In vitro testing to investigate the anticoagulant/antithrombotic and antidiabetic biological activity of *Leonotis leonurus*

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List of Abbreviations

ACC	acetyl- coenzyme A carboxylase
ACE	angiotensin-converting enzyme
ADP	adenosine phosphate
AEC	3-amino, 9-ethyl carbozol
Akt	protein kinase B
AMPK	AMP-activated protein kinase
AT$_4$	angiotensin receptor 4
AT III	antithrombin III
AL	aqueous extract
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate
BrdUrd	bromodeoxyuridine
BSA	bovine serum albumin
B2M	beta-2-microglobulin
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium ion
Ca^{2+}c	cytosolic Ca^{2+}
Ca^{2+}i	intracellular Ca^{2+}
CAM	cellular adhesion molecules
CAP	casitas B-lineage lymphoma 1 associated protein
Cb1	casitas B-lineage lymphoma 1
cDNA	copy deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
Ct	threshold cycle
CoA	co-enzyme A
COX-1	cyclooxygenase-1
Cyclo A	cyclophilin A
DAG	diacylglycerol

DIO	diet-induced obesity
DM	diabetes mellitus
dUTP	terminal deoxynucleotidyl transferase
EC	endothelial cells
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acid
ER	endoplasmic reticulum
FFAs	free fatty acids
FI	fibrin indices
FITC	fluorescein isothiocynate
FPA	fibrinopeptide A
FS	forward scatter
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GLUTs	glucose transporters
GP	glycoprotein
GPRP	glycyl-L-prolyl-L-arginyl-L-proline
GS	glycogen synthase
GSIS	glucose stimulated insulin secretion
GSK-3	glycogen synthase kinase-3
G-6-Pase	glucose-6-phosphatase
HBMS	hydroxymethylbilane synthase
HBSS	hank's balanced salt solution
HepG$_2$	human hepatoma cells
HNF	hepatocyte nuclear factor
HPLC	high performance liquid chromatography
I	insulin
IAPP	islet associated polypeptide
IC$_{50}$	inhibitor concentration required to yield 50% inhibition

IEC	ion exchange chromatography
IEC-6	intestinal cell line
IFN-γ	interferon- γ
IGF	insulin growth factor
IL-1 ß	interleukin-1 ß
IL-6	interleukin-6
IKK	IκB kinase
IP$_3$	inositol triphosphate
IR	insulin resistance
IRE	insulin responsive elements
IRE-1	inositol-requiring enzyme-1
IRS	insulin receptor substrate
JAK/STAT	janus kinase 2/signal transducer and activator of transcription
JNK/p38	Jun-N-terminal kinase/p38
LDLs	low density lipoproteins
M	marrubiin
MAPK	mitogen-activated-protein kinase
MODY	maturity onset diabetes of youth
mRNA	messenger RNA
mTOR	mammalian Target of Rapamycin
NC	negative control
NO	nitric oxide
nPKC	novel class of protein kinase C isoforms
NF-κB	nuclear factor-kappa B
OL	organic extract

PA 28	proteasome activator 28
PAC-1	monoclonal antibody directed at glycoprotein IIb/IIIa platelet complex
PAD	peripheral artery disease
PAI-1	plasminogen activator inhibitor-1
PAI-Ag	plasminogen activator inhibitor antigen
PAP pen	pen used for retaining reagents on a glass slide and preventing their waste by a water repellent circle around sections
PAR	protease-activated receptor
PBS	phosphate buffered saline
PC	positive control
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK 1	phosphoinositide-dependent kinase 1
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1	peroxisome proliferator activated receptor γ co-activator-1
PIC	pro-inflammatory cytokines
PI3′K	phosphoinositide-3′ kinase
PIP$_2$	phosphatidyl-inositol-3,4-bisphosphate
PIP$_3$	phosphatidyl-inositol-3,4,5-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PLA$_2$	phospholipase A$_2$
PPARγ	perixisome proliferator activated receptor γ
PPP	platelet poor plasma
PRP	platelet rich plasma
PTP1B	protein tyrosine phosphatase 1B
RAS	renin angiotensin system
Reagent A	serum blocking solution
Reagent B	biotinylated secondary antibody solution

Reagent C	streptavidin-peroxidase conjugate solution
Reagent D	3-amino, 9.ethyl carbozol single solution chromogen
$\mathbf{R_f}$	retardation factor
RIA	radioimmunoassay
RLT	RNeasy lysis buffer
ROS	reactive-oxygen species
ROX	6-Carboxyl-X-rhodamine
RP	reverse phase
RPE	RNeasy guanidine salt containing buffer
RT	room temperature
RT-PCR	real-time polymerase chain reaction
RW1	RNeasy guanidine salt containing buffer
S2238	α-Phe-L-Pipecolyl-L-Arg-p-nitroanilide
S2251	H-D-Val-Leu-Lys-p-nitroanilide
Sem	standard error of the mean
Ser	serine
SOCS 3	suppressor of cytokine signalling-3
SS	side scatter
S-Thr-P	serine-threonine phosphorylation
STZ	streptozotocin
SUs	sulfonylureas
TAT	thrombin-antithrombin complex
TATA	TATA box binding protein
TBA	tetrabutylammonium phosphate
TGF-β	transforming growth factor-β
Thr	threonine
TLC	thin layer chromatography
TLR	toll like receptors
TNF-α	tumor necrosis factor- α
tPA	tissue plasminogen activator

TR	transferring receptor
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
TXA$_2$	thromboxane A$_2$
TXB$_2$	thromboxane B$_2$
Tyr	tyrosine
Tyr-P	tyrosine phosphorylation
TZDs	thiazolidinediones
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
uPA	urokinase-type plasminogen activator
VLDLs	very low density lipoproteins
VSMCs	vascular smooth muscle cells
vWF	von Willerbrand factor
WHO	World Health Organisation
XBP-1	deletion of x-box-binding protein-1
Ywhaz	tyrosine-3-monooxygenase/tryptophan-5-monooxygenase-activation protein, zeta polypeptide

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Abstract

The rising costs of prescription drugs in the maintenance of personal health and well-being have increased the interest in medicinal plants. The World Health Organization estimates that 65%-80% of the world's population use traditional medicine as their primary form of health care. In this project the focus has been on the use of *Leonotis leonurus* extracts as a traditional medicine. The major chemical constituent of this plant is marrubiin, which is a diterpenoid labdane lactone formed from a precursor called premarrubiin. Aqueous and acetone extract (AL and OL extract, respectively) of this plant has been found to have an antithrombotic effect, with IC_{50} values of 3mg/ml and 6mg/ml, respectively. The extracts also have an effect on fibrinolysis, where the lysis time was decreased by more than 50% by the organic extract and standard marrubiin. In whole blood ADP-induced platelet aggregation, the organic extract inhibited aggregation by 68% at a final concentration of 138μg/ml (equivalent to 7.2μg/ml marrubiin). Marrubiin has also been screened for antithrombotic/anticoagulant activity; no antithrombotic activity has been observed but it increased the rate of fibrinolysis, by decreasing lysis time by 64% and also decreasing fibrin formation. From these findings it can be concluded that marrubiin has a fibrinolytic effect and antiplatelet aggregation effect.

In the diabetic studies, in hyperglycemic condition, the OL (10μg/ml) extract and standard marrubiin significantly increased insulin secretion by 200% (2-fold) and 400% (4-fold), respectively, with respect to the control. The OL extract and standard marrubiin stimulated the release of insulin, the stimulatory index was significantly increased by 450% (4.5-fold) and 500% (5-fold), respectively, with respect to the control. In the apoptotic studies, in the normoglycemic and hyperglycemic conditions, the OL extract decreased the occurrence of apoptosis, in a dose-dependent manner, with the lower concentrations inducing apoptosis significantly higher than the relevant controls. Standard marrubiin did not have an effect on apoptosis in hyperglycemic condition, but it decreased the occurrence of apoptosis by 200% (2-fold) under normoglycemic conditions. The OL extract increased proliferation by 148% (1.48-fold) and 155% (1.55-fold) in normoglycemic and hyperglycemic conditions,

respectively. The same effect was observed for standard marrubiin, where, proliferation was increased by 180% (1.8-fold) and 200% (2.0-fold) in normoglycemic and hyperglycemic conditions, respectively. RT-PCR displayed that standard marrubiin inhibited the expression of insulin by 50% under normoglycemic conditions.

CHAPTER 1: Literature Review

1.1 Introduction

According to the World Health Organisation (WHO), diabetes mellitus (DM) is a chronic condition that occurs when the body does not effectively use the insulin it produces or when the pancreas does not produce enough insulin. The basic two types of diabetes are: (a) type 1 diabetes mellitus (T1DM) which occurs when the body produces very little or no insulin, and (b) type 2 diabetes mellitus (T2DM) which occurs when the body does not effectively use insulin. The third type of DM is gestational DM that occurs during pregnancy, but disappears after pregnancy (http://www.who.int/diabetes/BOOKLET_HTML/en/index4.html, 3 January 2006).

T2DM is a multifactorial genetic syndrome which is determined by several genes and environmental factors. More than 150 million people are affected by DM and complications of the disease are increasing because some people cannot meet the resulting medical and financial burdens. The other secondary factors that cause a high incidence rate of T2DM are a lack of physical activity, and obesity. It is predicted that more than 300 million people will be affected by DM in 2025 with the vast majority being cases of T2DM (figure 1.1) (Tilburg *et al*., 2001; Leahy, 2005; Simpson *et al*., 2003; Cheng, 2005).
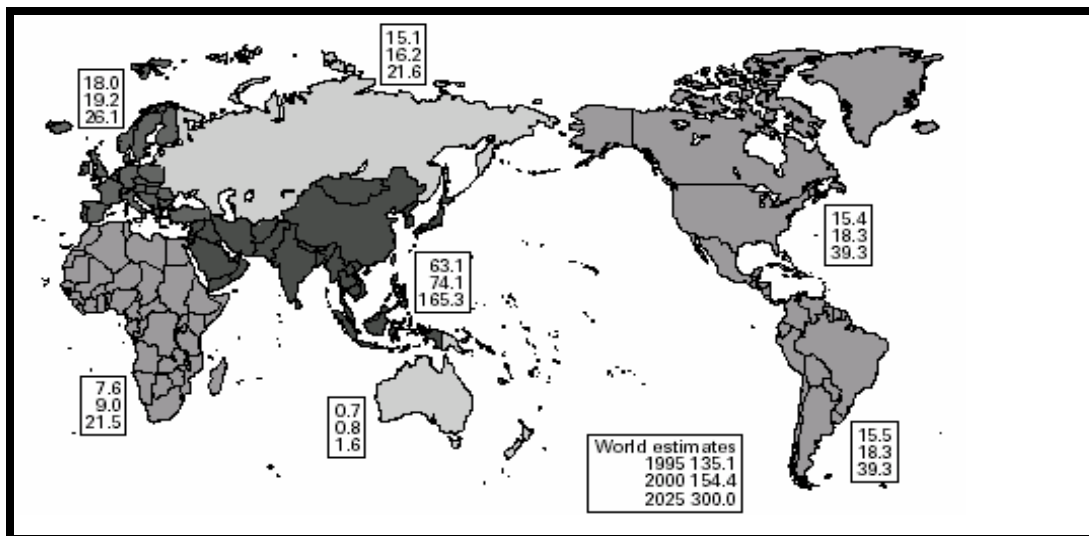
Figure 1.1: **Regional estimates of people with DM (in millions) for 1995, 2000, 2025 (Tilburg *et al*., 2001).**

T2DM accounts for 90% of all cases of DM. The normal glucose homeostasis is controlled by a balance between production of glucose by liver and kidneys, and the glucose uptake by the muscle, adipose tissue, liver, kidney, and brain. The predominant hormone involved in the uptake of glucose is insulin. This hormone enhances the conversion of glucose to glycogen and triglycerides, increases the oxidation of glucose, and inhibits hepatic glucose synthesis. Plasma glucose levels are kept within a small range by multiple mechanisms (figure 1.2) (Tilburg *et al.*, 2001).
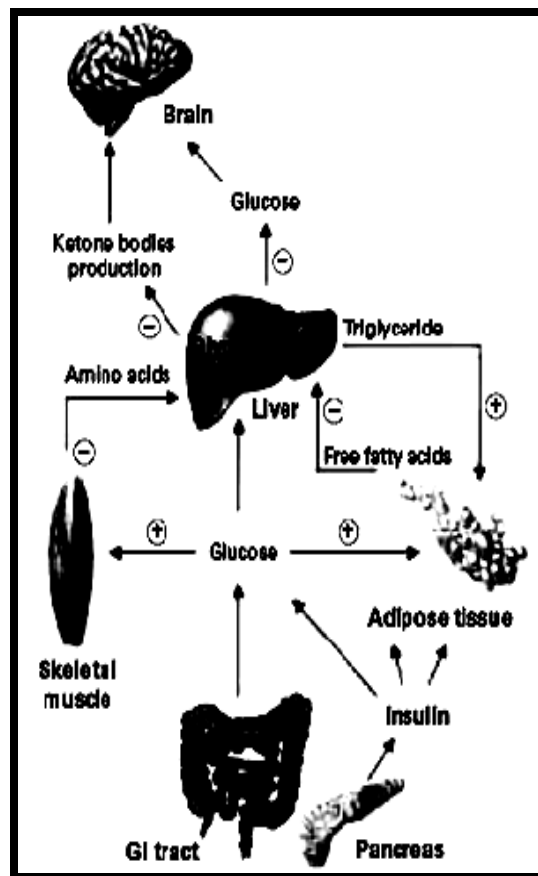
Figure 1.2: Insulin action after a meal. Selected actions of insulin are indicated with + (up regulation) or – (down regulation). Insulin activates transport of glucose to muscle and adipose tissue, and also promotes synthesis of glycogen and triglycerides by the liver. Increased insulin levels inhibit glucose production by the liver, lipolysis in adipose tissue and proteolysis in muscle. They also inhibit ketogenesis by the liver. Although the brain uses glucose as its main source, it can also use ketone bodies when glucose levels are insufficient (for example during fasting) (Tilburg *et al.*, 2001).

