the highest correlation coefficients obtained using the correlation method. Since α-amyrin and β-amyrin which were known to be antiplatelet (Aragao et al., 2007; Ching et al., 2010) were

common in both results, the MVDA methods were effective in identifying them as antiplatelet. The consensus method could identify α-amyrin and β-amyrin in all the four tests. The correlation method also identified α-amyrin and β-amyrin as the fourth and tenth potential antiplatelet compounds respectively. Further investigations should be done to verify the activity of other compounds listed with potential antiplatelet activity.

Table 4.4 Correlation list of potential antiplatelet compounds (top ten compounds with the highest correlation coefficients)

No.	Potential antiplatelet compound	Extract/fraction compound is present in*	Correlation coefficient	Match factor with Wiley MS Library
1	Octadecanoic acid,2,3-bis[(trimethylsilyl)oxy]propyl ester (CAS) 1-monostearin-DITMS	BF, EE, HF, ME, WE, WF	0.499	99
2	Mercaptoacetic acid, bis(trimethylsilyl)-	ME	0.444	99
3	3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]-, (R*,S*)- (CAS) Erythritol, 1,2,3,4-tetrakis-O-(trimethylsilyl)-	BF, ME	0.390	99
4	α-amyrin	EE, HF, ME	0.364	98
5	Bis-O-trimethylsilyl-palmitinic acid-glycerin-(1)-monoester	ME, WF	0.276	99
6	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester (CAS) 1-monopalmitin-DITMS	BF, EE	0.263	99
7	2-Isopropoxy-Propanenitrile	EE, HF, ME	0.250	99
8	Dodecanoic acid, trimethylsilyl ester (CAS) lauric acid-MONOTMS	HF	0.250	99
9	Adenine, Bis-N-(trimethylsilyl)-	ME,WE	0.248	91
10	β-amyrin trimethylsilyl ether	BF, EE, HF, ME	0.244	98

*BF, butanol fraction; EE, ethanol extract; HF, hexane fraction; ME, 70% v/v methanol extract; WE, water extract; WF, water fraction

According to literature, 10 phytoconstituents have so far been reported in *A. elliptica*. They are α-amyrin (Ahmad et al., 1977), β-amyrin (Ahmad et al., 1977; Chow et al., 1991), bauerenol (Ahmad et al., 1977; Chow et al., 1991), bergenin (Liu et al., 1993), isorhamnetin (Phadungkit and Luanratana, 2006), quercetin (Phadungkit and Luanratana, 2006), rapanone (Chow et al., 1991), syringic acid (Phadungkit and Luanratana, 2006), 5-(*Z*-heptadec-4'-enyl)resorcinol (Jalil et al., 2004) and 5-pentadecylresorcinol (Jalil et al., 2004). Of these 10 compounds, 6 of them (α-amyrin (Aragao et al., 2007; Mazura et al., 2007; Ching et al., 2010), β-amyrin (Aragao et al., 2007; Ching et al., 2010), quercetin (Tzeng et al., 1991; Kobzar et al., 2005; Mazura et al., 2007), syringic acid (Yang et al., 2002), 5-(*Z*-heptadec-4'-enyl)resorcinol (Jalil et al., 2004) and 5-pentadecylresorcinol (Jalil et al., 2004) have been reported to have antiplatelet activities. As there are 2 positive predictions, this equates to a precision of 28.6% and 20% for the consensus method and correlation method respectively. The recall for both was 33% since 2 out of the 6 known antiplatelet compounds were detected in *A. elliptica*.

In this study, four analytical methods were used to construct a consensus list of predicted bioactive compounds. The reason for using multiple methods is because in extracts and fractions with many different compounds, it is inevitable that an analytical method may wrongly identify some inactive compounds as bioactive. However, these errors are expected to be different for each analytical method because each uses a different algorithm. For example, OPLS and PLS-DA differs in the manner in which they treat classorthogonal variation (Bylesjö et al.,

2006). This creates a slightly different list of potential bioactive compounds from both methods, even though both methods are extensions of the partial leastsquares (PLS) methodology. Thus, generating a consensus list of bioactive compounds from the lists of potential bioactive compounds produced by the four analytical methods should reduce the chance of identifying inactive compounds as bioactive.

4.4.2.7 Prediction of anticoagulant compounds using MVDA

The plasma coagulation assay showed that the various extracts and fractions shortened PT (Figure 4.9), and thus it was assumed there were no anticoagulant compounds that acted on the extrinsic pathway. On the other hand, all extracts and fractions, with the exception of the water fraction, prolonged aPTT significantly (Figure 4.10). Hence, anticoagulant compounds that acted on the intrinsic pathway could be present in the relevant extracts and fractions. For this test, only noise, sugars and compounds that were not present in at least four out of the six samples were removed during pre-processing. This gave a consensus list shown in Table 4.5. Six compounds were identified to have anticoagulant activity in at least three out of the four tests. A correlation list shown in Table 4.6 was obtained on performing the correlation method.

Table 4.5 Consensus list of potential anticoagulant compounds (compounds identified as the top ten hits in at least three of the four tests).

No.	Potential anticoagulant compound	No. of tests the compound appeared as top 10 hits	Match factor with Wiley Mass Spectral Library
1	Octadecanoic acid,2,3-bis[(trimethylsilyl)oxy]propyl ester (CAS) 1-monostearin-DITMS	4	99
2	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester (CAS) malic acid 3TMS	4	99
3	Erythrose,)-methyloxim, tris-O-(trimethylsilyl)-	4	99
4	Acetamide, 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)-(CAS)N-methyl-N-trimethylsilyl trifluoroacetamide	3	99
5	Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone, D- (CAS) 2,3,5-tri-o-trimethylsilyl-xylono-1,4-lactone	3	97
6	Tetronicacid, tetrakis-O-(trimethylsilyl)-	3	99

Table 4.6 Correlation list of potential anticoagulant compounds (top ten compounds with the highest correlation coefficients).

No.	Potential anticoagulant compound	Correlation coefficient	Match factor with Wiley Mass Spectral Library
1	Tetradecanoic acid, trimethylsilyl ester (CAS) myristic acid-MONOTMS	0.661	90
2	Octadecanoic acid,2,3-bis[(trimethylsilyl)oxy]propyl ester (CAS) 1-monostearin-DITMS	0.660	99
3	Silane, [[(3.beta.)-lanosta-8,24-dien-3-yl]oxy]trimethyl-(CAS) lanosterol trimethylsilyl ether	0.648	94
4	.alpha.-Phenyl-.beta.-trimethylsiloxystyrene	0.647	96
5	ethyl 6-phenyl-2-(trifluoromethyl)-4-oxohex-5-enoate	0.628	
6	β-amyrin trimethylsilyl ether	0.610	98
7	Phosphine, tris[4-(trimethylsilyl)phenyl]- (CAS) Phosphine, tris[p-(trimethylsilyl)phenyl]-	0.577	99
8	6,6-dimethyl-2-[(trimethylsilyl)methyl]-4-oxo-2,3,4,5,6,7-hexahydrobenzofuran	0.540	98
9	N,O-bis(acetyl)-S-[2-(4-ethenylphenyl)-2-hydroxyethyl]-L-cysteine-methyl ester	0.536	95
10	2-Isopropoxy-Propanenitrile	0.499	96

Only octadecanoic acid,2,3-bis[(trimethylsilyl)oxy]propyl ester was common in both results. However, there were no reports on its anticoagulant effects. β-amyrin was identified by the correlation method to be anticoagulant although it was found not to be so in the plasma coagulation assay (Section 3.4.12). This prediction could be a false positive. One possible reason for the false positive could be due to the effects of the extracts and fractions being not significantly different from one another ($p > 0.05$). The anticoagulant compounds in the various extracts and fractions might be present at comparable concentrations, rendering the anticoagulant compounds not reliably identified. For the correlation method to be useful, there should be significant differences in the activities between the extracts/fractions. No reports on the anticoagulant effects of other potential anticoagulant compounds as listed in Tables 4.3 and 4.4 can be found.

4.4.2.8 Confirmation of antiplatelet activity of β-amyrin

The isolation of β- amyrin was reported in Chapter 3. An independent platelet aggregation assay was repeated using the isolated β-amyrin at a concentration of 10 μg ml^{-1} and a 13.2% inhibition of platelet aggregation was obtained. At the same concentration, aspirin showed a 26.1% inhibition of platelet aggregation.

The antiplatelet activity of the amyrins accounted in part for the activities seen in the 70% v/v methanol extract, the ethanol extract, the hexane and butanol fractions, but not in the water extract and water fraction. It can be seen from

Figure 4.6 that the amyrins are not present in both the chromatograms of the water extract and water fraction.

4.4.2.9 Advantage of using MVDA for natural product drug discovery

An advantage of using MVDA for discovery of bioactive compounds over bioassay-guided fractionation is the significant reduction in the number of repeated chromatography required. Jalil et al., 2004 reported a bioassay-guided fractionation method leading to the discovery of a potent platelet activating factor receptor antagonist. Repeated chromatography and bioassays were needed to obtain the pure compound. Using MVDA, 2 steps are required. After determining the activity of the fractions obtained by liquid—liquid partitioning, all samples are analyzed by GC-MS and MVDA, which can point out the putative bioactive compounds immediately. If the identities of the compounds are known, bioassays can be performed to confirm the activities. Otherwise, a targeted isolation and structural elucidation should be carried out prior to further bioassays.