Glucose counter-regulatory hormones such as glucagon, cortisol, epinephrine and norepinephrine raise plasma glucose levels and therefore, counteract hypoglycaemia. The balance between insulin and these hormones regulate glucose homeostasis (Tilburg *et al.*, 2001).

1.1.1 Genetic Predisposition

It has been identified that T2DM is a genetic disease. The genetic basis for many monogenic forms of diabetes has been discovered. These monogenic forms include mitochondrial genome defects and the association with diabetes and deafness, Wolfram's syndrome, several syndromes of extreme insulin resistance, and maturity onset diabetes of youth (MODY). These only account for a small proportion of diabetics (Leahy, 2005).

1.1.2 Environment

Environmental factors determine the development of diabetes phenotype. A good example is the positive association between obesity and lack of physical activity in the development of T2DM. Our modern lifestyle also has an effect on the development of T2DM, for example, there is an association between television watching, high calorie diets, and the lack of physical activity with risk of diabetes. The worldwide epidemic of diabetes is caused by these disadvantageous environmental factors. Improvement of the lifestyle can reverse the diabetes trend (Leahy, 2005).

1.1.3 Pancreatic Dysfunction

T2DM arises when the endocrine pancreas fails to secrete insulin to cope with the metabolic load because of the β-cell secretory dysfunction and/ or decreased β-cell mass (Leahy, 2005). This acquired organ dysfunction will be discussed in detail in subsequent sections.

1.2. Insulin Resistance in Type 2 Diabetes

Insulin resistance is defined as impaired insulin-mediated glucose clearance into target tissues. It is a pathological state in which cells fail to respond to ordinary levels of circulating insulin. As a result normoglycemia is maintained by higher than normal concentrations of insulin. The regulation of the secretion of insulin from the pancreatic ß-cells is vital for the control of blood glucose. Under normal conditions, insulin

regulates hepatic glucose production and also stimulates the uptake of glucose into the peripheral tissues, therefore, most of the insulin-mediated clearance of a glucose load goes into skeletal muscle, while the insulin response to a meal shuts down hepatic glucose production (Mlinar *et al.*, 2007; LeRoith and Gavrilova, 2005). In insulin resistance, there is a defect in the liver function, whereby the liver continues to produce glucose and the uptake of glucose into muscle is impaired (Mlinar *et al.*, 2007). High glucose levels may be involved in the etiology of ß-cell failure which leads to the onset of diabetes (figure 1.3). T2DM follows as a consequence if pancreatic cells are unable to compensate for this insulin resistance (Mlinar *et al.*, 2007; LeRoith and Gavrilova, 2005; Leahy, 2005).
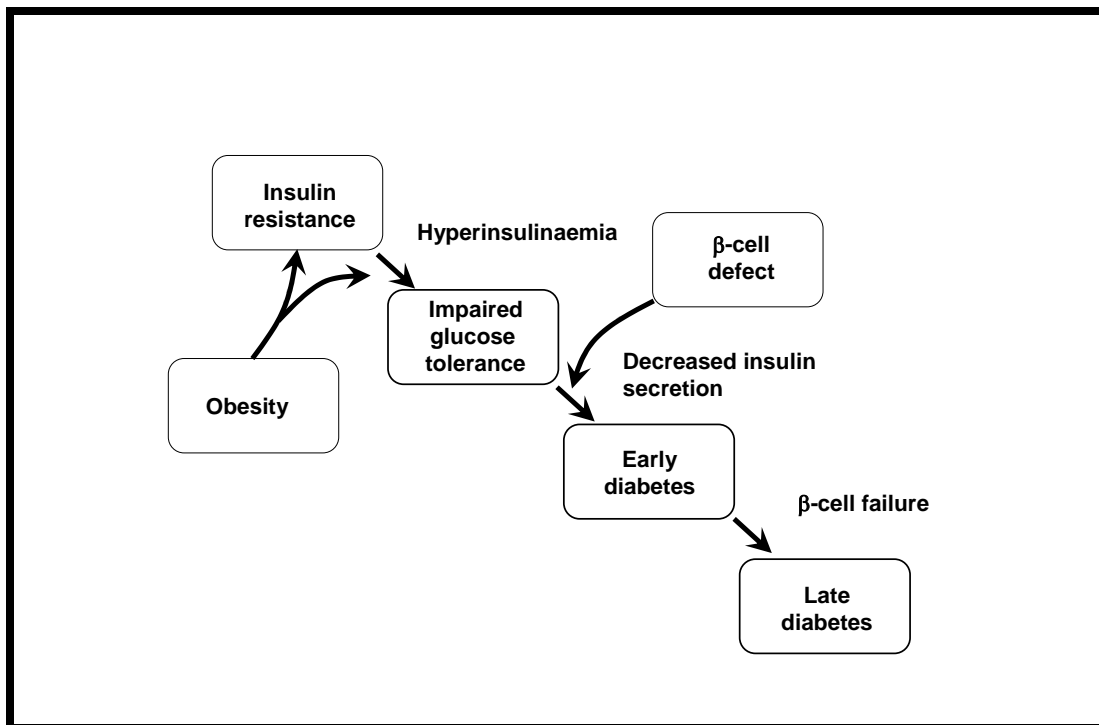
Figure 1.3: Underlying causes of type 2 diabetes (Saltiel, 2000).

1.2.1 Insulin Signalling

The insulin receptor is a member of the tyrosine (Tyr) kinase family. It is a heterotetramer expressed on liver, skeletal muscle, and adipose cells (Mlinar *et al*., 2007; LeRoith and Gavrilova, 2005; Leahy, 2005; and Schinner *et al.,* 2005). When insulin binds to its receptor, it causes activation of the receptor through its autophosphorylation. Scaffolding proteins including insulin receptor substrate (IRS) proteins, Cb1 (casitas B-lineage lymphoma) or Cb1 associated protein (CAP) bind to

intracellular receptor sites as a result of this autophosphorylation (Mlinar *et al*., 2007; LeRoith and Gavrilova, 2005; Leahy, 2005; Schinner *et al.,* 2005; Luna, 2004).

1.2.1.1 Insulin Receptor Substrate Proteins

IRSs belong to a family of proteins that mediate the action of insulin and the insulin growth factors (IGFs), both IGF-I and IGF- II (LeRoith and Gavrilova, 2005; Leahy, 2005; Luna, 2004). In a study conducted by Casellas *et al.* (2006), streptozotocin (STZ)-treated transgenic mice regenerated endocrine pancreas by expressing IGF-1. Expression of IGF-1 increased β-cell replication and neogenesis, and it protected islets from autoimmune destruction, hence, it was concluded that β-cell death may be prevented in mice susceptible to T1DM. A decrease in the levels of IGF-II in neonatal rats has been associated with an increase in β-cell apoptosis, suggesting that IGF-II may have an anti-apoptotic function in β-cells. Overexpression of IGF-II in β-cells, not in the liver, resulted in DM, presumably through insulin production at the same time causing downregulation of insulin sensitivity (Rossetti *et al*., 1996; Petrik *et al*., 1999).

There are four identified members, IRS-1 to 4, but IRS-1 and 2 are the most important IRS proteins in the regulation of carbohydrate metabolism. IRS-1 is the first downstream member of the insulin action cascade, and plays a key role in insulin signalling in skeletal muscle, while IRS-2 is primarily active in the liver (Schinner *et al.,* 2005). Mutations of IRS-1 in humans result in insulin resistance, inactivation of the same proteins in mice resulted in insulin resistance mainly of muscle and fat without diabetes, while mutations of IRS-1 in mice result in growth retardation due to resistance to insulin and IGF-1, β-cell hyperplasia, and impaired glucose tolerance (Zhang, 2002, Bruning *et al*., 1996). In IRS-1 knockout mice, similar results were observed, with mild insulin resistance and no diabetes, suggesting that IRS-1 plays a role in IGF insulin receptor (IGF-IR) signalling (Araki *et al*., 1994). In IRS-2 knockout mice, fasting hyperglycemia and impaired insulin secretion was observed. The IRS-2 knockout mice did not only show insulin resistance of muscle, fat and liver, but also develop evidence of diabetes. From these findings it was concluded that dysfunction of IRS-2 and its downstream targets might represent a common feature of both ß-cell failure and peripheral insulin resistance (Previs *et al*., 2000; Almind *et al*., 1999). Mutations of both IRS-1 and IRS-2 in mice lead to severe insulin resistance in skeletal muscle and liver with marked β-cell hyperplasia (Zhang, 2002).

IRS proteins are not catalytically active themselves. At least two signal transduction pathways are activated by IRS proteins (figure 1.4), one is IRS-phosphoinositide-3′ kinase (PI3′K)-Akt pathway and the other is Ras-mitogen-activated protein kinase pathway (MAPK) (Schinner *et al.,* 2005 and Luna, 2004).

1.2.1.2 Phosphoinositide-3′-kinase/Akt Signalling

In tissues that are involved in whole body fuel metabolism, such as the liver, muscle, and fat, insulin′s effects are mediated through the IRS-PI3′K-Akt pathway (LeRoith and Gavrilova, 2005; Schinner *et al.,* 2005). There are three classes of the PI3′K enzyme. Class Ia is the major effector of insulin signalling and activates Akt pathway by generating phosphatidyl-inositol-3,4-bisphosphate (PIP_2) and phosphatidyl-inositol-3,4,5-triphosphate (PIP_3). Class Ib is a G-protein-regulated kinase. Class II can be activated by insulin, but is unable to generate PIP_2 and PIP_3, therefore, it is unlikely that it would mediate common insulin effects as Class I. Class III appears not to play a role in insulin signalling (Mlinar *et al*., 2007; Schinner *et al.,* 2005).

PI3′K is activated once it binds to phosphorylated sites on IRS proteins. When PI3′K is activated, it generates 3′-phosphoinositides, PIP_2 and PIP_3. PIP_2 and PIP_3 bind to phosphoinositide-dependent kinase 1 (PDK 1). Known substrates of the PDKs are protein kinase B (PKB) and also typical forms of protein kinase C (PKC). Akt (also known as PKB) is a serine/threonine (Ser/Thr) kinase. The Akt pathway mediates the effects of insulin on glycogen synthesis, protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis. The Akt pathway regulates both, glucose uptake via facilitated glucose transporters (GLUTs) and intracellular glucose metabolism in insulin sensitive tissues such as the skeletal muscle. When Akt is inactive, it is located in the cytoplasm, and when it is stimulated with insulin it is translocated to the plasma membrane. The insulin signalling mechanisms are summarized in figure 1.4 (LeRoith and Gavrilova, 2005; Schinner *et al.,* 2005).
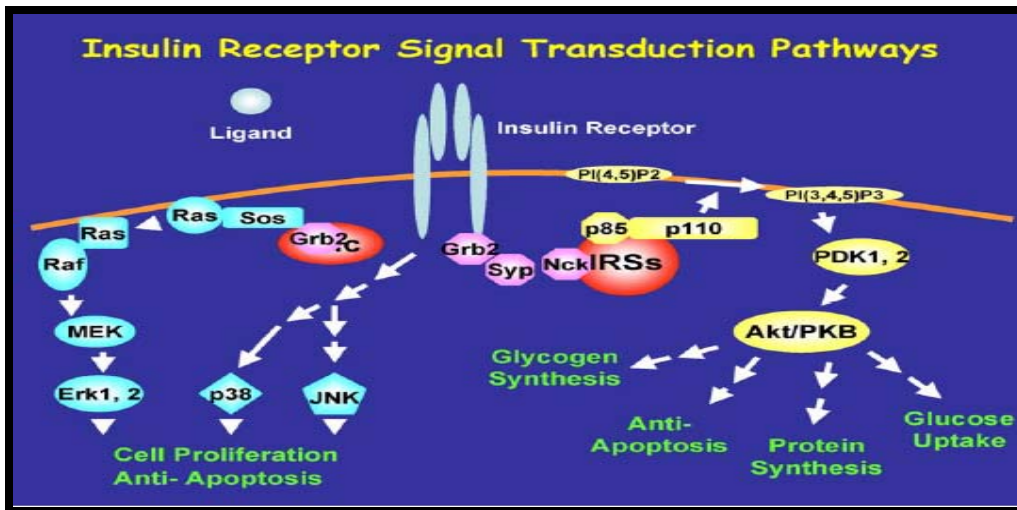
Figure 1.4: Signalling pathways of the insulin receptor. After autophosphorylation of insulin receptor, Tyr (tyrosine) phosphorylation in insulin receptor substrates 1 to 4 (IRS-1 to 4) takes place and subsequently two different pathways transduce the signal: the PI3′K-dependent pathway mediating metabolic responses, including glucose/lipid/protein metabolism and insulin-stimulated glucose uptake, and the mitogen-activated protein kinase (MAPK) pathway that results in cell proliferation and differentiation. IRS—insulin receptor substrate; PI3′K—phosphoinositide-3-kinase; PDK-1—phosphoinositide-dependent kinase 1; PKB—protein kinase B (LeRoith and Gavrilova, 2005).

1.3 Glucose Metabolism

About 75% of insulin-dependent postprandial glucose disposal occurs into the skeletal muscle. When PI3′K is activated, GLUT4 is translocated from the cytoplasm to the plasma membrane with a consecutive increase of glucose transport into muscle and fat. Inhibition of PI3′K with pharmacological inhibitors causes defects in the insulin-stimulated glucose uptake, while overexpression with several PKB constructs causes an increase in GLUT4-mediated glucose uptake. The potential role of PKB in the pathogenesis of insulin resistance has been of central interest in recent years. In one study, the function of PKB was found to be impaired in muscle and adipocytes of diabetic patients (Krook *et al.*, 1998; Rondinone *et al*., 1999), while in a different study, no impairment of PKB activity was observed in muscles of diabetic patients despite the reduced PI3′K activity (Kim *et al.,* 1999). This might indicate that only a small fraction of cellular PI3′K is required to induce full activation of PKB. Three different isoforms of PKB have been identified in mammals (α, β, and γ) (Vanhaesebroeck *et al*., 2000). In an *in vitro* study in human skeletal muscle strips, all three isoforms were found to be

activated by insulin in muscles from lean controls, whereas only PKBα (Akt1) was activated in muscle from obese insulin resistant patients (Brozinick *et al*., 2003). Mice with a knock out of the PKBß (Akt2) show insulin resistance ending up with a phenotype closely resembling T2DM in humans (Cho *et al*., 2001; Cho *et al*., 2004). At the beginning, these PKBß knock-out mice have impaired insulin-mediated glucose disposal and suppression of hepatic glucose production in response to insulin. Finally, they progress to develop a relative ß-cell dysfunction and consecutively manifest diabetes. Therefore, these findings show the essential role played by PKBß in maintaining glucose homeostasis. The function of PKBγ (Akt3) is not elucidated (Schinner *et al.,* 2005; Shulman, 2000).

1.4 Glucose oxidation

The insulin resistance appears to be caused in part by the presence of high levels of lipids in cells such as skeletal muscle where this would not normally be found. The presence of excess lipid stores in skeletal muscle cells interferes with energy metabolism, impairing glucose oxidation and insulin response. Skeletal muscle is one of the primary glucose-consuming tissues, giving it a central role in insulin resistance (Mullen *et al*., 2007). The increased risk of DM associated with obesity may be caused by increased lipid deposits in skeletal muscle and liver, creating insulin resistance (Mullen *et al*., 2007).

Leptin is a peptide hormone secreted by adipose tissue that has been associated with many processes. One of the target tissues of leptin is the hypothalamus where it can act to regulate feeding behavior and metabolism. Another leptin target is skeletal muscle. Activation of leptin signaling in skeletal muscle activates the AMP-activated protein kinase (AMPK). AMPK phosphorylates and inactivates the enzyme acetyl- coenzyme A (CoA) carboxylase (ACC). ACC catalyzes the production of malonyl-CoA from acetyl-CoA. Malonyl-CoA in turn is an inhibitor of the importotion of fatty acids into mitochondria by carnitine palmitoyl-transferase I for oxidation and energy production. In the presence of leptin, AMPK is activated, ACC is inhibited, and malonyl-CoA levels fall, increasing the oxidation of fatty acids and reducing the lipid content of cells. The reduced lipid content in skeletal muscle allows insulin signaling and glucose consumption to return to their normal levels, reducing insulin resistance (Mullen *et al*., 2007).

In genetically obese mice, the stimulatory effect of AMPK by adiponectin in muscle is lost (Mullen *et al*., 2007). The majority part of glucose taken up from blood after insulin stimulation is stored as glycogen in skeletal muscle (Shulman, 2000). Glycogen synthase kinase-3 (GSK-3) is a critical enzyme regulating glycogen synthesis (Shulman, 2000). Dysregulated glycogen synthesis is a critical feature in diabetes mellitus (Shulman, 2000). Glycogen synthesis rates are 50% lower in diabetic patients compared to healthy individuals. Glycogen synthase (GS) is inhibited when it is phosphoryled by GSK-3. PKB inhibits GSK-3 by phosphorylation (Summers *et al*., 1999). Expression of GSK-3 mutant that is insensitive to PKB phosphorylation results in a reduction of insulin-mediated glycogen synthesis. In insulin resistance, impaired hepatic glycogen storage and GS activity are common findings (Schinner *et al.,* 2005).

1.5 Hepatic Glucose Production

The increase of glucose production by the liver because of insulin resistance is correlated to the fasting hyperglycemia in patients with T2DM. This is a result of the lack of inhibition of the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) catalytic subunit. Normally, these enzymes are inhibited by insulin at a transcriptional level via the activation of PKB. The promoters of both PEPCK and G-6-Pase genes contain insulin-responsive elements (IRE) (Schinner *et al.,* 2005). *In vitro* studies have shown that three Foxo-family transcription factors (Foxo1a, Foxo3a and Foxo4) bind to IRE of these genes, and phosphorylation of Foxo-proteins by PKB, results in inactivation and consequently inhibition of target gene expression (Hall *et al*., 2000; Wolfrum *et al*., 2003). Foxo-proteins have been shown to be involved in the insulin-dependent regulation of gluconeogenic gene expression and insulin resistance *in vivo*. The partial knockout of the Foxo1 gene in insulin resistant mice resulted in reduced G-6-Pase-mRNA and insulin levels comparable with metabolically unaffected control animals. Therefore, these findings show a relationship between Foxo1 regulation by insulin and glycemic control *in vivo* (Nakae *et al*., 2002). In addition, the peroxisome proliferators activated receptors-γ (PPARγ) co-activator-1 (PGC-1), a factor intergrating the effects of glucocorticoids and cyclic adenosine monophosphate (cAMP) on gluconeogenic gene expression in the liver is also regulated by PKB and Foxo1 (Puigeserver *et al*.,

2003). This provides additional evidence that PKB and Foxo1 are critical parts of the network integrating hepatic glucose production (Schinner *et al.,* 2005).

In addition to the Foxo-transcription factors, members of the hepatocyte nuclear factor (HNF) family of transcription factors may be involved in regulation of glucose metabolism by insulin. Interaction of HNF1 with IRE enhances the inhibitory effect of insulin on the promoter of the G-6-Pase. Mice with defective or inactivated HNF1 develop diabetes due to defective glucose-stimulated insulin secretion (GSIS), as a result of impaired glycolysis. The insulin content of these mice is diminished although the number of ß-cells is not reduced. HNF4 is involved in the PI3′K-Akt-dependent stimulation of glucokinase gene expression by insulin, which is an important mechanism to increase glycolysis (Streeper *et al*., 1998). On the molecular level, HNF4 may directly interact with Foxo1, and Foxo1 may act as an inhibitor of HNF4. In this setting, insulin stimulates HNF4-transcriptional activity by sequestrating Foxo1 from HNF4 (Hirota *et al*., 2003). However, although genetic defects of some HNF-transcription factors (HNF1α, and HNF4α) are the basis of some forms of MODY, the role of HNF-transcription factors in the pathogenesis of T2DM remains unclear (LeRoith and Gavrilova, 2005; Schinner *et al.,* 2005; Hirota *et al*., 2003).

1.6 The Role of Adipose Tissue in Insulin Resistance

Dysfunction of adipose tissue plays a critical role in the development of insulin resistance and T2DM. Li *et al.* (2002) showed that there is a difference in gene expression of rat adipose tissue at the onset of obesity. After 2 weeks of diet-induced obesity (DIO) (high fat diet), there was a 300% (3-fold) increase in wet weight of the epididymal fat pads, which could lead to hyperplasia of white adipose tissue if the diet was prolonged. After 1 week of high fat diet, 96 genes were differentially upregulated when compared to control rats (normal diet). The largest group of genes that were upregulated were involved in mitochondrial functions, these included stearyl-CoA desaturase, cytochrome c oxidase subunits, and cytochrome b. Genes that were involved in carbohydrate and protein metabolism (cytosolic malate dehydrogenase, carboxyl esterase, glycerol 3-phosphate dehydrogenase, ribosomal proteins elongation factor-1, proteasome activator (PA) 28, and a gene similar to human regulator of non-sense transcripts 2), thermogenesis (70-kDa and 27-kDa heat shock proteins), TNF-α

which is involved in insulin resistance, adipocyte-specific proteins (leptin and resistin), and cell growth or differentiation proteins (lipogenin and IGF-1) were also upregulated.

Obesity leads to whole body insulin resistance. By regulating the levels of circulating free fatty acids (FFAs) and by secreting adipokines, adipose tissue can modulate whole body glucose metabolism. The accumulation of fatty acids or their metabolites result in an impairment of signalling through IRS/PI3′K, also potentially involving PKCθ finally resulting in reduced translocation of GLUT4 into the plasma membrane (White, 2002). The accumulation of FFAs may increase Ser phosphorlyation of IRS proteins, thereby impairing insulin signal transduction (Schinner *et al.,* 2005; Leahy, 2005; Kahn, 2003; Leiter and Lewanczuk, 2005; Shulman, 2000; White, 2002).

The most known studied adipokines are tumor necrosis factor-α (TNF-α), IL-6, leptin, adiponectin and resistin. TNF-α is a cytokine produced by adipocytes (Schinner *et al.,* 2005). TNF-α can impair insulin action on glucose metabolism and increase lipolysis. TNF-α increases serine (Ser) phosphorylation of IRS-1 and downregulates GLUT4 expression, thereby contributing to insulin resistance (Saltiel and Kahn, 2001). The role of leptin in the regulation of food intake and energy expenditure is well established. Mutations in the leptin receptor cause obesity in humans. Leptin has a direct effect on insulin sensitivity, it reverses insulin resistance in mice with congenital lipodystrophy (Shimomura *et al*., 1999). The adipokine adiponectin has insulin-sensitizing effects as it enhances inhibition of hepatic glucose output, while increasing glucose uptake and utilization in fat and muscle (Stumvoll and Haring, 2002). In obese individuals, the expression of adiponectin is decreased. In humans, adiponectin levels correlate with insulin sensitivity (Kubota *et al*., 2002). Mice that are deficient in adiponectin are insulin resistant, this state is reversed by administration of adiponectin. Adiponectin also plays a role in the regulation of body weight (Maeda *et al*., 2002). In rodents, resistin decreases insulin-dependent glucose transport *in vitro*, while it increases fasting blood glucose levels and hepatic glucose production *in vivo*. The latter was reflected by reduced levels of mRNA of G-6-Pase and PEPCK in livers from resistin knockout mice (Steppan *et al.,* 2001; Pravenec *et al*., 2003; Rajala *et al*., 2003), however, the physiological significance of resistin in humans is less clear (Degawa-Yamauchi *et al*., 2003).

1.7 β-Cell Dysfunction in Type 2 Diabetes

Although insulin resistance and the resulting impairment in glucose tolerance are early signs of diabetes, malfunction or even death of the insulin-producing ß-cells also contribute to the disease. Ultimately about a third of T2DM patients are required to take insulin (Bergman, 1989). The disruption of the normal relationship between ß-cell function and insulin sensitivity is central to the pathogenesis of hyperglycemia in T2DM patients. When islet ß-cell function is impaired, underproduction of insulin occurs, leading to overproduction of glucose by the liver and under-utilization of glucose in the peripheral tissues. Therefore, the goal of therapeutic interventions is to reduce glucose levels by (a) improving ß-cell function to increase insulin secretion, (b) reducing hepatic glucose production, and (c) improving glucose uptake in the peripheral tissues (Kahn, 2000; Fujimoto, 2000; Leahy, 2005; Kahn, 2003; Leiter and Lewanczuk, 2005).

1.7.1 Cellular Mechanisms Controlling Adult β-Cell Mass

At least four independent mechanisms regulate the mass of β-cells, namely (a) β-cell replication, (b) β-cell size, (c) β-cell neogenesis, and (d) β-cell apoptosis. The sum of the rates of the first three mechanisms minus the rate of β-cell apoptosis equals the net rate of β-cell growth. The contribution made by each mechanism toward β-cell growth changes with the different stages of life or when β-cell adapts to changes in metabolic load (Cerf, 2006; Rhodes, 2005; Bernard-Kargar and Ktorza, 2001).

It is difficult to measure the changes in β-cell mass because some entities can be overlooked. Markers of cell division such as Ki-67 may underestimate the incidence of β-cell replication because they correspond to a small transient window in the cell cycle. Likewise, apoptotic and necrotic cells are efficiently cleared by macrophages *in vivo*, therefore, the extent of β-cell apoptosis analyzed in pancreatic sections may be underappreciated. Multiple pancreatic sections are required for detection of pancreatic β-cell neogenesis. It then becomes difficult to judge whether insulin positive cells actually mature into fully differentiated β-cells or are alternative cell types that have been misclassified without the use of specific markers for precursor β-cells. These technical difficulties notwithstanding, a model of postnatal pancreatic β-cell growth in humans is emerging from studies of both humans and rodents. In normal conditions, there is a transient burst in β-cell replication just after birth, followed by a transitory

rise in β-cell neogenesis. In the later stage of this neonatal burst of β-cell neogenesis there is also a modest amount of apoptosis that parallels pancreatic islet rearrangement. Because the rate of apoptosis is low, there is a net increase in β-cell growth early in life. The early burst of β-cell growth is observed in rodents, although it is speculated the same mechanism occurs in humans. Elderly people are susceptible to T2DM because in senior years of life the rate of β-cell apoptosis increases and outweighs the rate of β-cell replication and neogenesis (Cerf, 2006; Rhodes, 2005).

1.7.2 Adaptation of β-Cell Mass to Metabolic Load

During adulthood, pancreatic β-cell mass adapts to changes in metabolic homeostasis. A good example is pregnancy. During pregnancy in rodents, the β-cell mass can almost double to compensate for the increase in metabolic load, because of the developing fetus. A similar adaptation likely occurs in humans. In a study conducted by Persson e*t al.* (1995) on pregnant women, adequate glucose control during gestation was observed. Maternal insulin response to glucose increased significantly with gestation and was much above that observed in non-pregnant people, hence, it was speculated that, this increase in β-cell mass is driven by two pregnancy hormones, prolactin and placental lactogen. The concentration of these two hormones dramatically increased during the last trimester. After birth, the β-cell mass returns to normal levels due to an increase in β-cell apoptosis and a decrease in β-cell replication (Rhodes, 2005; Persson e*t al.* 1995).