There is much potential for future developments using MVDA for natural product discovery using the current method, In this study, all compounds in the plants were assumed not to have any synergistic or antagonistic effects. In other words, the compounds were assumed to be acting independently and do not promote platelet aggregation. As plant extracts contain a myriad of components, further experiments can be done to include this parameter in the study. Further more, analysis using liquid chromatography can also be used to analyse compounds not detected by GC-MS.

4.5 Conclusion

A rapid and simple method of identifying bioactive compounds from crude plant extracts and their partially purified fractions using MVDA has been developed and demonstrated. Successful prediction of α-amyrin and β-amyrin as potential antiplatelet agents in *A. elliptica* corroborated with independent bioassay results. While the amyrins have been successfully predicted, other hits should also be further evaluated to ensure the reliability of the method.Using MVDA to identify potential bioactive components may reduce time and cost of drug discovery and increase efficiency. GC-MS has been demonstrated to be useful in the current work for rapid identification of compounds with antiplatelet activity in *A. elliptica*. This simple method using easily available commercial software (SIMCA-P, RapidMiner and Microsoft Excel) can be optimised and applied to other plant extracts and other biological activities.

CHAPTER 5

Antiplatelet, anticoagulation and pharmacokinetic studies of *A. elliptica* and its isolated bioactive component in rats

5.1 E*x vivo* and *in vivo* antiplatelet and anticoagulant activities of *A. elliptica* and β-amyrin in rats

5.1.1 Introduction

A. elliptica is a medicinal plant used traditionally by the Malays for conditions such as pains in the region of the heart, fever, diarrhoea and liver poisoning. The traditional uses and scientific studies of the plant have been reviewed in Section 1.3.2. It has been shown in Section 3.1.4.7 that α- and β-amyrins are the major components in the *A. elliptica* plant extract. In Chapter 3, collagen-induced platelet aggregation and plasma coagulation assays performed *in vitro* also suggest that the amyrins could be partly responsible for the activities of the leaf extract (Ching et al., 2010). α- and β-amyrins are triterpenes found naturally occurring in a variety of plants (Xu et al., 2004). The biological activities of the amyrins, such as anxiolytic, antidepressant (Aragao et al., 2006), antinociceptive (Otuki et al., 2005), liver-protective (Oliveira et al., 2005), gastroprotective (Oliveira et al., 2004), antiplatelet (Aragao et al., 2007; Ching et al., 2010) and anti-inflammatory (Akihisa et al., 1996) have been reported. As the amyrins exhibit a wide range of biological activities, they could be promising lead

compounds for further pharmaceutical development. In addition, α- and β-amyrin can be commonly found in vegetables such as peas (*Pisum sativum*) (Morita et al., 2000) and cabbages (*Brassica oleracea*) (Martelanc et al., 2007), thus there could be potential food-drug interaction when drugs are consumed together with such vegetables.

Previous investigations of the antiplatelet activities of *A. elliptica* elucidated three components responsible for the activity. 5-(*Z*-heptadec-4'-enyl)resorcinol was shown to inhibit platelet activating factor receptor binding on rabbit platelets (Jalil et al., 2004), while α- and β-amyrin were shown to inhibit collagen-induced platelet aggregation in rabbits *in vitro* (Ching et al., 2010). Since only β-amyrin was successfully isolated and purified as described in Section 3.1.4.4, the isolated β-amyrin will be studied *in vivo* in this chapter. In this study, platelet aggregation and plasma coagulation tests were performed using rats, and results from this study will be useful for future clinical studies of the compounds.

5.1.2 Objectives

The overall objective of the work in this section is to investigate the *in vivo* and *ex vivo* antiplatelet and anticoagulant activity of *A. elliptica* and its isolated component, β-amyrin. The specific objectives are to

1. Investigate the effects of the *A. elliptica* 70% v/v methanol extract and its isolated component, β-amyrin on haemostasis using the rat model by the tail bleeding assay.

2. Investigate the antiplatelet and anticoagulation effects of the *A. elliptica* 70% v/v methanol extract and its isolated component, β-amyrin using the rat model by a collagen-induced platelet aggregation test and a plasma coagulation test.

5.1.3 Materials and Methods

5.1.3.1 Plant material and extraction

Leaves of *A. elliptica* were extracted using 70% v/v methanol by Soxhlet extraction as described in Section 3.1.3.3. The solvent was evaporated to dryness *in vacuo* and the dried extract was reconstituted in appropriate solvents for chemical analysis or bioassays.

5.1.3.2 Chemical analysis of plant extract using HPLC and GC-MS

The analysis of the leaf extract was carried out using both HPLC and GC-MS. Details of the methods are as in Section 3.1.3.5 and 3.1.3.6 respectively.

5.1.3.3 Isolation of β-amyrin

β-amyrin was isolated from *A. elliptica* leaf extract as described in Section 3.1.3.7. Briefly, the 70% v/v methanol extract (21.5 g) was reconstituted in HPLC grade methanol and fractionated using a preparative HPLC system and a preparative Zorbax Eclipse XDB-C18 column using methanol as the mobile phase. The fraction collected was further purified using a semi-preparative

column. Purity of the isolated β-amyrin was checked by analyzing the compound against its standard (Extrasynthese, Genay, France) using both HPLC and GC-MS.

5.1.3.4 Animals

Male Sprague-Dawley rats (200–300 g) were purchased from the Laboratory Animal Centre (Singapore), and housed at the Animal Holding Unit of National University of Singapore. The experiments were carried out in accordance to internationally accepted guidelines on laboratory animal use (NIH publication No.85-23, revised in 1985) and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. The animals were maintained in an air-conditioned room at a temperature of 22 ± 1 ^{o}C and a humidity of 62 ± 2%, with unrestricted access to food and water. Three days of acclimatisation were allowed before any experimentation.

5.1.3.5 *In vivo* tail-bleeding assay

The rats were divided into four treatment groups (six rats per group) and orally administered with 0.5% CMC (vehicle control), aspirin (30 mg kg^{-1}), leaf extract (300 mg kg^{-1}) or β-amyrin isolated from the leaf extract (30 mg kg^{-1}). They were anaesthetised intraperitoneally with a mixture of ketamine (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}) at 90 min after dosing. The tail-bleeding model was

based on previous methods with minor modifications (Beviglia et al, 1993; Lau et al., 2009). Briefly, the tail was pre-warmed for 5 min in phosphate buffered saline (PBS; 8 g 1^{-1} NaCl, 0.2 g 1^{-1} KCl, 1.44 g 1^{-1} Na_2HPO_4, 0.24 g 1^{-1} KH_2PO_4) at 37 ^oC. The bleeding was induced by transection of the rat tail at 2 mm from the tip. The distal portion (3 cm) of the tail was immersed vertically into PBS at 37 ^oC. The time between the start of transaction to bleeding cessation was recorded as the bleeding time. Bleeding cessation was considered to be the time when the flow of blood stopped for at least 30 s. If the bleeding did not cease by 15 min, the experiment was ended with pressure being applied to the wound to stop the bleeding.

5.1.3.6 *Ex vivo* platelet aggregation assays

Blood (4 ml) from each rat was collected, intra-cardiacally at 110 min after dosing, directly into citrated bottles (Tapval, 4 ml blood collection tubes, 0.106 M citrate, Deltalab S.A., Barcelona, Spain). The whole blood aggregation assay was carried out by the measurement of impedance using a whole blood aggregometer (Chronolog Corporation, Havertown, PA) as described in Section 3.2.3.5.

5.1.3.7 *Ex vivo* plasma coagulation assays

The collected blood from the rats was centrifuged at 1500 *g* for 15 minutes to obtain clear plasma as the supernatant. For the prothrombin time (PT) test, 50 µl of plasma was mixed with 100 µl of Thromborel S (Human placental

thromboplastin, Calcium added, DADE BEHRING, Marburg, Germany) and incubated for 180 s. The maximum time for detection of a clot formation was standardised at 120 s. For the aPTT determination, 50 µl of plasma was mixed with 50 µl of aPTT reagent (DADE, Actin FSL, activated PTT reagent, DADE BEHRING, Marburg, Germany) and incubated for 60 s. This was followed by an addition of 50 µl of calcium chloride (Calcium chloride solution, 0.025 M, DADE BEHRING, Marburg, Germany) and a further incubation of 240 s. The time for the plasma to clot was determined by the Automated Blood Coagulation Analyzer CA-500 Series (Sysmex, Kobe, Japan). The maximum time for detection of a clot formation was set at 190 s. All tests were carried out in triplicates.

5.1.3.8 Statistical analysis

The percentage inhibition of platelet aggregation, PT and aPTT are reported as means ± standard deviations of results obtained in triplicates. Analyses of significance of values compared with controls, where appropriate, were done using the Student's t-test at 5% level of significance in Microsoft Excel 2007.

5.1.4 Results and Discussion

5.1.4.1 Isolation of β-amyrin

From the soxhlet extraction, 42.9 g of dried leaf extract was obtained from 517.5 g of fresh leaves. This equates to a yield of 8.3% w/w of the leaves. 21.5 g of dried leaf extract was reconstituted in 40 ml of methanol and used for the isolation of β-amyrin. The total amount of β-amyrin isolated and purified was 96.8 mg. The isolated compound was a white powder. The identity of β-amyrin in the extract was verified by running the standard for comparison as well as by mass spectrometry and an autolibrary search using the Wiley mass spectral library in GC-MS.

5.1.4.2 Tail bleeding assay

The tail bleeding times of rats administered with either the vehicle, leaf extract, β-amyrin or aspirin are shown in Table 5.1. The doses of the drugs and extract were estimated from a previous study by Chua (2005). The rats treated with either the leaf extract or β-amyrin had longer bleeding times compared to the control rats. The results suggest that both β-amyrin and the leaf extract have antithrombotic effects as demonstrated by the prolonged bleeding times compared to the control.