In obesity and insulin resistance (rodents and humans), β-cell mass adapts because of an increase in β-cell replication and neogenesis, and also hypertrophy. A 50% increase in β-cell mass of obese humans was observed when compared to normal-weight control humans (Butler *et al*., 2003; Ritzel *et al.*, 2006). A small increase in β-cell apoptosis is observed in nondiabetic obesity (humans), but this is compensated for by the net increase in β-cell replication, neogenesis, and cell size (Ritzel *et al*., 2006; Bernard-Kargar and Ktorza, 2001).

1.7.3 Failure of β-Cells Mass to Compensate for Metabolic Load

Although there might be an initial compensatory increase in β-cell mass, the onset of T2DM is accompanied by a progressive decrease in β-cell mass in rodents and humans (Butler *et al*., 2003). As a result, the body can no longer adapt to changes in metabolic

load, including insulin resistance associated with obesity. As T2DM state progresses, the decline in β-cell mass occurs, because β-cell apoptosis outweighs β-cell replication and neogenesis (Rhodes, 2005; Bernard-Kargar and Ktorza, 2001).

1.7.4 Lowered β-Cell Mass in Type 2 Diabetes

ß-cells compensate for insulin resistance by increasing their cell mass, but diabetic patients have a low ß-cell mass (Butler *et al*., 2003). This is because of the development of amyloid plaques on the islets of diabetic patients. The replacement of ß-cells by islet amyloid plaques may be important. Glucose desensitisation or glucotoxicity and lipotoxicity may be associated with the development of these structures. The islet amyloid plaques develop from a normal ß-cell secreted protein that is co-packaged with insulin in insulin granules. This 37 amino acid peptide is called Islet Associated Polypeptide (IAPP) also called amylin. The reason why this protein spontaneously forms amyloid fibrils in patients with T2DM but not in healthy individuals is unclear. The amyloid fibrils cause ß-cell destruction in early stages of T2DM. The development of amyloid fibrils induces apoptosis, which is the cause of the lowered ß-cell mass in T2DM. Amyloid also appears in elderly normal people (Butler *et al*., 2003; Cerf, 2006).

Some studies state that amyloid deposits accumulate in the pancreatic islets of the majority of people with T2DM and are associated with the decreased islet ß-cell mass and function which characterize the disease, hence, amyloid does not cause T2DM but is a consequence of T2DM (Butler *et al*., 2003; Clark *et al*., 1988). For islet amyloid to occur, an amyloidogenic form of IAPP is required; thus, humans, non-human primates and a few other species may spontaneously develop islet amyloidosis (Westermark *et al*., 1990). In contrast, rats and mice do not develop islet amyloid due to differences in the amino acid sequence of rodent IAPP which render it non-amyloidogenic. Thus, transgenic mice expressing the amyloidogenic human IAPP in their islet ß-cells have been developed by a number of groups as models of islet amyloid formation (Yagui *et al*., 1995). The contribution that genetic background strain makes in amyloid formation in human IAPP is not known. Previous studies failed to show islet amyloid formation in human IAPP transgenic mice (Yagui *et al*., 1995; Hoppener *et al*., 1993). It was later discovered that genetic manipulations were required to form amyloid. Interbreeding of human IAPP mice with mice carrying mutations associated with β-cell dysfunction together with severe obesity and insulin resistance that leads to T2DM was required in

order to observe amyloid deposition similar to that observed in T2DM (Hoppener *et al*., 1999; Soeller *et al*., 1998). More research is required to fully understand the role that the amyloid plays in T2DM.

1.7.5 The Role of IRS-2 Signalling in β-Cell Survival

Both IRS-1 and IRS-2 are expressed in pancreatic β-cells, however, IRS-1 is involved in cellular calcium ion (Ca^{2+}) homeostasis while IRS-2 plays a critical role in β-cell growth (Whithers *et al*., 1999). Suppression of the expression of IRS-2 may lead to an increase in the incidence of β-cell apoptosis and consequently the onset of T2DM. An increase in β-cell mass occurs when IRS-2 is phosphorylated. However, IRS-2 contains multiple sites for Ser/threonine (Thr) phosphorylation, subsequently resulting in IRS-2 ubiquitination, proteosomal degradation, and ultimately β-cell apoptosis (figure 1.5) (Rhodes, 2005). Inactivation of insulin during its circulation in blood, and defects at the receptor and postreceptor signalling levels occurs when insulin action is impaired. Insulin inactivation may be caused by products of hemolysis (Steinke and Driscoll, 1967). A minority of insulin resistant cases is characterized by a single genetic or acquired trait. The abnormal production of anti-insulin receptor antibodies may cause insulin resistance, this incidence is quite low (Rosenstein *et al*., 2001). In an insulin resistance state there is an increase in the degradation of insulin receptor. McElduff *et al*. (1984) studied the degradation rate of the insulin receptor in several genetic syndromes of extreme insulin resistance, and they found that cells from patients with insulin resistance with either low-normal or absolutely low binding have accelerated rates of receptor degradation. Normal degradation rates from cell lines derived from patients with autoantibodies to insulin receptor were used to demonstrate the specificity of accelerated receptor degradation in genetic syndrome. In the genetic syndrome, the presence of the autoantibodies results in extreme insulin resistance *in vivo*. Normal receptor concentrations are maintained by an increase in receptor synthesis during receptor degradation, but, in patients with a genetic syndrome, synthesis does not occur. About 90% reduction in receptor concentration was observed in these patients. Defects in post-translational synthesis of the receptor could be a cause of this state. Abnormalities in LDL receptor were found in patients with hypercholesterolemia due to deficiency in LDL binding sites (Tolleshaug *et al*., 1982). Mutations in insulin receptor gene at the ATP binding site reduce the binding of insulin to its receptor and receptor autophosphorylation (Xie *et al*., 2002). A decrease or lack of receptor Tyr kinase

activity is caused by the disruption of autophosphorylation, leading to a failure of postreceptor effects exerted via IRS-1 (Chou *et al.*, 1987). This dysfunction in the insulin transduction cascade may cause a drastic fall in glucose metabolism and contributes to the development of insulin resistance (Xie *et al.*, 2002). Fibroblast from obese individuals that were transfected with DNA of mutant insulin receptors showed a 90% reduction in high-affinity insulin binding sites (Maassen *et al.*, 2003). Leprechaun patients have a mutation near the carboxyl terminal of the insulin receptor, Asp707 is changed into Ala. Fibroblasts of these patients had no high-affinity binding sites. Hart *et al.* (1996) showed that Ala707 insulin receptor is processed and transported to the cell surface but this mutation distorts the insulin binding sites.

1.7.5.1 Involvement of Endoplasmic Reticulum Stress and Inflammatory Pathways in Insulin Resistance

The increase in Ser/Thr phosphorylation of IRS-1/2 contributes to insulin resistance in mammals (Jager *et al.*, 2007). It is evident that FFA directly or by the intermediate of TNF-α causes this increase in Ser/Thr phosphorylation. The basic mechanism for inflammatory mediators to induce insulin resistance is to interfere with the normal signalling downstream events of the insulin receptor (Ruan and Lodish, 2003; Ruan and Lodish, 2004; Fantuzzi, 2005; Kovacs and Stumvoll, 2005; Tzeng *et al.*, 2005; Bjornholm and Zierath, 2005). PKCs, PI3′K-downstream kinases, and MAPKs are thought to be responsible for Ser/Thr phosphorylation which is followed by an enhanced proteosomal degradation of IRS-1/2. Successful Tyr phosphorylation and insulin signaling requires a certain basal Ser/Thr phosphorylation state of IRS-1. TNF-α and FFAs induce inhibitory phosphorylation on Ser/Thr residues of IRS-1/2 which becomes unable to bind to PI3′K. The interference of TNF-α and FFAs with the insulin signaling pathway also induces other inflammatory pathways (figure 1.5). These pathways can be induced through endoplasmic reticulum (ER) stress (Ozcan *et al.*, 2004). Ozcan *et al.* (2004) tested whether experimental manipulations of ER stress responses could lead to modifications of insulin receptor signaling and insulin action. ER stress was induced in cultured cells by tunicamycin or thapsigargin exposure or by deletion of x-box-binding protein-1 (XBP-1), a critical transcription factor regulating unfolded protein response. Generation of ER stress led to an increase in IRS-1 Ser phosphorylation and suppressed insulin-induced insulin receptor signal transduction. Inositol-requiring enzyme-1 (IRE-1)-dependent activation of Jun-N-terminal kinase

(JNK) mediated cellular insulin resistance caused by ER stress. Insulin resistance could be refracted by overexpression of XBP-1 leading to a reduction of ER stress. The relationship between ER stress and insulin resistance was also demonstrated in whole animals. Mice with XBP-1 haplo-insufficiency were introduced to a high-fat diet, which induced ER stress, insulin resistance, and hyperglycemia compared to wild-type controls. In liver and adipose tissues of these animals, there was an increase in ER stress markers including an increase in JNK activity and IRS-1 Ser phosphorylation, thus, these animals had an impaired insulin receptor signaling in liver and adipose tissues. In lean animals, there was no change in insulin sensitivity or insulin receptor signalling capacity in liver or adipose tissues (Ozcan *et al.*, 2004). These studies show that there is a link between ER stress responses, insulin receptor signaling and insulin action. Modifications in ER stress responses have an impact on systemic insulin sensitivity.

The metabolically overloaded ER leads to the activation of the pro-inflammatory intracellular pathways. The inflammatory pathways are activated via the overproduction of the reactive-oxygen species (ROS) by an increase in glucose metabolism inducing mitochondrial stress. FFA induces the activation of IκB kinase (IKK), JNK, and PKCθ converging to the activation of nuclear factor-kappa B (NF-κB). TNF-α and FFAs also induces the activation of Suppressor of Cytokine Signalling-3 (SOCS-3), which binds with the insulin receptor and suppresses receptor autophosphorylation and IRS-1 phosphorylation on Tyr. Ubiquitination and proteosomal degradation of IRS-1/2 is induced by SOCS-3. TNF-α and FFAs also stimulate the activation of the protein tyrosine phosphatase 1B (PTP1B). PTP1B terminates the normal insulin signaling. TNF-α and FFA also interfere with the activation of PPARγ which is a direct antagonist of NF-κB (Mlinar *et al.*, 2007; Fulop *et al.*, 2006; Jager *et al.*, 2007). The activation of these inflammatory pathways leads to phosphorylation of IRS-1/2, ultimately causing insulin resistance (Ozcan *et al.*, 2004).
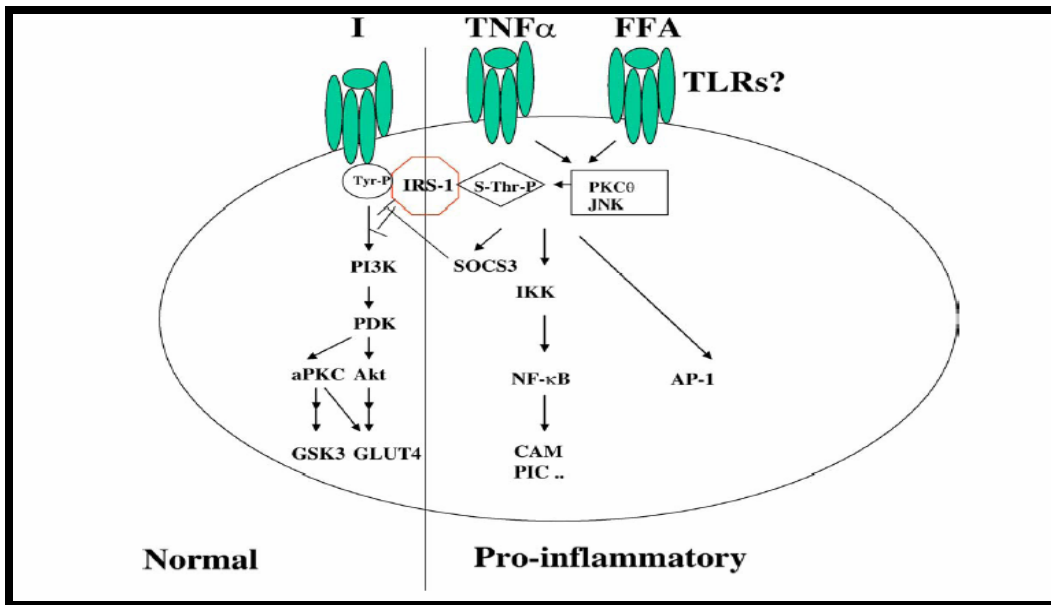
Figure 1.5: Signalling pathways leading to the TNFα- and FFAs-induced insulin resistance. Normally insulin induces the tyrosine phosphorylation (Tyr-P) of the IRS-1 which leads to the translocation of the GLUT4 to the membrane via PI3′K, PDK, activated PKC and Akt. When there is a pro-inflammatory status TNFα and FFA induce the serine-phosphorylation (S-Thr-P) of the IRS-1 and SOCS3 leading to the inhibition of the normal insulin receptor signalling. Concomitantly, the inflammatory mediator transcription factors, NF-κB, and AP-1, are activated via JNK, PKCθ and IKK activation. This results in the production of cellular adhesion molecules (CAM) and pro-inflammatory cytokines (PIC). I: insulin; TLRs: toll like receptors (Fulop *et al*., 2006).