Table 5. 1 Tail-bleeding times after oral administration of test samples (n denotes the number of rats being analysed for each test sample). * $p < 0.05$; ** $p < 0.01$ compared with the control

Treatment (Dose)	Dose (mg/ kg, oral)	Bleeding time/ s	No. of rats with bleeding times > 15 min
Control (n=6)	–	398 ± 54	0
Leaf Extract (n=6)	300	741 ± 150**	2
β-amyrin (n=6)	30	628 ± 200*	1
Aspirin (n=5)	30	864 ± 81**	4

The mean bleeding time of the rats treated with 300 mg kg^{-1} of leaf extract was not statistically different from that of rats dosed with 30 mg kg^{-1} (166.5 μmol kg^{-1}) of aspirin. This shows that the leaf extract could affect haemostasis by prolonging bleeding. There are also no significant statistical differences between the bleeding times elicited by the same dosage of 30 mg kg^{-1} of either aspirin or β-amyrin, implying the efficacy of β-amyrin to prolong bleeding. However, 30 mg kg^{-1} of β-amyrin is equivalent to 70.3 μmol kg^{-1}, which is less than half that of the dosage of aspirin. The results could imply that β-amyrin is more efficacious at prolonging bleeding. The results were in agreement with *in vitro* results shown previously. In Section 3.2.4.2, it was reported that β-amyrin had an IC_{50} value for inhibiting collagen-induced platelet aggregation that is six times lower than aspirin when the effects were studied *in vitro* on rabbit's blood (Ching et al., 2010).

The tail-bleeding time assay is a model of haemostasis and affected by different factors such as platelet aggregation and plasma coagulation (Mackman, 2004). Further *ex vivo* assays were performed to investigate the platelet aggregation and plasma coagulation effects of the leaf extract and β-amyrin.

5.1.4.3 *Ex vivo* platelet aggregation assay

The percentage inhibition of platelet aggregation of the different treatment groups are presented in Figure 5.1.

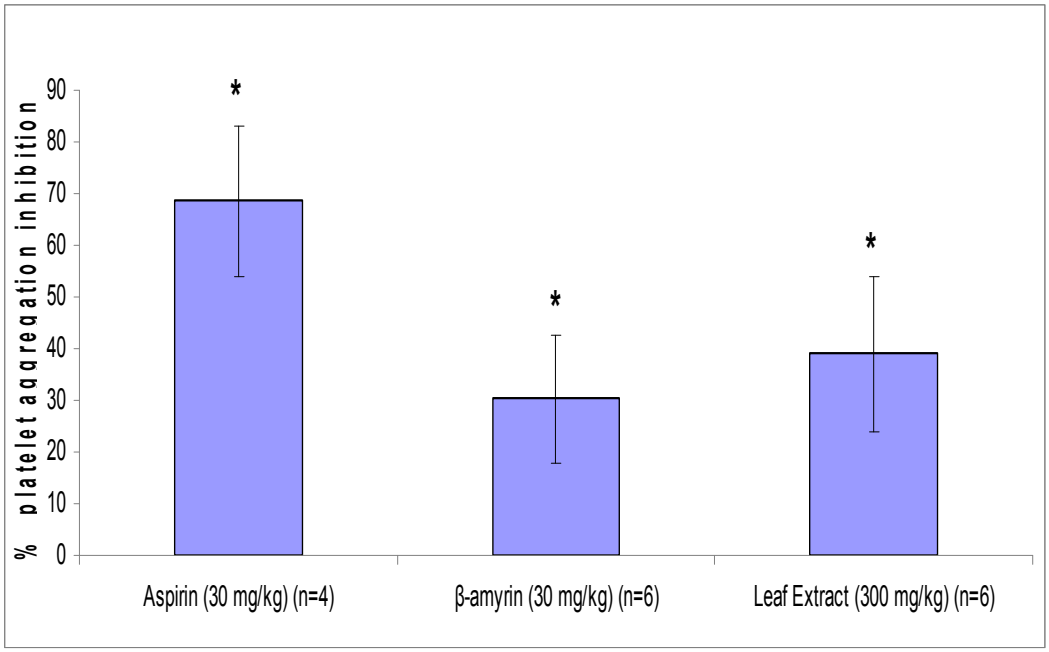


Figure 5.1 *Ex vivo* comparison of percentage inhibition of collagen-induced platelet aggregation after treatment with different test samples in SD rats. Error bars represent standard deviation and experiments on each animal were done in triplicates. Doses of test samples indicated in brackets; n denotes the number of rats being analysed for each test sample. * $p < 0.05$ compared to control.

Approximately 30 to 40% inhibition of collagen-induced platelet aggregation was observed in the two groups of rats treated with β-amyrin (30 mg kg^{-1} or 70.3 μmol kg^{-1}) and the leaf extract (300 mg kg^{-1}). Aspirin (30 mg kg^{-1} or 166.5 μmol kg^{-1}) resulted in greater inhibition of platelet aggregation (69%). While aspirin showed a greater inhibition, it has to be noted that on a molar basis, the rats were treated with a lower dose of β-amyrin (70.3 μmol kg^{-1}) compared to aspirin (166.5 μmol kg^{-1}). These results agree with the tail-bleeding time assay, which showed that β-amyrin was able to prolong bleeding at the studied dosage. The results also implied that β-amyrin is orally active, and could affect collagen-induced platelet aggregation *in vivo* as well as *in vitro* (Aragao et al., 2007; Ching et al., 2010). Investigations of a α- and β-amyrin mixture *in vitro* showed that it is able to inhibit ADP-induced platelet aggregation but only weakly inhibit arachidonic acid-induced platelet aggregation (Aragao et al., 2007). It is known that ADP exerts its platelet activating effects via two receptors, the $P2Y_1$ and $P2Y_{12}$. Binding of ADP to $P2Y_1$ causes changes in platelet shape and transient aggregation, while binding of ADP to $P2Y_{12}$ induces signalling which results in platelet aggregation and eventually, growth and stabilisation of the thrombus (Dorsam and Kunapuli, 2004). $P2Y_{12}$ also takes part in the amplification of platelet aggregation caused by other agonists such as thromboxane A2 (TXA_2) and thrombin (Offermanns, 2006). The other important platelet agonist, collagen, mediates platelet adhesion under high shear stress by interacting indirectly with $GPIb_\alpha$ via the von willebrand factor (Varga-Szabo et al., 2008). This interaction activates GPIIb/IIIa, which allows platelets to aggregate. At low shear stress

conditions, GPIa/IIa and GPVI on the platelet surface bind collagen (Varga-Szabo et al., 2008). GPVI has an important role in activating the platelet, which leads to a chain of events involving adhesion, aggregation and degranulation of the platelet (Varga-Szabo et al., 2008). Based on existing knowledge, further work should be done to elucidate the mechanism of α- and β-amyrin in inhibiting platelet aggregation involving these pathways and receptors.

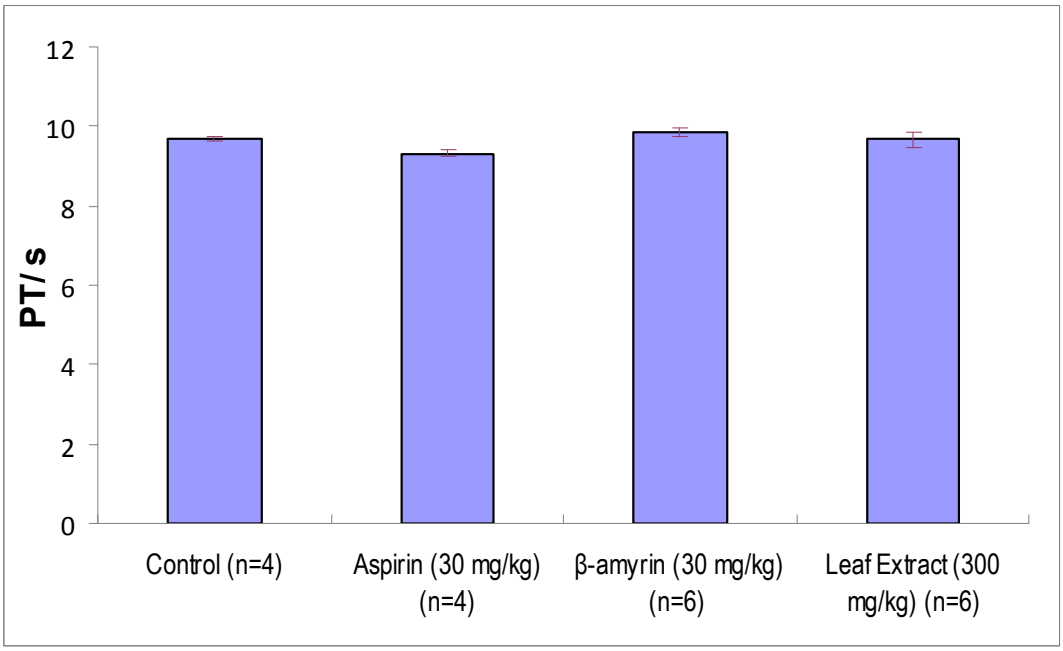
α- and β-amyrin mixtures had been studied for various activities *in vivo* and showed promising effects. The activities studied include analgesia (Aragao et al., 2007), anti-inflammatory (Aragao et al., 2007), anti- colitis (Vitor et al., 2009), anxiolytic (Aragao et al., 2006), antidepressant (Aragao et al., 2006), antinociceptive (Holanda Pinto et al., 2008), antitubercular (Higuchi et al., 2008), gastroprotective (Oliveira et al., 2004), protection against liver injury (Oliveira et al., 2005) and scratching suppression (Oliveira et al., 2004). α- and β-amyrin have also been individually assessed for their anti-inflammatory in mice (Akihisa et al., 1996). When α-amyrin was studied for its anti-inflammatory activity on mice, it was shown that while α-amyrin had no effect on the cyclooxygenase (COX)-1 and COX-2 activities *in vitro*, the compound was able to dose-dependently inhibit 12-*O*-tetradecanoylphorbol-acetate -induced COX-2 expression in the mouse skin (Medeiros et al., 2007). Aspirin is known to act on COX by reducing the synthesis of TXA_2 in platelets, thereby preventing aggregation. More investigations should be carried out to find out if the amyrins are able to act in a similar fashion.

5.1.4.4 *Ex vivo* plasma coagulation assay

Figure 5.2 shows the PT and aPTT of rats after treatment with the vehicle, leaf extract, β-amyrin or the positive control, aspirin. The results of the *ex vivo* plasma coagulation assay showed that the leaf extracts and β-amyrin did not exhibit any anticoagulant activities.

On the contrary to our previous investigations which showed that the leaf extract prolonged the aPTT but not PT *in vitro*, the *ex vivo* studies here showed that the leaf extract did not prolong plasma coagulation at an oral dose of 300mg kg^{-1}. Such an observation could be due to the active component being administered at too low a dosage, low bioavailability or poor absorption via the oral route, metabolic inactivation or simply inter-species variation in drug response in which the active compound in the leaf extract might only be effective in human blood. Similar to the results of *in vitro* plasma coagulation assay in Section 3.2.4.4, β-amyrin did not show anticoagulation effects *in vivo*. Although it was reported that β-amyrin possesses antithrombin activity *in vitro* when a chromogenic bioassay was performed (Medeiros et al., 2002), β-amyrin did not show any anticoagulant activity in this study as seen from the PT and aPTT obtained. These results imply that β-amyrin had increased the bleeding time of rats by affecting platelet aggregation, but not plasma coagulation.

(A)



(B)

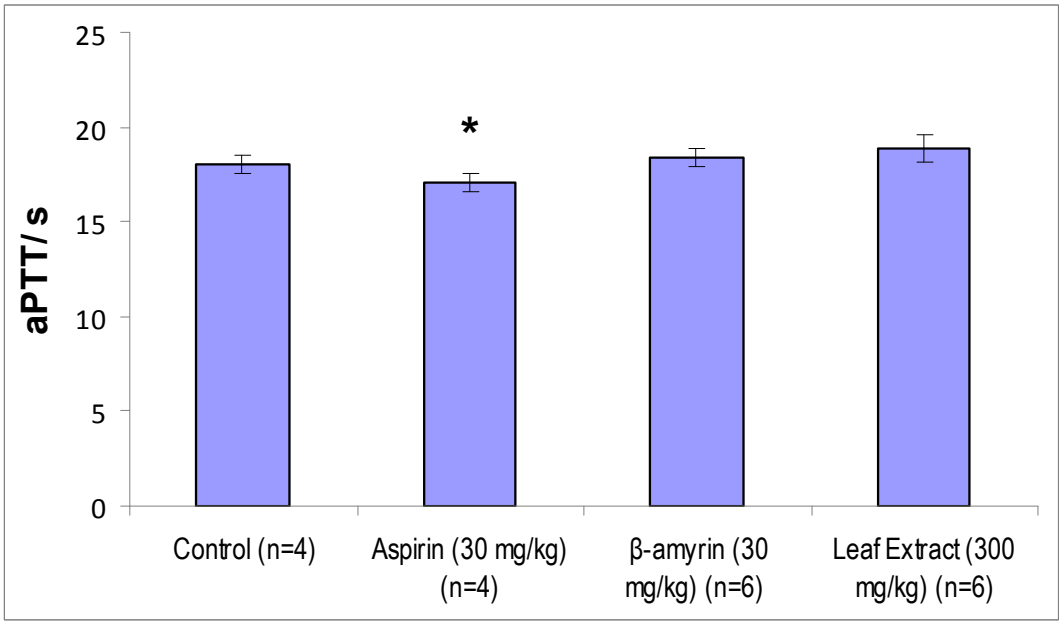


Figure 5.2 (A) PT and (B) aPTT after treatment with different test samples in SD rats. Error bars represent standard deviation and experiments on each animal were done in triplicates. Doses of test samples are indicated in brackets; n denotes the number of rats in each treatment group. * $p < 0.05$ compared to control.

5.1.5 Conclusion

This is the first report of *A. elliptica* leaf extract and its isolated component β-amyrin on haemostasis, platelet aggregation and plasma coagulation *in vivo* in rats. In summary, the results showed that the leaf extract of *A. elliptica* has antiplatelet effects, but not anticoagulant effects. It is shown from the assays that β-amyrin is one of the components in the leaf extract responsible for the antiplatelet effects. Both the extract and β-amyrin, when dosed orally to rats, could increase tail bleeding times and inhibit platelet aggregation. However as opposed to the *in vitro* studies conducted, the extract did not result in the prolongation of PT or aPTT. The lack of anticoagulant activity is probably due either to the concentration or inactivation of the active components by metabolism. Further work is required to elucidate the other active components in the leaf extract and the mechanisms of action of the isolated compound, β-amyrin.

5.2 Pharmacokinetic study of *A. elliptica* and its bioactive components, α-amyrin and β-amyrin in rats

5.2.1 Introduction

Pharmacokinetics plays a vital role in drug discovery and development. In drug development, good *in vitro* activities might not translate to good *in vivo* activities if the drug does not have good bioavailability and suitable duration of action. According to a review by Lin and Lu (1997), many failures of drug candidates were due to unacceptable pharmacokinetic properties, for example too long or short half lives, poor absorption and extensive metabolism. It is thus important to understand the pharmacokinetics of the drug early in development so that a decision could be made to either exclude a compound from further screening or to optimise the pharmacokinetic properties of the compound. It is important to note that approximately 40% of failures of drug candidates in clinical development were due to unfavourable pharmacokinetics (Kennedy, 1997; Kubinyi, 2003; Prentis et al., 1988). From the high failure rate, it can be seen that pharmacokinetics play an important role in drug discovery.

In Chapter 3, it was shown that the 70% v/v methanol *A. elliptica* leaf extract and its isolated component, β-amyrin is antiplatelet *in vivo*. However to the best of our knowledge, the pharmacokinetic profiles of α- and β-amyrin have not been reported. In order for the compounds to be investigated as a drug, it would be useful to understand its absorption and clearance profiles, for estimation of the dosages and to further improve the pharmacokinetic properties of the compound.

The lack of a reliable analytical method could have hindered the pharmacokinetic studies of the amyrins. To facilitate further investigations of these compounds, this study aims to develop and validate a GC-MS method to quantify α- and β-amyrin in rat plasma. Since only β-amyrin was isolated and studied *in vivo* previously, the pharmacokinetic profile of β-amyrin was studied in this section. This is the first report on the pharmacokinetic profile of β-amyrin. The information obtained from this study will be useful for further investigations on the potential medicinal application of the amyrins.

5.2.2 Objectives

The overall objective of this section is to assess the pharmacokinetic profiles of α- and β-amyrin in Sprague-Dawley rats. The specific objectives are to

1. Develop and validate a GC-MS method for the detection and quantification of α- and β-amyrin in rat plasma.
2. Investigate the pharmacokinetics of α- and β-amyrin in the 70% v/v methanol extract of *A. elliptica* and the pure β-amyrin.

5.2.3 Materials and methods

5.2.3.1 Reagents

α- and β-amyrin (≥98.5%) were purchased from Extrasynthese (Genay Cedex, France) while the sodium salt of carboxymethyl cellulose (CMC) was purchased from Hopkin and Williams (Chadwell Heath, UK). HPLC grade methanol was obtained from Tedia Company (Fairfield, OH). Methyltestosterone was obtained from U.S Pharmacopeia (Rockville, MD).

5.2.3.2 Preparation of plant extract

The 70% v/v methanol extract was prepared as described in Section 3.1.3.3. The plant extract was dried *in vacuo* before suspending in 0.5% (w/w) CMC. The concentrations of α- and β-amyrins in the plant extract were quantified using GC-MS.

5.2.3.3 GC-MS method development for detection of the amyrins and internal standard methyltestosterone

A Shimadzu (Kyoto, Japan) GC-MS system (gc2010 and qp2010 MS) was used for quantifying both α- and β-amyrins in the rat plasma. Chromatographic separation was achieved with a DB5 column of film thickness 0.25 μm, length 30.0 m and diameter 0.25 mm (Agilent Technologies, Santa Clara, CA). The initial oven temperature was 180 ^{o}C and increased to 280 ^{o}C at a rate of 50 ^{o}C min^{-1}. This temperature was held for another 19 min. The helium flow rate was 1.6 ml min^{-1}. The interface and ion source temperatures were 250 ^{o}C and 200 ^{o}C

respectively. At the beginning of the assay development, the mass spectrometer was operated in the scan mode to obtain the mass spectra of α-amyrin, β-amyrin and methyltestosterone. After the identification of the major ions, the selected-ion monitoring (SIM) mode was applied, detecting ions with m/z 203, 218 and 426 for both α- and β-amyrins and ions with m/z 43, 124 and 302 for the internal standard, methyltestosterone. Quantification was done using the total ion chromatogram (TIC) of the quantifier ions with m/z 218 and 302 for the amyrins and methyltestosterone respectively.