1.7.5.2 Glucotoxicity, Lipotoxicity, and β-cell Function

High glucose levels impair one or more key aspects of ß-cell physiology and/or gene expression. In conditions whereby the ß-cells are exposed to abnormally high levels of glucose, essentially every major pathway, key enzymes, and important genes in the ß-cells are altered. The mammalian Target of Rapamycin (mTOR) in β-cells which is a nutrient-sensing Ser/Thr protein kinase is activated in chronic hyperglycemia (Zhande *et al*., 2002; Briaud *et al*., 2005). The activation of this protein kinase triggers the Ser/Thr phosphorylation of IRS-2 and its subsequent proteosomal degradation, leading to increased β-cell apoptosis (Zhande *et al*., 2002; Briaud *et al*., 2005). Chronic hyperglycemia triggers β-cell apoptosis by additional mechanisms referred to as glucotoxicity (Briaud *et al*., 2005). During glucotoxicity, potentially damaging ROS are generated. Ca^{2+} levels are also raised to cytotoxic levels, and the expression of IAPP is also increased (Weyer *et al*., 1999). Some of these metabolic stresses can activate the

JNK/p38 stress protein in turn leading to Ser/Thr phosphorylation of IRS-2 and its degradation (Zhande *et al.*, 2002; Briaud *et al.*, 2005).

Lipotoxicity is when an excess of FFAs are detrimental to ß-cell function and viability. The ceramides which are metabolic products of FFAs and precursors of oxidative stress induce ß-cell dysfunction and death (Song *et al.*, 2003; Unger, 1995). For lipotoxicity to occur, high glucose levels must be present for production of high levels of malonyl-CoA that would inhibit fatty acid oxidation; otherwise, the fatty acids would be oxidized and thus detoxified (Leahy, 2005). Fatty acids activate a novel class of PKC isoforms (nPKC) through the production of acyl-CoA. Activation of nPKC triggers the Ser/Thr phosphorylation of IRS molecules that in ß-cells would promote IRS-2 degradation and ultimately leads to ß-cell apoptosis (Prentki *et al.*, 2002). Another lipotoxic mechanism that can lead to IRS-2 degradation is through the generation of ceramide from palmitate (Prentki *et al.*, 2002). The ceramide induces a mitochondrial apoptotic pathway and inhibition of PKB. The JNK/p38 stress protein is also activated (Mlinar *et al.*, 2007).

The cytokines induce ß-cell apoptosis through signalling pathways that activate the transcriptional factor NFκB. Some cytokines activate the JNK 2/Signal Transducer and Activator of Transcription (JAK/STAT) post-receptor signalling pathway. This leads to increased expression of the SOCS-1 and SOCS-3 proteins which normally binds to leptin, interleukin-6 (IL-6), and interferon-γ (IFN-γ) receptors and inhibit the JAK/STAT signalling. SOCS-1 and SOCS-3 bind to the C-terminal of IRS proteins thereby leading to their ubiquitination and subsequent degradation (Kile *et al.*, 2002). The mechanisms by which IRS-2 causes loss in β-cell mass is explained in figure 1.6.
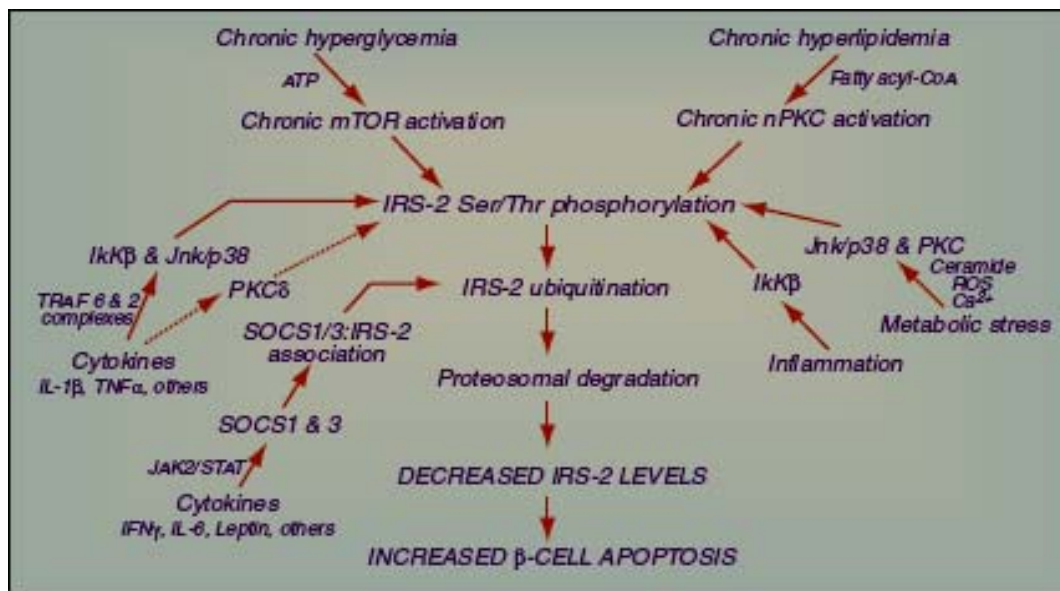
Figure 1.6: Potential mechanisms that trigger IRS-2 degradation and apoptosis of β cells. IRS-2 expression in β-cells is vital for normal β-cell growth, survival, and turnover. Chronic hyperglycemia by means of mTOR activation and hyperlipidemia by means of fatty acyl-CoA-mediated activation of nPKC can lead to increased serine/threonine phosphorylation of IRS-2 that then leads to its ubiquination and subsequent proteosomal degradation. In addition, certain cytokines, including Il-1 β and TNF-α, activate IκKβ and JNK/p38 kinases and/or PKCδ, which in turn also leads to IRS-2 Ser/Thr phosphorylation. Other local inflammatory responses can activate IκKβ and other metabolic stresses can induce expression of SOCS-1 and SOCS-3, which can then bind to IRS-2, leading to its ubiquination and subsequent proteosomal degradation. This can significantly lower IRS-2 levels in the β-cell. The resultant increase in β-cell apoptosis is thought to be a key factor contributing to the loss of β-cell mass in T2DM (Rhodes, 2005).

1.8 Thrombosis and Diabetes

T1DM and T2DM are associated with high risk thrombotic complications, and abnormalities of the inflammatory response. DM patients are at high risk to develop cardiovascular disease, where DM is also considered to be a hypercoagulable state. Hypercoagulability in a broad sense includes platelet function and fibrinolysis, these both play a critical role in the pathogenesis of cardiovascular disease and thrombosis in diabetic patients (Lyon and Hsueh, 2003; Colwell, 2001; Kwaan, 1992).

The main coagulation reactions are divided into two reactions, intrinsic and extrinsic systems. The initial step in coagulation is the activation of tissue factor. The intrinsic coagulation system is initiated by the activation of factor XII on contact with a

negatively charged surface. The formation of a complex composed of tissue factor and factor VII is induced by the extrinsic coagulation system. The enzyme complex called prothrombinase forms thrombin from its derivative or zymogen prothrombin. The activation of the intrinsic and extrinsic coagulation cascades leads to the generation of thrombin, the activation of fibrinogen, the release of fibrinopeptides, the formation of soluble fibrin, and finally, the formation of factor XIII-mediated, cross linked, insoluble fibrin. In the fibrinolytic system, the proteolytic enzyme plasmin which is formed from plasminogen by tissue plasminogen activator (tPA), lyses the fibrin deposists and thrombi. The activators of the fibrinolytic system are of the urokinase-type plasminogen activator (uPA) and tPA. The catalytic action of these proteases is modulated by the inhibitors of plasmin and plasminogen activators. The inhibitors of fibrinolysis are plasminogen activator inhibitor-1 (PAI-1), α_2-antiplasmin, and α_2-macroglobulin (figure 1.7) (Lyon and Hsueh, 2003; Colwell, 2001; Kwaan, 1992).
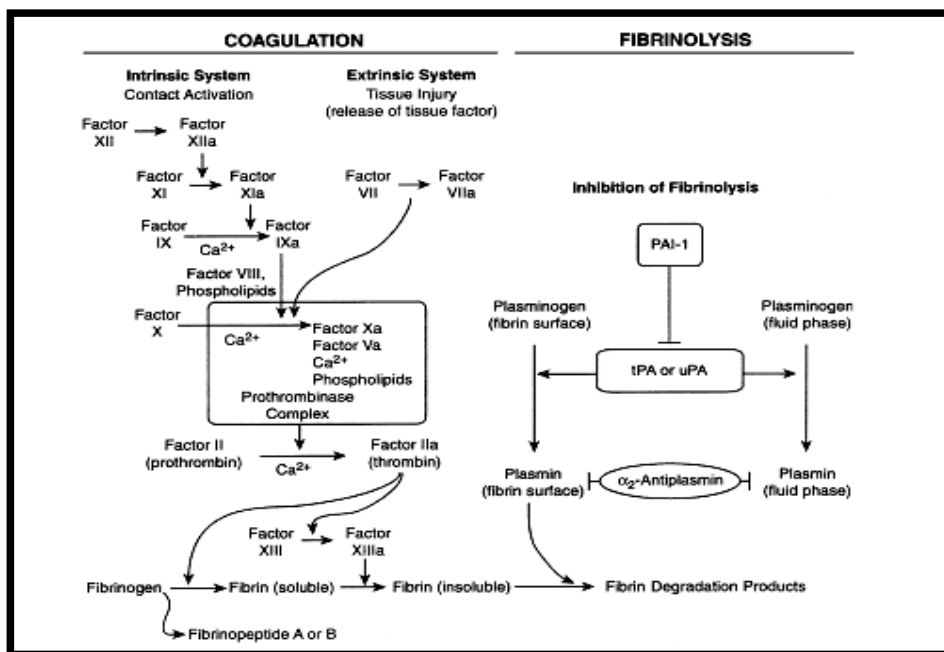
Figure 1.7: Coagulation (*left*) and fibrinolytic (*right*) pathways (Lyon and Hsueh, 2003).

1.8.1 Changes in the Coagulation System

As has already been mentioned, the activation of the coagulation pathway leads to the generation of a fibrin clot from fibrinogen by the action of thrombin. Most of the factors in the intrinsic pathway have been shown to be altered in diabetic patients (Ceriello, 1990). The changes in plasma levels of glucose or insulin affect the plasma levels of the coagulation factors. For instance, plasma fibrinogen levels correlate with

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glycemic regulation in cross-sectional studies in DM, these levels may fall during insulin therapy in humans (Ceriello, 1997). There is evidence of a correlation between plasma levels of fibrinopeptide A (FPA), thrombin-antithrombin complex (TAT), and hyperglycemia. A rise in plasma glucose levels causes a rise in plasma levels of FPA and a decrease in plasma levels of TAT (Jokl and Colwell, 1997; Jokl and Colwell, 1997). The inhibitors of the coagulation system in diabetic patients are depressed as the coagulant activity increases. These inhibitors include antithrombin III, a physiological inhibitor of factors X and II, and protein C, which inhibits factor V and VIII. The levels of TAT in plasma reflects the amount of thrombin formed, therefore, increased plasma levels of this complex suggests the activation of the coagulation cascade in the bloodstream. In diabetic patients high levels of the complex were observed (Jokl and Colwell, 1997). At the cellular level, monocytes of diabetic patients show a greater procoagulant activity. At a systemic level, because of hyperglycemia, non-enzymatic glycation may occur. Likewise, the activators of plasminogen may activate an impaired plasminogen because of the glycation of plasminogen, and the glycation of the platelet membrane protein complex IIb-IIIa, which is the platelet receptor for fibrinogen, may account for the increased platelet aggregation in diabetic patients. An infusion of insulin reverses these changes in coagulation (Kwaan, 1992; Matsuda *et al*., 1996).

1.8.2 Fibrinolytic System

Under physiological conditions, several pathways modulate the fibrinolytic activity of the endothelium. Two of the pathways are found to be altered in diabetic patients (Juhan-Vague *et al*., 1991). One pathway is the thombomodulin-protein C reaction, a mechanism by which protein C is activated by the secretion of thrombomodulin by the endothelial cells. In addition to the inhibition of clotting factors V and VIII, activated protein C, enhances fibrinolytic activity through its inhibition of PAI-1. The second pathway involves lipoprotein A. Lipoprotein A has a binding domain homologous to that of plasminogen, the activation of which causes fibrinolysis, and thus, lipoprotein A can compete with plasminogen in impairing several fibrinolytic functions, including binding with fibrin, with plasminogen receptors on the endothelial surface, and with heparin-bound tPA. PAI-1 is found to be increased in diabetic patients (Kwaan, 1992; Matsuda *et al*., 1996).

The balance between tPA and PAI-1 is a critical factor determining whether a clot is formed or lysed. The expression of uPA, tPA, and PAI-1 in the endothelial cells and smooth muscle cells are relevant to their role in thrombosis (Kwaan, 1992; Colwell, 2001; Johnstone and Veves, 2001). A major mechanism of action of the components of the fibrinolytic system is through the proteolysis of the extracellular matrix (ECM) proteins. The primary catalytic activators of the metalloproteinases are uPA and plasmin. These metalloproteinases include, collagenase, which is responsible for the proteolysis of the ECM proteins. In diabetic patients it has been observed that the capillary membrane which consists of type IV collagen, thickens. Through the activation of collagenase, uPA can modulate collagen deposition and the resulting basement membrane thickening. Therefore, high levels of uPA would be expected to restrict the thickening of the basement membrane. The reduced fibrinolytic activity of diabetic patients may be responsible for the basement membrane thickening in the microagiopathy, and the increased uPA locally expressed in the capillaries, may stop this process. Therefore, the alterations in the fibrinolysis system not only increase the risk of thrombosis in diabetes but also play a role in the pathogenesis of vascular complications, including atherosclerosis and microangiopathy (Kwaan, 1992).