5.2.3.4 Sample preparation

Stock solutions of α- and β-amyrins and the internal standard, methyltestosterone were prepared in methanol and stored at 4 ^{o}C, away from light. The final concentrations of calibration standards for α- and β-amyrin were 1, 5, 10, 50, 100, 500, 1000 and 2000 ng ml^{-1}. To assess the accuracy of this assay, quality control samples (1, 200 and 1500 ng ml^{-1}) were also prepared independently. All samples were prepared freshly by spiking 5 µl of appropriate concentrations of amyrin into 100 µl of pooled rat plasma. For the processing of plasma samples, 5 µl of internal standard, methyltestosterone (20 µg ml^{-1}) was spiked into 100 µl of plasma in a clean 1.5 ml microcentrifuge tube. The samples were mixed well before 300 µl of ice cold methanol was added, vortexed for 1 min and centrifuged at 10,000 *g* for 10 min. After the protein precipitation, the supernatant was transferred to a clean microcentrifuge tube and evaporated to dryness. The residue was reconstituted in 100 µl of methanol and centrifuged for

another 5 min at 10,000 *g*. 1 µl of the resulting supernatant was injected into the GC-MS.

5.2.3.5 GC-MS assay validation for pharmacokinetic study

The assay was validated for its selectivity, sensitivity, linearity, precision, repeatability, accuracy and absolute recovery. The post preparative, freeze-thaw, short term and long term stability profiles of α- and β-amyrins were also assessed.

Selectivity was initially investigated by comparing the chromatograms of plasma pooled from 8 individual rats to the same pool of plasma spiked with α- and β-amyrin and the internal standard methyltestosterone. A confirmation was performed by the collecting pre-dosing samples from the 10 individual rats used in the pharmacokinetic study. The selectivity of the assay was determined by the chromatographic separation of the compounds between the pre-dosing and post-dosing plasma samples. In the mass spectra obtained by SIM, the ratios of the detected ions of each compound were used as a secondary control for determining selectivity.

The sensitivity of the GC-MS assay was represented by the limit of detection (LOD) and limit of quantitation (LOQ), defined by comparing measured signals from samples with known concentrations of analyte with those of blank samples, and by establishing the minimum concentration at which the analyte can be reliably detected or quantified. A signal to noise ratio of 3 and 10 is used for estimating the LOD and LOQ respectively.

The ratio of the peak area of the amyrins to methyltestosterone was defined as the analytical response. GraphPad Prism Version 3.03 (San Diego, CA) was used to assess the linearity of the calibration curve, where x was the concentration of amyrin and y was the analytical response. A weighting factor of $1/x^2$ was applied in the linear regression (Lin and Ho, 2009; Lin et al., 2009). The calibration was executed on 5 consecutive days. For intra-day analysis, 5 replicate of samples were prepared; for inter-day assay, duplicate samples were prepared. Similarly, quality control (QC) samples (1, 200 and 1500 ng ml^{-1}) were also prepared and analyzed. The intra- and inter-day relative standard deviation (RSD) at individual concentration was used as a precision / reproducibility indicator.

The absolute recovery (%) was calculated by comparing the peak areas of the quality control samples with the peak areas of plasma-free samples containing the same concentration of amyrins. The analytical recovery (%) was calculated by comparing the amounts of amyrin detected to that spiked into plasma.

The stability profiles of α- and β-amyrin solutions were obtained by storing the sample solutions of the standards at 24 ^{o}C for 7 days. Stability of the standards in plasma under different conditions was measured using the QC samples. Post preparative stability of the samples was assessed by re-measuring the processed samples after storage at 24 ^{o}C for 24 h. Freeze-thaw stability of the samples was evaluated by subjecting the samples to 3 freeze (-80 ^{o}C)-thaw (24 ^{o}C) cycles. Short term stability of the samples was investigated by storing the samples at 4 ^{o}C for 24 h and long term stability by storing the samples at -80 ^{o}C for 14

days. Stabilities of the above conditions were calculated by comparing the amount of amyrins left before and after storage.

5.2.3.6 Pharmacokinetic study design

This pharmacokinetic study was carried out following the "Guidelines on the Care and Use of Animals for Scientific Purposes" (National Advisory Committee for Laboratory Animal Research, Singapore, 2004). The study design and animal handling procedures of this study were reviewed and approved by the Institutional Animal Care and Use Committee of the National University of Singapore (NUS).

Ten Sprague-Dawley rats (male, 300-320 g) were purchased from the Comparative Medicine Center of NUS. The rats were maintained in a specific pathogen free animal facility (24°C, 60% relative humidity) at the Comparative Medicine Center and kept on a 12-h light/dark cycle with free access to food and water. On the day before the pharmacokinetic study, a polyethylene tube (I.D. 0.58 mm, O.D. 0.965 mm, Becton Dickinson, Sparks, MD 21152, USA) was implanted into the right jugular vein under isoflurane anaesthesia. This cannula was used for intravenous drug administration as well as for blood sampling. The rats were divided into three groups. Group 1 (n = 4) received single oral administration of 300 mg kg^{-1} crude plant extract through oral gavage (equivalent of 1.9 mg kg^{-1} and 3 mg kg^{-1} of α- and β-amyrin respectively); group 2 (n = 3) received single oral administration of β-amyrin standard (3 mg kg^{-1}) suspended in 0.5% (w/w) carboxymethyl cellulose sodium through oral gavage; group 3 (n=3)

received single intravenous administration of β-amyrin (1 mg kg^{-1}) dissolved in ethanol at a concentration of 5 mg ml^{-1}, through a 30-sec infusion. Serial blood samples were collected before dosing and at 45, 90, 110, 180, 300, 480, 720 and 1440 min after oral gavage and 5, 15, 30, 60, 90, 120, 180, 300, 480, 720, 1440 min after intravenous administration. After the intravenous dosing or blood sampling, the cannula was flushed with 0.2 ml heparin-saline (10 I.U. ml-1). After centrifuging at 3,000 *g* (4 °C) for 10 min, the plasma was collected and stored at -80 °C. The protocol of this pharmacokinetic study was modified from two previous reports (Lin and Ho, 2009; Lin et al., 2009).

5.2.3.7 Pharmacokinetic analysis

The pharmacokinetic parameters in this study were calculated using the non-compartmental model with the software WinNonlin standard version 1.0 (Scientific Consulting Inc., Apex, NC). For rats that received oral administration of the plant extract or β-amyrin standard (Groups 1 and 2), the plasma exposure (area under the plasma amyrin concentration *versus* time curve, $AUC_{0\rightarrow t}$) was calculated by the linear trapezoidal rule with the time point from 0 to the last time point (Lin and Ho, 2009; Lin et al., 2009). The $AUC_{0\rightarrow t}$ for rats that received the intravenous administration (Group 3) was calculated using the same rule, but with a logarithmic scale (Lin and Ho, 2009; Lin et al., 2009). Clearance (*Cl*) values for the rats given intravenous administration (Group 3) was calculated using non-compartmental methods (Cl = Dose / $AUC_{0\rightarrow t}$). The bioavailabilities (*F*) of β-amyrin after oral administration in Groups 1 and 2 were calculated as follows:

F (%) = [$AUC_{0\rightarrow t}$ (Groups 1 or 2)/ 3 mg kg^{-1}]/ [$AUC_{0\rightarrow t}$ (Group 3)/ 1 mg kg^{-1}] × 100%

Due to the presence of a secondary peak in the plasma β-amyrin concentration *versus* time curve in rats that received intravenous administration, the apparent volume of distribution (V) was calculated with the first 3 data points (up to 30 min) using one-compartmental first order elimination model as described in Das et al. (2008).

5.2.3.8. Statistics

Statistical analyses were performed using GraphPad Prism Version 3.03. All experimental values were reported as mean ± standard deviation (SD). A two-tailed *t*-test was used to compare the pharmacokinetic parameters between the different groups. A p value < 0.05 was adopted to indicate statistical significance.

5.2.4 Results and discussion

5.2.4.1 GC-MS assay development and validation

α-amyrin, β-amyrin and methyltestosterone were eluted only at high temperatures. The oven temperature of the GC-MS was thus held at 280 ^{o}C for 19 min in order for complete elution of the 3 compounds. The selectivity of the assay is determined. Figure 5.3 shows the GC-MS chromatograms of a pre-dosing plasma sample, a blank plasma sample spiked with methyltestosterone and amyrin standards, as well as a plasma sample taken from a rat dosed with the plant extract. The internal standard, methyltestosterone, eluted at 5.9 min. No interfering peak was observed in the blank pooled plasma samples or in the pre-dosing samples at these timings (Figure 5.3a). Although α- and β-amyrin are structural isomers, they were well separated and eluted at 17.2 min and 15.9 min respectively (Figure 5.3b). Moreover, no interfering peak was identified in the chromatograms from the post-dosing samples (Figure 5.3c).

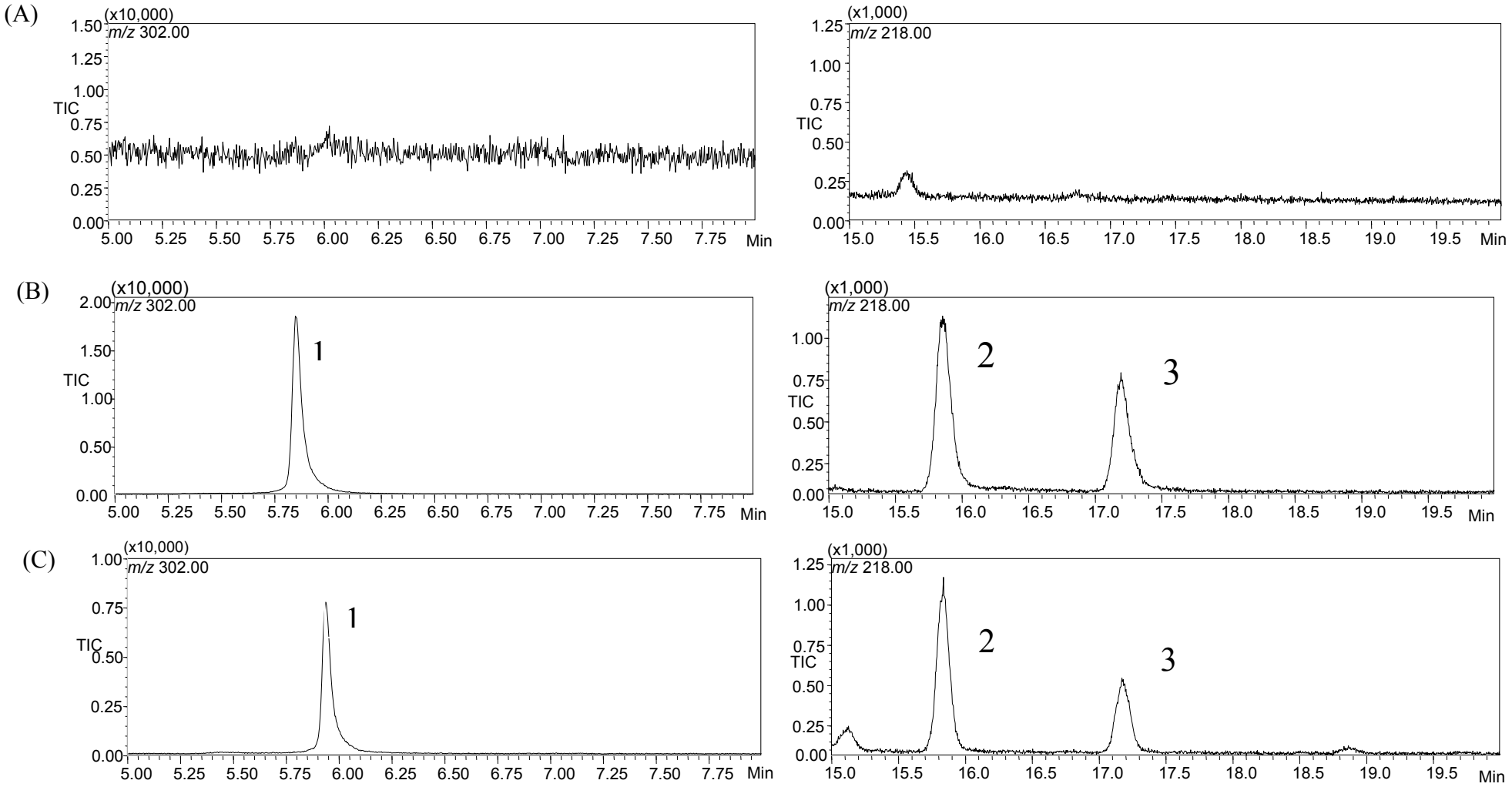


Figure 5.3 GC-MS chromatograms of (A) a pre-dosing plasma sample (B) a blank plasma sample spiked with 1 μg ml^{-1} methyltestosterone (peak 1; 5.944 min) and 100 ng ml^{-1} each of β-amyrin (peak 2; 15.854 min) and α-amyrin (peak 3; 17.193 min) (C) methyltestosterone (peak 1; 5.937 min), β-amyrin (peak 2; 15.836 min) and α-amyrin (peak 3; 17.170 min) in a plasma sample taken from a rat 5 h after being dosed with 300 mg kg^{-1} of the plant extract.

The calibration curves for α- and β-amyrins were obtained by spiking the standards of the two amyrins into pooled rat plasma. Data for the linearity, LOD and LOQ is presented in Table 5.2.

Table 5.2 Linearity, LOD and LOQ data of α-amyrin and β-amyrin standard calibration curves.

Sample	Linear range (ng mL^{-1})	R^2 value	LOD (ng ml^{-1})	LOQ (ng ml^{-1})
α-amyrin	1 – 2000	0.9963 – 0.9976	0.4	1.0
β-amyrin	1 – 2000	0.9976 – 0.9990	0.4	1.0

The intra-day and inter-day RSD were less than 12% and 9% respectively for both α- and β-amyrin (Table 1). Recoveries of the amyrins were good. The absolute recoveries of both amyrins were more than 70 % while the intra-day and inter-day analytical recoveries were greater than 95% (Table 5.3). Both α- and β-amyrins appeared to be stable at all tested storage conditions (Table 5.4). In summary, a reliable GC-MS method has been developed and validated to quantify α- and β-amyrin in rat plasma.

Table 5.3 Absolute and analytical recoveries of α-amyrin and β-amyrin.

	Spiked concentration (ng ml^{-1}) of α-amyrin					
	1	RSD (%)	200	RSD (%)	1500	RSD (%)
Absolute Recovery (%)	85.1 ± 9.34	9.82	72.7 ± 8.48	11.7	70.7 ± 3.13	4.43
Intra-day Analytical Recovery (%)	95.3 ± 10.5	11.0	96.7 ± 11.6	12.0	99.1 ± 4.39	4.43
Inter-day Analytical Recovery (%)	95.9 ± 5.70	5.94	95.1 ± 8.60	9.04	98.1 ± 4.82	4.91

	Spiked concentration (ng ml^{-1}) of β-amyrin					
	1	RSD (%)	200	RSD (%)	1500	RSD (%)
Absolute Recovery (%)	88.5 ± 7.00	7.91	87.7 ± 6.25	7.13	73.8 ± 6.39	8.66
Intra-day Analytical Recovery (%)	95.2 ± 7.53	7.91	95.4 ± 6.80	7.13	96.2 ± 8.25	8.58
Inter-day Analytical Recovery (%)	96.5 ± 12.6	13.1	95.5 ± 7.11	7.45	95.0 ± 5.07	5.34

Results are presented as mean ± SD

Table 5.4 Stability of α-amyrin and β-amyrin.

Stability (% Remained)	Spiked concentration (ng ml^{-1}) of α-amyrin			Spiked concentration (ng ml^{-1}) of β-amyrin		
	1	200	1500	1	200	1500
Stock solution stored at 24ºC for 7 days	101 ± 8.5	104 ± 5.4	101.8 ± 7.7	99.4 ± 7.3	105 ± 13.5	97.0 ± 7.0
Plasma samples stored at 4ºC for 24 h	106 ± 5.3	93.9 ± 3.2	90.3 ± 11.2	97.3 ± 3.3	104 ± 9.7	89.7 ± 9.0
Plasma samples stored at -80ºC for 14 days	107 ± 6.2	90.0 ± 4.3	94.4 ± 4.0	90.5 ± 9.5	92.2 ± 2.8	90.2 ± 5.6
Plasma samples after 3 Freeze-thaw cycles	101 ± 7.6	91.6 ± 6.4	98.0 ± 13.8	109 ± 11.2	110 ± 6.1	106 ± 9.1
Post-preparative samples stored at 24ºC for 24 h	99.5 ± 8.8	107.8 ± 8.9	102 ± 6.3	101 ± 3.7	104 ± 8.9	101 ± 1.5

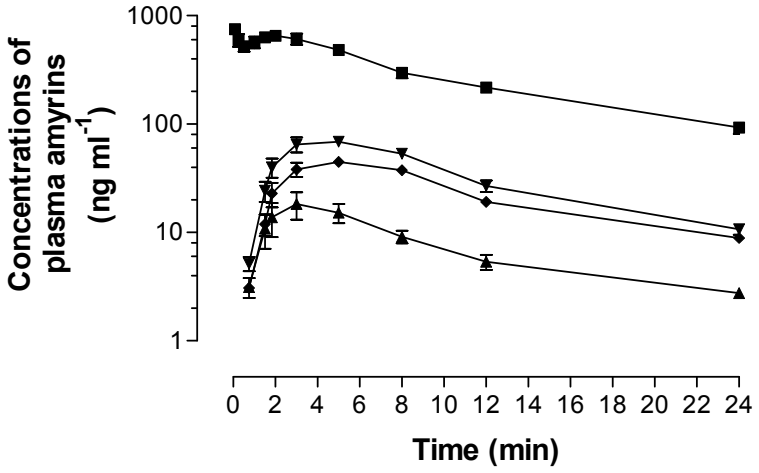
Results are presented as mean ± SD

5.2.4.2 Pharmacokinetic profiles of α- and β-amyrin

The pharmacokinetic profiles of amyrins were subsequently assessed in Sprague-Dawley rats using the validated GC-MS method. To our knowledge, this was the first attempt to measure amyrins in biological samples. Earl et al. (2002) previously reported that the majority of the amyrins in Shea oleine were excreted unchanged in rats and humans when administered orally, which implied that there was little or no metabolism of the amyrins after oral absorption. Figure 5.4 shows the pharmacokinetic profile of β-amyrin after a single intravenous or oral administration. When administered intravenously, plasma β-amyrin concentration declined rapidly over the first 30 min, but the concentration increased subsequently in the following 2 h. The appearance of this secondary peak suggested the presence of enterohepatic recirculation. A moderate apparent volume of distribution was found (V = 1317 ± 253 ml kg^{-1}). The clearance of β-amyrin was extremely slow (Cl = 2.04 ± 0.24 ml min^{-1} kg^{-1}). Similarly, the terminal half-life of β-amyrin was found to be very long ($t_{1/2\,\lambda z}$ = 610 ± 179 min).