Since PAI-1 is the major inhibitor of fibrinolysis, a great deal of work has been completed to understand this inhibitor's action. The prothrombotic state exists when plasma levels of PAI-1 are found to be elevated. PAI-1 is produced by a variety of cells, including hepatocytes, vascular smooth muscle cells (VSMCs), endothelial cells, fibroblast, mesangial cells and adipocytes. The gene transcription of PAI-1 is stimulated by several growth factors including insulin, angiotensin II, and TNF-α, FFAs, triglycerides, and glucose. The stimulation of the expression of PAI-1 by insulin and angiotensin II is through the MAPK pathway. Physiological concentrations of glucocorticoids have been shown to stimulate PAI-1 expression *in vitro* (Lyon and Hsueh, 2003; De Taeye *et al.*, 2005).

 The blood platelets carry about 90% of the total PAI-1 in humans in the α-granules, in an inactive form, plasminogen activator inhibitor antigen (PAI-Ag). After aggregation platelets release PAI-1, and this is physiologically active at the local vascular site to inhibit fibrinolysis (Colwell, 2001; Johnstone and Veves, 2001).

Plasma levels of PAI-1 are usually elevated in T2DM (Jokl *et al*., 1994; Juhan-Vague *et al*., 1991; Juhan-Vague *et al*., 1989) and are normal in T1DM (Carmassi *et al*., 1992; Juhan-Vague *et al*., 1991; Juhan-Vague *et al*., 1989) Several mechanisms have been suggested to explain the high plasma PAI-1 levels. *In vitro*, the transcription of the gene that codes for PAI-1 in human endothelial cell cultures is increased in the presence of high glucose concentrations (Wiman, 1995). Non-enzymatic glycation of fibrinogen, tPA, and plasminogen will impair plasminogen activation. Further, the rate of plasmin formation is decreased by plasminogen and tPA isolated from diabetic donors. Plasma insulin levels correlate with PAI-1 activity, and the synthesis of PAI-1 in messenger RNA (mRNA) is increased in cultured human hepatocytes and 3T3-L1 adipocytes. PAI-1 expression in cultured bovine aorta endothelial cells is stimulated by insulin and proinsulin. Endothelial release of PAI-1, *in vitro*, is increased by oxidized or glycated (LDLs) and by very low density lipoproteins (VLDLs) (Colwell, 2001).

1.8.3 The Renin Angiotensin System and Fibrinolysis

The renin angiotensin system (RAS) is a hormonal system that protects against fluid volume loss. In addition to causing vasoconstriction and the retention of salt and water, angiotensin inhibits fibrinolysis, by doing so, it promotes clot formation and protects against hemorrhage and volume loss. This function is lost in diabetic people (Agirbasli and Vaughan, 2000; Varughese *et al*., 2005; Kirpichnikov and Sowers, 2001).

The balance of the fibrinolytic system can be disturbed by the activation of RAS, through excess production of PAI-1 which is stimulated by RAS. t-PA production is down-regulated by angiotensin-converting enzyme (ACE) via its degrading action on bradykinin, which is a stimulator of t-PA production *in vivo*. In contrast, ACE up-regulates endothelial cell and smooth muscle cell PAI-1 production by the action of angiotensin II, via its degradation product angiotensin IV, which acts on a specific angiotensin receptor 4 (AT_4 receptor) (figure 1.8) (Agirbasli and Vaughan, 2000; Huber, 2001).
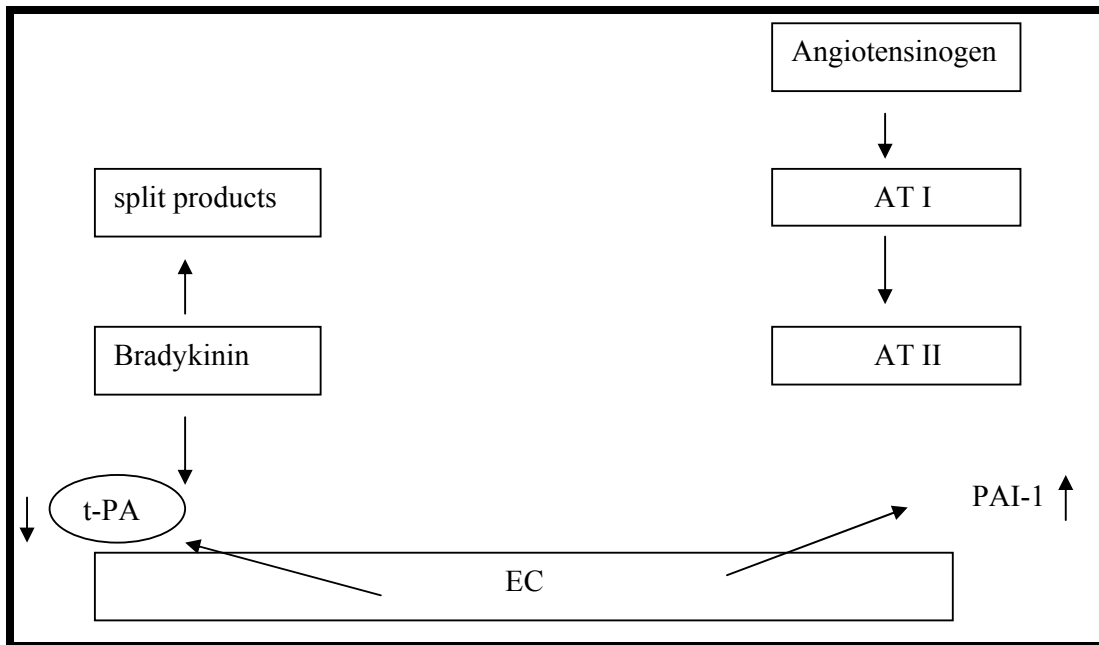
Figure 1.8: The renin-angiotensin system (RAS) and the regulation of fibrinolytic factors. **Schematic illustration of the central role of the angiotensin-converting enzyme (ACE) at the** **interface between RAS and the fibrinolytic system. Conversion of angiotensin (AT) I to AT II by** **ACE leads to increased endothelial expression of PAI-1, whereas degradation of bradykinin by** **ACE inhibits endothelial production of t-PA. EC = endothelial cells (Huber, 2001).**

1.8.4 Platelet Function

Platelets play a fundamental life-saving role in haemostasis at the site of vascular injury. Platelets are small, anuclear cellular bodies that are formed from megakaryocytes, and they circulate in the blood for eight to ten days. Because of their small size, platelets are pushed to the edge of the vessels, hence, placing them in an optimal position required to constantly survey the integrity of the vasculature. Platelets are involved in a number of pathophysiological processes which are outlined in figure 1.9, but the focus of platelet function has been on their involvement in haemostasis (Harrison, 2005; Clemetson, 1999; Vinik *et al.*, 2001).
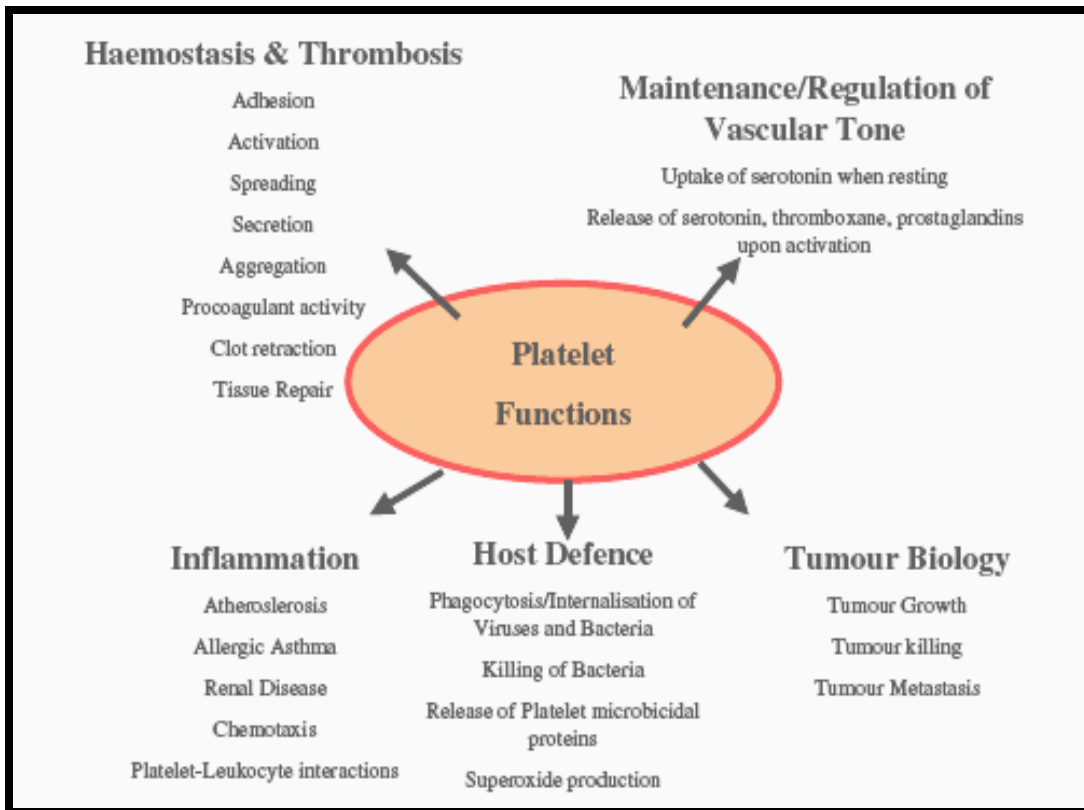
Figure 1.9: The multifunctional platelet. Platelets are involved in many pathophysiological processes, in addition to haemostasis and thrombosis, namely: maintenance of vascular tone, inflammation, host defence and tumour biology (Harrison, 2005).

1.8.4.1 The Role of Platelets in Primary Haemostasis

When a vessel is injured, platelets adhere, are activated and become adhesive for other platelets so that they aggregate (figure 1.10). In figure 1.10, endothelial denudation and binding of collagen and von Willebrand factor (vWF) to glycoproteins is the first step (figure 1.10, no.1) in platelet activation. The activation of glycoprotein (GP)IIb/IIIa (figure 1.10, no. 2) results in the exposure of the fibrinogen binding site (figure 1.10, no. 3) and fibrinogen binds to the complex of the GPs and platelets. The resulting aggregate or thrombus is stabilized by formation of fibrin. The development of the initial haemostatic plug by platelets is called primary haemostasis. Thrombosis is the pathological version of haemostasis, it occurs because of a disequilibrium between the positive and negative feedback mechanisms that control the growth of a thrombus (Clemetson, 1999).
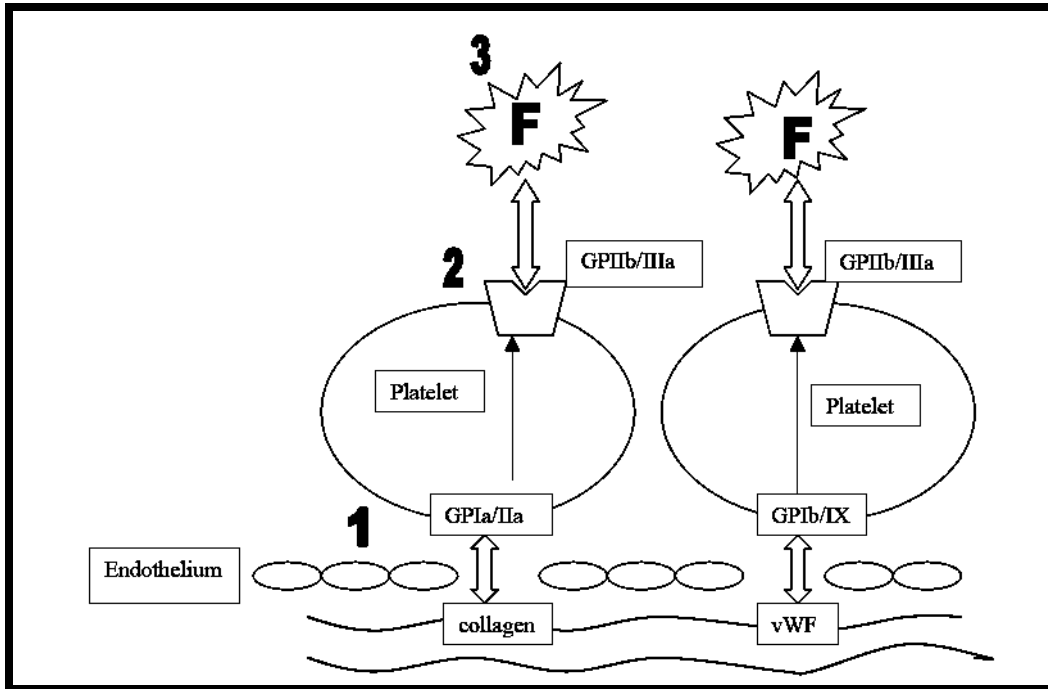
Figure 1.10: Platelet adhesion, activation and aggregation on an exposed wound site (Cassar *et al.*, 2003).

Once the platelets are activated, degranulation of the platelets occur, which involves the discharge of platelet granule contents such as α-granule proteins β-thromboglobulin, platelet factor 4, and platelet-derived growth factor (PDGF) as well as adenosine diphosphate (ADP) from the platelet dense granules. ADP is an inducer of platelet aggregation. It does this by amplifying the platelet response induced by other platelet agonists, and it also stimulates the generation of thromboxane A_2 (TXA_2) which is a potent platelet activator. Platelets adhere directly to collagen or indirectly via the binding of vWF to GPIb/IX. A thrombus forms as platelets aggregate via the binding of bivalent fibrinogen to GPIIb/IIIa (figure 1.11). During the activation of platelets, P-selectin, which is found in the membrane of α-granules in resting platelets, rapidly appears in the outer membrane. This serves as a receptor for oligosaccharides within the membranes of monocytes and neutrophils, causing such cells to adhere locally (Clemetson, 1999; Cassar *et al.*, 2003; Rand *et al.*, 2003; Clutton *et al.*, 2001; Coller, 1995).

After the endothelial cell is disrupted, the coagulation cascade is activated and thrombin is generated at the site of damage. Generation of fibrin leads to further platelet aggregation as well as fibrin production. Thrombin binds to two protease-activated receptors (PAR) termed PAR1 and PAR4 (figure 1.11, no.1). Through these receptors, thrombin activates phospholipase C which hydrolyses PIP_2 into IP_3 and diacylglycerol (DAG) (figure 1.11, no. 2) (Cassar *et al.*, 2003; Rand *et al.*, 2003; Clutton *et al.*, 2001; Coller, 1995).
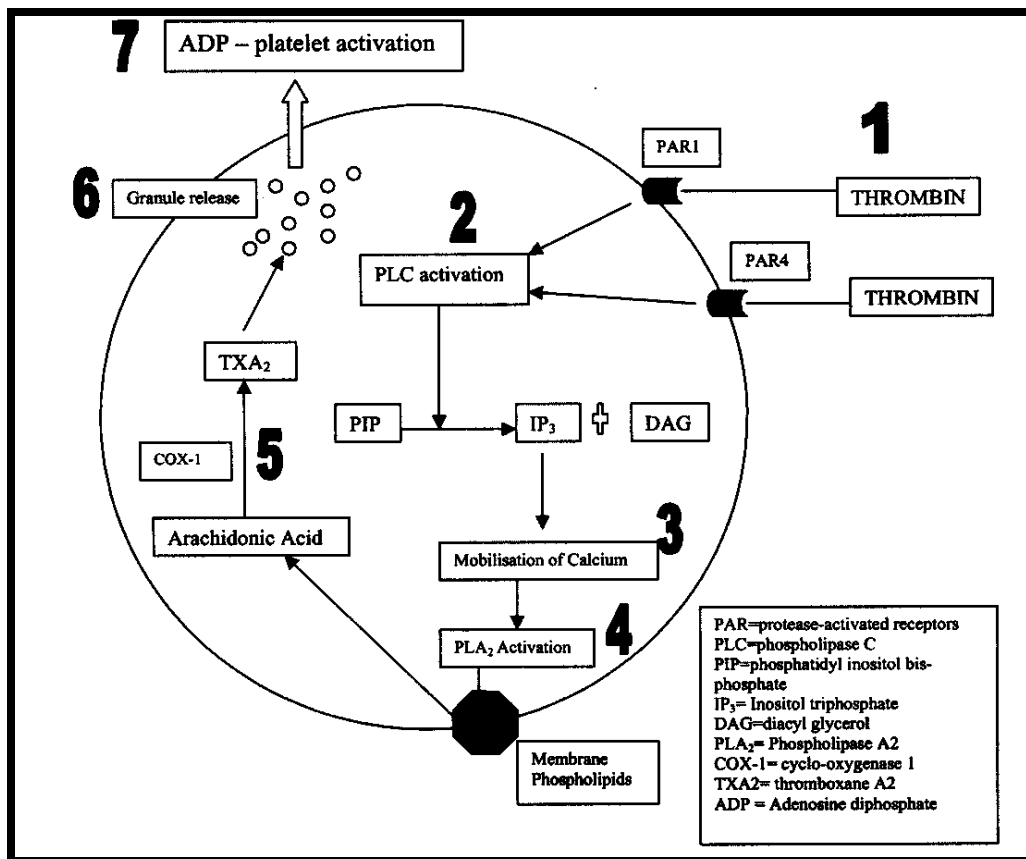
Figure 1.11: Thrombin-induced platelet activation via IP_3 pathway (Cassar *et al.*, 2003).

The mobilization of calcium stores (figure 1.11, no. 3) is then activated by the formation of IP_3, which in turn activates phospholipase A_2 (PLA_2) which leads to the production of arachidonic acid (figure 1.11, no. 4). Further platelet activation and aggregation is caused by the formation of TXA_2 (figure 11, no. 5) from arachidonic acid and this reaction is catalysed by cyclooxygenase-1 (COX-1). DAG, stimulates protein kinase C (figure 1.12) which mediates various platelet responses most important

of which is activation of the fibrinogen receptor GPIIb/IIIa (Cassar *et al.*, 2003; Rand *et al.*, 2003; Clutton *et al.*, 2001; Coller, 1995).
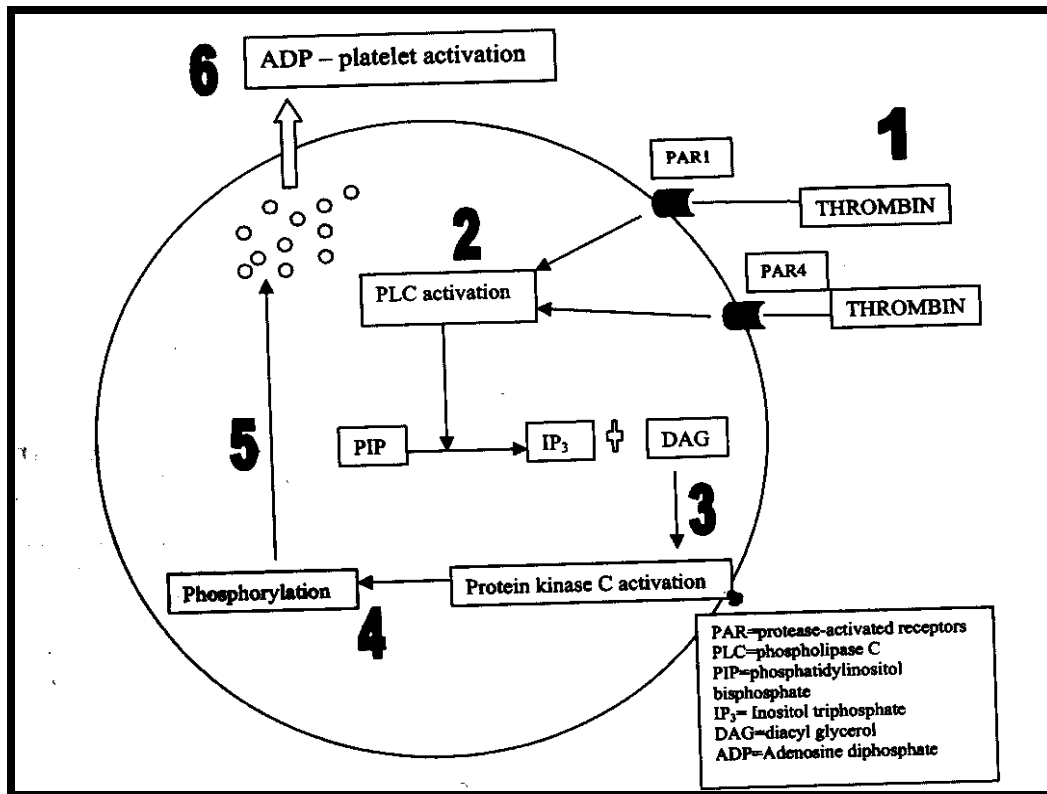
Figure 1.12: Thrombin mediated platelet activation via DAG pathway (Cassar *et al.*, 2003).

1.8.4.2 Platelet Function in Subjects with Diabetes Mellitus

There is an increased sensitivity of platelets *in vitro* to a variety of aggregating agents found in people with T1DM and T2DM as well as animal models of diabetes (Winocour *et al.*, 1985; Winocour *et al.*, 1984). There is evidence of increased synthesis of prostaglandin metabolites, with increased release of thromboxane, a proaggregant and a vasoconstrictor. There is an increase in degranulation in response to diverse stimuli in platelets from people who have diabetes (Halushka *et al.*, 1981). The capacity to promote growth of smooth muscle cells *in vitro* is high, this is shown by exposure of smooth muscle cells to platelets from people with poorly controlled diabetes as compared with those who have well controlled diabetes (Winocour *et al.*, 1983). Since α-granules release growth factors once the platelets attach to them, the enhanced growth promoting activity of platelets from people with poorly controlled

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diabetes appears to be secondary to increased α-granule degranulation. The threshold for induction of release of substances residing in dense granules in response to thrombin is lower from diabetic people compared with nondiabetic people (Winocour *et al.*, 1990). Thus, the generation of coagulant factor Xa and of thrombin is increased by three to sevenfold in samples of blood containing platelets from diabetic people compared with those from nondiabetic people (Colwell, 2001; Johnstone and Veves, 2001).

There is evidence of platelet activation *in vivo*, and diabetic people have elevated platelet turnover (Winocour *et al.*, 1984). Measurements in blood or urine samples for components and metabolites of components released from activated platelets can be used to assess platelet activation *in vivo*. Alpha-granules contain platelet factor 4 and β-thromboglobulin, which are platelet specific proteins. Their concentrations in blood are increased in diabetic people, consistent with a steady state increase in activation of platelets reflected by degranulation (Winocour *et al.*, 1990). In addition, thromboxane B_2 (TXB_2), a metabolite of TXA_2 that is produced by platelets, show increased concentrations in blood samples and urine samples of diabetic people. This has shown to be a marker of a steady state increase in activation of platelets reflected by increased flux through the thromboxane synthase pathway (Halushka *et al.*, 1981). In diabetes, the non-enzymatic glycation of platelet membranes alters fluidity. People with diabetes have an enhanced expression of adhesive receptors on the platelet surface, including P-selectin, fibrinogen, and vWF receptors. Increased binding of fibrinogen to platelet receptors has been described in diabetes (Tschoepe *et al.*, 1995; Tschoepe *et al.*, 1993). Activated platelets release mitogens, such as PDGF, and transforming growth factor-β (TGF-β) (Colwell, 2001; Johnstone and Veves, 2001).

1.8.4.3 Mechanisms Responsible for Hyper-reactivity of Platelets in Patients with Diabetes

Patients with T1DM and T2DM have been shown to have increased expression of the surface GPs Ib and IIb/IIIa in their platelets (Winocour *et al.*, 1985). GPIb/IX binds to vWF in the subendothelium and is responsible for adherence of platelets at the injured site. Therefore, platelets are activated by the interaction between GPIb/IX and vWF. The binding of fibrinogen and platelet aggregation is induced by the activation of GPIIb/IIIa. Thus, increased expression of either or both of these two surface

glycoproteins is likely to contribute to the increased reactivity that has been observed in platelets from diabetic people. Winocour and his colleagues (1990) have shown an association between decreased membrane fluidity and hypersensitivity of platelets to thrombin. When platelets are incubated in media containing concentrations of glucose similar to those seen in blood from diabetic people, a reduction of membrane fluidity occurs (Winocour *et al*., 1990). Since membrane fluidity is likely to alter membrane receptor accessibility to ligands, reduced membrane fluidity may contribute to hypersensitivity of platelets. Improved glucose control, would be expected to decrease glycation of membrane proteins, increase membrane fluidity, and decrease hypersensitivity (Johnstone and Veves, 2001; Winocour *et al*., 1990).

Arachidonic metabolism in platelets of diabetic patients is altered. This causes an increase in the production of TXA_2, thus, contributing to platelet sensitivity. (Varughese *et al*., 2005).

The intracellular mobilization of calcium is important in several steps involved in the activation of platelets. People with T2DM show increased levels of basal concentrations of calcium (Clemetson, 1999). The increase in the activity of second messengers may contribute to the hypersensitivity of platelets in diabetic people (Johnstone and Veves, 2001; Kirpichnikov and Sowers, 2001).

Insulin alters the reactivity of platelets. The exposure of platelets to insulin causes an increase in synthesis of nitric oxide (NO) which then leads to the decrease in platelet aggregation (Stamler *et al*., 1989). The production of NO increases intraplatelet concentrations of the cyclic nucleotides, cyclic guanosine monophosphate (cGMP), and cAMP. Both of these cyclic nucleotides are known to inhibit the activation of platelets. Insulin deficiency typical in T1DM diabetes and seen in later stages of T2DM may contribute to increased platelet reactivity (Johnstone and Veves, 2001; Kirpichnikov and Sowers, 2001).

1.8.5 Therapy for the Procoagulant State

The activity of protein C, which is the inhibitor of the coagulation system through factor V or factor VIII is depressed in diabetes (Ceriello, 1993). The conversion of

fibrinogen to fibrin is inhibited by antithrombin III (AT III). Therefore diabetes is correlated with low plasma levels of AT III, and by this mechanism thrombosis can be promoted. High blood glucose levels can be used to increase plasma levels of factor VII, and a relationship between hyperglycemia and plasma and urinary fibrinopeptide A has been described (Colwell and Jokl, 1996). Thus, there is a relationship between diabetes and a number of the coagulation factors. Insulin treatment has a favourable effect on plasma factor VII, AT III activity and plasma protein C concentration and activity (Ceriello, 1993; Ceriello *et al.*, 1990), therefore it can be used to improve the metabolic control in T1DM. Acutely administered insulin therapy can be used to lower fibrinogen levels. In T2DM, exercise can be used to lower plasma fibrinogen levels (Jokl and Colwell, 1997; Jokl and Colwell, 1997). Fibrinogen has been identified as a risk factor that can cause peripheral arterial disease (PAD) in diabetic people and non-diabetic people. Fibrinogen synthesis is increased in diabetes and its plasma levels are increased in insulin resistance syndrome (Jokl and Colwell, 1997). Therefore, therapy directed at fibrinogen levels or factor VII would seem to be appropriate for addressing procoagulant diseases, diabetes and PAD. Short-term insulin therapy lowers fibrinogen levels (Ceriello, 1997). There are also drugs that do lower fibrinogen levels by decreasing its synthesis. These drugs include anabolic steroids and fibrate drugs. Fibrinogen level is decreased by 10% to 20% in individuals who have T1DM and T2DM by decreasing its synthesis (Colwell, 2001).