The major pharmacokinetic parameters of β-amyrin are shown in Table 5.5.

(A)



(B)

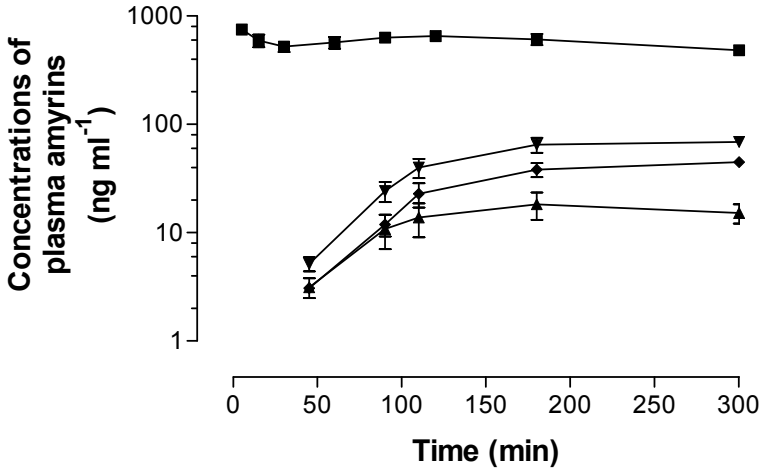


Figure 5.4 (A) Plasma concentration versus time profiles of amyrins in rats after receiving: a single intravenous administration of 1 mg kg^{-1} β-amyrin standard (■) (*n* = 3); a single oral dose of β-amyrin standard at 3 mg kg^{-1} (▲) (*n* = 3); a single oral dose of 300 mg kg^{-1} plant extract equivalent of 3 mg kg^{-1} of β-amyrin (▼) and 1.9 mg kg^{-1} of α-amyrin (♦) (*n* = 4). (B) Plasma concentration versus time profiles of amyrins for the period 5 to 300 min. Data is presented as mean ± SD.

Table 5.5 Pharmacokinetic parameters of α-amyrin and β-amyrin in rats.

	β-amyrin standard IV (Group 3)	β-amyrin standard oral dose (Group 2)	Plant extract oral dose (Group 1)	
Dose (mg kg^{-1})	1	3	300 (equivalent of 3 mg kg^{-1} β-amyrin)	300 (equivalent of 1.9 mg kg^{-1} α -amyrin)
V (ml kg^{-1})	1317 ± 253			
$AUC_{0\rightarrow 24h}$ (min µg ml^{-1})	411 ± 61.9	10.6 ± 2.84	47.3 ± 4.46*	32.2 ± 2.3
Cl (ml min^{-1} kg^{-1})	2.04 ± 0.24			
$t_{1/2\,\lambda Z}$ (h)	10.2 ± 3.0	10.3 ± 1.6	8.3 ± 2.3	9.2 ± 1.1
$MRT_{0\rightarrow 24h}$ (h)	8.0 ± 0.4	8.5 ± 1.4	8.7 ± 0.4	9.2 ± 0.6
C_{max} (ng ml^{-1})		18.7 ± 8.4	75.0 ± 10.2*	46.8 ± 4.13
t_{max} (h)	-	3 (n = 2) or 5 (n = 1)	3 (n = 3) or 5 (n = 1)	3 (n = 1) or 5 (n = 3)
F (%)		0.86 ± 0.23	3.83 ± 0.36*	

Results are presented as mean ± SD
n= 4 for Group 1 and n=3 for Group 2 and 3
*$p < 0.001$ between this group and Group 2

From Figure 5.4, it can be seen that after oral gavage, β-amyrin was absorbed gradually and the plasma concentration peaked at either 3 or 5 h post-dosing in both Group 1 and 2. When β-amyrin was administered in the crude plant extract, the maximal plasma concentration (C_{max}) was about 3-fold higher than that acquired with the suspension of pure compound (75.0 ± 10.2 *v.s.* 18.7 ± 8.4, two-tailed *t*-test: $p < 0.001$). Similarly, the bioavailability of β-amyrin administered with the plant extract matrix was about 3-fold higher than the pure compound (3.8 ± 0.36 *v.s.* 0.86 ± 0.23, two-tailed *t*-test: $p < 0.001$). Clearly, the plant extract matrix was able to enhance the absolute oral bioavailability of β-amyrin. Similar phenomena have also been observed in other studies. A report on resveratrol in humans showed that the bioavailability of the compound was 6-fold higher when administered in the form of red wine or grape juice, compared to that administered as tablets enriched with resveratrol (Ortuno et al., 2010). Similarly, Mukinda et al. showed that luteolin derivatives in *Artemisia afra* extracts were more efficiently absorbed in Caco-2 cells compared to pure solutions of the compounds (Mukinda et al., 2010). The mechanism for this observation was unclear, but it was hypothesized that some constituents in the plant extract matrix could have inhibited efflux transporters of the compounds, while enhancing metabolism in the intestinal cells so that they were more easily absorbed via basolateral transporters (Mukinda et al., 2010). Keung et al. (1996) found that the bioavailability of daidzin in the extract of Radix Puerariae was approximately 10 times greater than the pure compound when administered intraperitoneally. This was attributed in part, to the plant extract enhancing the solubility of daidzin in aqueous environments. Moreover, rutin was also observed to have a better absorption in the form of a buckwheat tea preparation compared to the pure form (Graefe et al., 2001). It was hypothesized that the tea preparation contained co-factors in the plant extract

matrix that increased the solubility of rutin, as well as decreased the viscosity of the mucus layer of the intestines, leading to increased absorption. Similarly, it could be postulated that a higher oral bioavailability of β-amyrin in crude plant extract was due to the increased solubility and/or competition in metabolism. However, the exact mechanisms are to be elucidated in future study.

Although the bioavailability of β-amyrin was increased by the plant extract matrix, it was still considered very low (3.77%). The low bioavailability observed is not surprising as the log P value of β-amyrin is 9.2, much higher than the recommended log P value of 5 for an orally active drug in the Lipinski's rule of 5. Solubility-enhancing drug delivery system may be useful for the oral delivery of β-amyrin.

After reaching C_{max}, the plasma β-amyrin concentration declined gradually, and still remained in the plasma (10.7 ± 2.3 ng ml^{-1} and 2.7 ± 0.2 ng ml^{-1} respectively for Group 1 and Group 2) 24 hrs after oral administration. Other than the AUC, C_{max} and bioavailability, the $t_{1/2\lambda z}$ and mean residence time (MRT) of β-amyrin in Group 1 and 2 were not significantly different from each other. Similarly, the $t_{1/2\lambda z}$ and MRT of β-amyrin in Group 3 were similar to those in Group 1 and 2. As the isomer of α-amyrin exists naturally in the *A. elliptica* extract, it is of interest to investigate the pharmacokinetic profile of α-amyrin as well. The pharmacokinetic profile of α-amyrin was compared to that of β-amyrin in Fig. 5.4. From Table 5.5, it can be seen that α-amyrin was absorbed to a similar extent as β-amyrin as the dose normalized $AUC_{0\rightarrow t}$ and C_{max} of both α- and β-amyrin were similar.

Although the pharmacokinetic profiles of both α- and β-amyrins have not been previously reported before, the pharmacokinetics of some other triterpenes has been investigated. For example, oleanolic acid, a compound belonging to the same

class (oleanane) as β-amyrin, possessed a very low oral bioavailability (0.7%). This was comparable to the oral bioavailability of β-amyrin reported here (0.86 ± 0.23%). The low oral bioavailability of oleanolic acid was attributed to its poor gastrointestinal absorption and hepatic first-pass metabolism. Studies of oleanolic acid conducted on Caco-2 cells suggested that the compound had low permeability and the absorption was mediated by passive diffusion (Jeong et al., 2007). Further investigations on the bio-membrane permeability and metabolic stability of α- and β-amyrin are warranted.

5.2.4.3 Application of pharmacokinetic study to antiplatelet and anticoagulant activity of *A. elliptica* extract in rats

In Section 5.1, blood sampling from the rats were taken at time points comparable to the pharmacokinetic study (tail bleeding as performed at 90 min and blood was collected intra-cardiacally at 110 min). In Sections 5.1.4.2 and 5.1.4.3, the results showed that rats treated with pure β-amyrin did not show longer tail bleeding times or stronger platelet aggregation inhibition compared to the group treated with leaf extract (Table 5.1).

From this study, the bioavailability of β-amyrin is found to be low and is also affected by the matrix which the amyrin is in. The bioavailability of β-amyrin was 3.83% when given in the form of the leaf extract, as compared to 0.86% when given as a suspension. β-amyrin concentration in rat plasma at time points 90 to 110 min was 20 to 40 ng ml^{-1} for rats dosed with 300 mg kg^{-1} of leaf extract and 10 to 14 ng ml^{-1} for rats dosed with 3 mg kg^{-1} of pure β-amyrin. In rats dosed with the leaf extract, there was also presence of 12 to 23 ng ml^{-1} of α-amyrin in the rat plasma. The rats in the *ex vivo* platelet aggregation assay were dosed with 30 mg kg^{-1} of pure β-amyrin or 300mg kg^{-1} leaf extract, thus there should be comparatively a higher concentration of

amyrin in rats treated with pure β-amyrin than those dosed with the leaf extract. However the results of the tail bleeding assay and *ex vivo* platelet aggregation assay were comparable between the rats treated with pure β-amyrin and those dosed with the leaf extract. This implies the presence of other bioactive components in the leaf extract that could contribute to the bleeding times and antiplatelet activity observed.

For the *in vitro* plasma coagulation assay in Section 3.2.4.4, 0.01 mg ml^{-1} of β-amyrin was used in the assay but prolongation of neither PT nor aPTT was observed. A higher concentration of amyrins would be attained in the blood of the rats in the *ex vivo* plasma coagulation assay, but no prolongation of the PT and aPTT was observed too. The results imply that the amyrins do not show any anticoagulant effect at the tested doses.

5.2.5 Conclusion

In this study, a reliable GC-MS method was developed and validated to quantify α- and β-amyrin in rat plasma. A preliminary pharmacokinetic study was subsequently carried out in Sprague-Dawley rats. It was found that β-amyrin displayed an extremely slow clearance, a very long terminal half-life and low bioavailability in rats. As β-amyrin shows a wide range of biological activity, future investigations on the amyrins may be focused on their detailed pharmacokinetics, metabolic stability as well as methods to improve the delivery and bioavailability.

CHAPTER 6

Conclusion

Medicinal plants have played an important role in the treatment of diseases since antiquity. Even in modern medicine, it has been reported that approximately 50% of the drugs introduced since 1994 are either natural product or natural product-derived compounds. From current research trends, natural products will continue to be an important source of lead compounds for new drugs. In the treatment of cardiovascular diseases caused by thromboembolism, there is a continual need for better drugs. Common adverse effects of current antiplatelet or anticoagulant drugs used to treat cardiovascular diseases include excessive bleeding and gastrointestinal toxicity. In view of the short comings of the current drugs, development of better drugs with fewer adverse effects is necessary.

In Chapter 1, an overview of antiplatelet and anticoagulant drugs, medicinal plants and metabolomics is presented. In particular, the herb of interest, *A. elliptica*, traditionally used to treat diarrhoea, liver poisoning, fever, parturition complications and pain in the region of the heart etc., is reviewed. In this work, one of its traditional usage namely for "pain in the region of the heart" is interpreted as symptoms related to problems in blood circulation. This in turn is related to platelet aggregation and plasma coagulation.

Chapter 2 states the hypothesis and objectives of this work. It is hypothesised that *A. elliptica* possesses phytoconstituents with antiplatelet and/or anticoagulant activities that may be potential lead compounds for development of new drugs. The

objective of the work is to investigate the potential antiplatelet and anticoagulant activities of *A. elliptica* and to identify and isolate the active compound(s) responsible for the activities.

In Chapter 3, it was successfully shown that the *A. elliptica* 70% v/v methanol extract has antiplatelet and anticoagulant activities *in vitro* on rabbit's blood and human plasma respectively. β-amyrin was successfully isolated from the 70% v/v methanol extract of *A. elliptica* and found to have antiplatelet activity. The 70% v/v methanol extract had an IC_{50} value of 167μg ml^{-1} in inhibiting collagen-induced platelet aggregation, while β-amyrin had an IC_{50} value of 10.5 μM. Its structural isomer, α-amyrin, was also present in *A. elliptica*. Unfortunately, it was not successfully isolated. However, it was also active and the commercial standard gave an IC_{50} value of 21.3 μM. The amyrins were also three to six times more potent than aspirin (IC_{50} = 62.7 μM) with regards to the antiplatelet activity. This is the first report of the antiplatelet activity of the individual amyrins.

A GC-MS method was developed and validated to quantify the amount of amyrins in the plant extract. Derivatised GC-MS methods have been popularly used for the analysis of triterpenes but derivatisation is required in the method to make the solutes volatile for analysis. However the steps for derivatisation are laborious and inefficient when large numbers of samples are required for analysis. In Chapter 3, the GC-MS method was streamlined to remove the derivatisation step. The concentrations of α- and β-amyrins were subsequently quantified to be 0.6% w/w and 1.0 % w/w respectively of the dried *A. elliptica* leaf extract. These correspond to 0.05% w/w and 0.08% w/w of α- and β-amyrin in the fresh leaves respectively. While the *A. elliptica* leaf extract showed anticoagulant activities, the isolated compound β-amyrin did not exhibit anticoagulant activities at the tested concentrations. Further work should

therefore include isolation of compounds responsible for the anticoagulant activity, and also other compounds that are antiplatelet in the leaf extract.

The isolation of the active compound, β-amyrin was performed by a conventional fractionation method. It is widely recognised that the conventional method has several disadvantages as discussed in Section 1.3.3. Conventional methods of detecting bioactive compounds are slow and tedious, involving repeated steps of fractionation and bioassays. An added drawback is its difficulty to detect compounds in low concentrations. Hence in Chapter 4, a novel MVDA drug discovery method has been successfully developed for identifying potential antiplatelet compounds from medicinal plant extracts, without the need for bioassay-guided fractionation. The modeling used in this work is performed using easily available commercial software. This MVDA method consists of two modeling. The first is named the consensus method, where Orthogonal Partial Least Squares (OPLS), Partial Least Squares projection of latent structures-Discriminant Analysis (PLS-DA), Chi-Squared weighting and InfoGain weighting were performed. The second is named the correlation method, where a correlation of the concentrations of constituents and biological activities in the various extracts and fractions for each compound was done. In total, 855 different compounds were detected by GC-MS analysis. Both the consensus and correlation methods identified α- and β-amyrin as potential antiplatelet compounds, among others. Successful prediction of α- and β-amyrin as potential antiplatelet agents in *A. elliptica* corroborated with independent bioassay results. The method may reduce time and cost of drug discovery and increase efficiency. This novel platform method can be applied to other plant extracts and biological activities, thus reducing time and cost of drug discovery.

In vitro results from earlier chapters showed that the 70% v/v methanol extract of *A. elliptica* had antiplatelet and anticoagulation properties, and both α- and β-amyrin were partly responsible for the antiplatelet activities. As β-amyrin was isolated successfully, further *in vivo* work on the extract and on pure β-amyrin was carried out. Thus in Chapter 5, an *in vivo* study in rats was performed to study the activities. In the study, rats were dosed orally either with 300 mg kg^{-1} of the 70% v/v methanol extract (equivalent of 1.8 mg kg^{-1} of α-amyrin and 3 mg kg^{-1} of β-amyrin), 30 mg kg^{-1} of isolated β-amyrin or 30 mg kg^{-1} of aspirin. After dosing, an *in vivo* tail-bleeding assay and *ex vivo* platelet aggregation and plasma coagulation assay were performed. Results of the tail-bleeding study showed that the bleeding times of rats dosed with the leaf extract were comparable to rats dosed with either 30 mg kg^{-1} of pure β-amyrin or aspirin. The *ex vivo* platelet aggregation assay done on the same rats showed that both the leaf extract and β-amyrin treated animals inhibited 30% of collagen-induced platelet aggregation. However anticoagulant effects were not seen in both groups of rats. This observation was different from that seen in *in vitro* plasma coagulation assays where 0.2 mg ml^{-1} of leaf extract was able to induce anticoagulation by increasing the aPTT in human plasma. Further investigations should be conducted to understand the reason behind, which could be either due to low concentrations of the active components, specie differences or metabolism and deactivation of the active compound when ingested *in vivo*.

In Chapter 5, the pharmacokinetic profile of β-amyrin in rats was studied. Due to the large amounts of compounds needed, a commercial source of β-amyrin was used. Both α- and β-amyrins in the 70% v/v methanol extract of *A. elliptica* were monitored in the rats after oral administration. β-amyrin was found to have a very long terminal elimination half-life ($t_{1/2\ \lambda z}$ = 610 ± 179 min) and slow clearance (*Cl* =

2.04 ± 0.24 ml min^{-1} kg^{-1}). The absolute oral bioavailability of β-amyrin in the plant extract was poor (3.83%) and was about 4-fold higher than that in the suspension of pure form (0.86%). When given in the plant extract, both α- and β-amyrin had a similar dose normalized C_{max}. When these results were applied in the *ex vivo* platelet aggregation tests in Chapter 4, it was calculated that there should be comparatively a higher concentration of amyrin in rats treated with 30 mg kg^{-1} of pure isolated β-amyrin than those dosed with 300 mg kg^{-1} the leaf extract. This implies the presence of other bioactive components in the leaf extract that could contribute to the bleeding times and antiplatelet activity observed since the 2 experimental groups have comparable activities. Thus it is worthwhile to investigate the effects of other hits identified in the MVDA method (Chapter 4) as well as to identify other unknown bioactive components in the active extracts and fractions (Chapter 3). Due to the poor solubility and low oral bioavailability, future research should focus on improving the solubility and bioavailability of the amyrins by exploring various formulations and appropriate drug delivery systems. A more extensive pharmacokinetic study should also be done by increasing the number of animals and monitoring the plasma concentrations over a longer period of time.

Since the amyrins have been identified to be active, more extensive studies of the toxicity of the amyrins are required for them to be further developed into drugs. As the amyrins were found to have antiplatelet effects, the exact mechanism of action could be elucidated. It was also reported that the amyrins have a wide range of pharmacological effects. The non-specificity, any adverse effects and potential for herb-drug interaction may pose a challenge in its further development and should be further evaluated. Clinical trials will be needed before the amyrins can be approved as new therapeutics.

In conclusion, the results presented in this thesis provide some scientific evidence for the traditional uses of *A. elliptica*. Further work is warranted to develop the lead compounds into useful therapeutics.

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