1.8.6 Therapy for the Fibrinolytic System

There has been great interest in exploring therapeutic approaches for lowering PAI-1 plasma levels, because PAI-1 is the major inhibitor of the fibrinolytic system in humans, and its plasma level is increased in T2DM (Winocour *et al.*, 1990). The high levels of PAI-1 in diabetes contribute to the high prevalence of PAD in diabetic people. PAI-1 levels correlate with body weight, so exercise and dieting help in controlling its levels. Given the association between PAI-1 levels and T2DM, studies of insulin, oral sulfonylureas (SUs), metformin, and the thiazolidinediones (TZDs) are relevant. The effect of hypolipidemic drugs have been studied because of the correlation between triglycerides and PAI-1 levels. Finally, the effect of ACE inhibitors on PAI-1 is now recognised (Colwell, 2001). The effect of exercise and weight loss will be discussed in sections 1.7.6.1 and 1.7.6.2 respectively and the oral antidiabetic agents in section 1.7.6.3.

1.8.6.1 Exercise

A number of studies have shown that exercise increases fibrinolytic activity in healthy people (Colwell, 1986; Homsby *et al.*, 1990). Many of these studies were done before it was known that PAI-1 is important in regulating the fibrinolytic system. *In vitro* methods such as the fibrin plate method were used to estimate blood fibrinolytic activity. Just by walking up and down the stairs or taking part in a marathon, the fibrinolytic activity is increased. One postulated mechanism explaining the increase of fibrinolytic activity after exercising is that tPA release from the vascular endothelium is increased (Colwell, 1986). There is also activation of the intrinsic system which regulates fibrinolysis. The studies that have been done previously, showed that the effect of exercise is not mediated by the decrease in plasma levels of inhibitors of this system (antiplasmin, α_2-microglobulin, AT III) (Johnstone and Veves, 2001; Colwell, 2001; Hilberg *et al.*, 2003).

1.8.6.2 Weight Loss

The effect of weight loss by dieting on PAI-1 levels has been studied. In a study conducted previously, plasma levels of PAI-1 fall after 24 hours of fasting in non-obese women (Charles *et al.*, 1998). Similar results could be obtained within 2 to 13 weeks in a strict hypocaloric diet (Charles *et al.*, 1998). Individuals who have the highest plasma levels of PAI-1 and triglycerides levels respond very well to dietary measures, therefore, weight loss is one of the main factors associated with a decrease in PAI-1 plasma levels (Hilberg *et al.*, 2003).

1.8.6.3 Oral Antidiabetic Agents

Generally, the action of a drug *in vivo* on platelet function is not observed *in vitro*. Insulin therapy has been shown to decrease platelet thromboxane release and reduce platelet sensitivity to aggregating agents both *in vitro* and *in vivo* (Vague *et al.*, 1987). If hypoglycemia is produced by insulin administration, platelet sensitivity to aggregation is increased, probably by the action of epinephrine release *in vivo* (Colwell, 2001).

1.8.6.3.1 Metformin

Some studies have reported that metformin may inhibit platelet aggregation (Vague *et al.*, 1987; Jarvi *et al.*, 1999). Others have argued that glycemic control rather than a drug is responsible for this effect. Metformin (figure 1.13) has been found to decrease plasma levels of PAI-1 in non-diabetic obese individuals. Metformin treatment in T2DM diabetic people lowered PAI-1 activity, increased glycemic control, and lowered insulin resistance (Yudkin, 1999; Colwell, 2001).
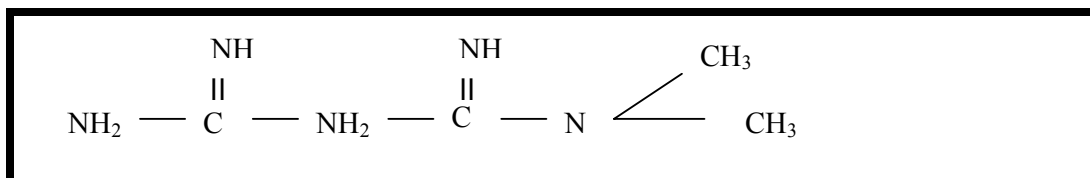
Figure 1.13: The chemical structure of metformin (Beisswenger *et al.*, 1999).

1.8.6.3.2 Thiazolidinediones

Studies which were done on women with polycystic ovarian syndrome (PCOS), which is characterized by insulin resistance and is often accompanied by DM, showed that when troglitazone (figure 1.14) is administered on a 12 week course, there is a significant fall in level and activity of PAI-1 and PAI-1 antigen (PAI-1 Ag) (Ehrmann *et al.*, 1997). Metformin therapy on women with PCOS yielded similar results (Wiman, 1995).
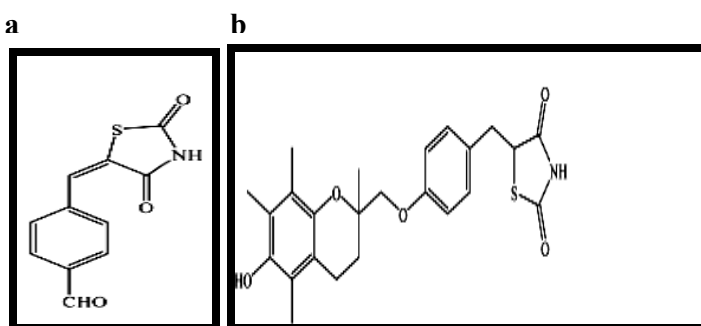
Figure 1.14: The chemical structures of (a) Thiazolidine-2,4-dione, (b) Troglitazone (Jung *et al.*, 2006).

1.8.6.3.3 Sulfonylureas

This agent has no effect on PAI-1 levels unless it is combined with rosiglitazone (figure 1.15) (Freed *et al.*, 2000). A significant effect of SUs on PAI-1 levels has not been convincingly shown (Colwell, 2001).
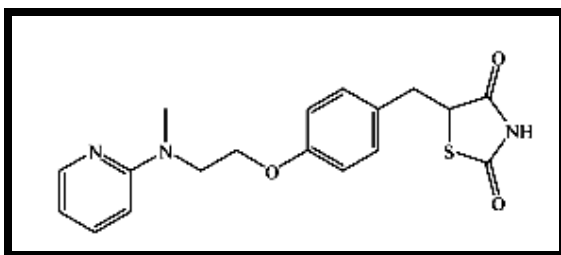
Figure 1.15: The chemical structure of rosiglitazone (Jung *et al.*, 2006).

1.8.6.3.4 Gemfibrozil

In vitro studies have shown that purified VLDL from hyperglycaemic patients can increase the level of PAI-1 production from endothelial cells. Therefore by lowering triglyceride levels through weight loss or gemfibrozil (figure 1.16) therapy, lower PAI-1 levels can be achieved (Colwell, 2001).
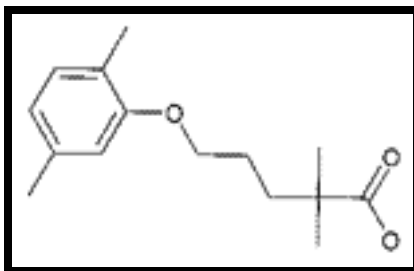
Figure 1.16: The chemical structure of Gemfibrozil (http://www.plantexusa.com/commercial./ gemfibrozil.htm, 20 May 2005).

1.8.6.4 ACE Inhibitors

RAS is involved in regulating the fibrinolytic balance. Angiotensin II increases PAI-1 production and ACE inhibitors lower plasma levels of PAI-1 (Yudkin, 1999; Tkac *et al.*, 2003).

1.9 Therapy Targeted at Platelets in Diabetes

1.9.1 Aspirin

Platelets can be permanently inactivated by using low doses of aspirin (Davi *et al.*, 1996). Low doses of aspirin inactivate platelet prostaglandin growth hormone (GH) synthase, the enzyme cyclooxygenase, which catalyses the conversion of arachidonic acid to prostaglandins G_2 and H_2 (Davi *et al.*, 1996). The prostaglandins are precursors of thromboxane. Aspirin (figure 1.17) therapy has been widely investigated because

platelet release of thromboxane is elevated in T1DM and T2DM, and this may lead to PAD (Davi *et al.*, 1996). The inhibition of thromboxane is immediate and begins shortly after absorption of aspirin from the gastrointestinal tract. Platelets have limited protein synthetic abilities, therefore, cyclooxygenase activity is inhibited throughout the lifespan of the platelets, which is about 10 days. Each day, new platelets enter the bloodstream at a rate of 10% per day, therefore, a low dosage of aspirin will inhibit COX-1 of these new platelets. Larger doses of aspirin are also effective but they cause gastrointestinal or cerebral bleeding (Colwell, 2001).
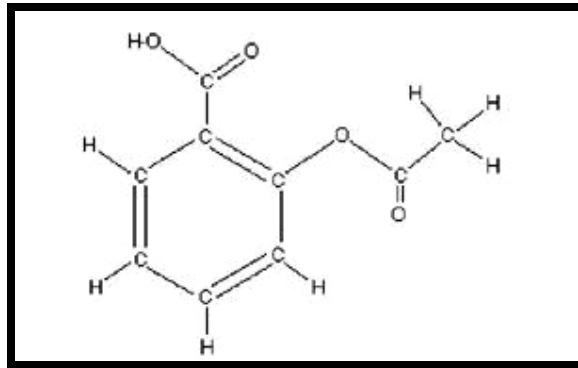
Figure 1.17: The chemical structure of Aspirin (Kauffman, 2000).

1.9.2 Clopidrogel and Ticlopidine

These two drugs inhibit platelet function by pathways which are distinct from the arachidonic acid to thromboxane route. Clopidrogel (figure 18a) and ticlopidine (figure 18b) are thienopyridine derivatives that inhibit ADP binding to the platelet type 2 purinergic receptor (Winocour, 1990). The effect lasts for the lifetime of the platelets. By blocking this receptor, the activation of GPIIb/ IIIa and the binding of fibrinogen to that receptor complex are impaired. Monoclonal antibodies against GP complex have been developed (Colwell, 2001, Winocour, 1992).
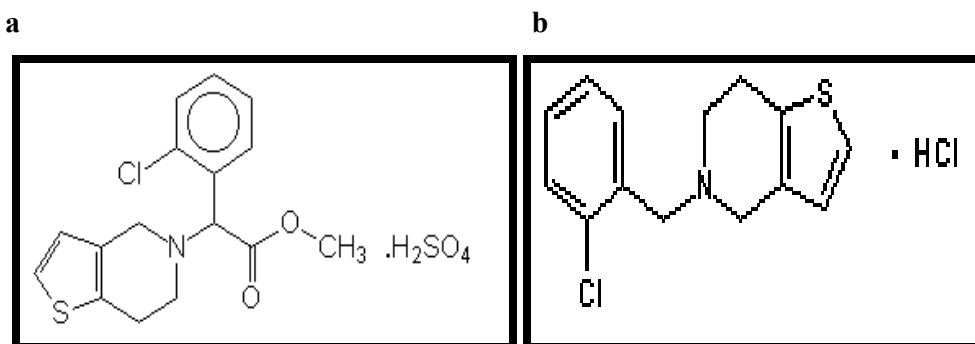
Figure 1.18: The chemical structure of (a) Clopidrogel (http://www.esculape.com/ drugsoft/plavix.html, 20 May 2005) and (b) Ticlopidine (http://www.moldb.nihs.go.jp/jp/ scripts/name_exact_e.asp? name _e= Ticlopidine Hydrochloride, 20 May 2005).

1.10 Using Plants as Alternatives

The rising costs of prescription drugs in the maintenance of personal health and well being have increased the interest in medicinal plants. Plants have played a role in the prevention and treatment of diseases since prehistoric times. With the improvement of scientific medicine in the 20th century, the discovery of new drugs has come to depend on the application of rational codified principles, providing an understanding of why some treatments are effective and others are not. It has been widely observed that in some countries, traditional medicines are used. Traditional medicines have been used at an extensive rate in industrialized societies because of the growing rate of extraction and development of new drugs and chemotherapeutics from these plants. As a result, traditional medicine is used for minor ailments because of the increasing costs of personal health maintenance (Drew and Meyers, 1997; Talalay and Talalay, 2001; Hoareau and Da Silva, 1999).

WHO estimates that 65%-80% of the world's population use traditional medicine as their primary form of health care (Scott *et al*., 2004; Stafford *et al*., 2005). Africa is a rich source of medicinal plants. Many drugs today trace their origin to plants. The indigenous flora of South Africa is notable for its species richness and high degree of endemicity. Traditional medicine is an integral part of the South African cultural life, with an estimated 70% of the population using traditional medicine on a regular basis (Scott *et al*., 2004). The South African Health Ministry is considering incorporation of

these medicines within its formal health care plan (Scott *et al.*, 2004). Although many plant agents have been used for centuries, some plant products that have been used have components that have not been well characterized, are difficult to quantify, and vary qualitatively and quantitatively from plant to plant, in different plant parts, and according to stages of plant development and conditions of growth. Plants can also have toxic effects. Adverse effects of traditional medicine may be intrinsic or extrinsic, (table 1.1). The risk and severity of adverse events may be affected by the patient's age, genetic constitution, nutritional state, or consumption of large amounts of the herbal preparation (Drew and Meyers, 1997; Talalay and Talalay, 2001; Hoareau and Da Silva, 1999; Masika and Afolayan, 2003; Scott *et al.*, 2004; Stafford *et al.*, 2005).

Table 1.1: Classification of adverse effects associated with herbal medicine (Drew and Myers, 1997).

Intrinsic (Type A reactions)	Predictable toxicity, overdosage, interaction with pharmaceuticals
Intrinsic (Type B reactions)	Idiosyncratic reactions (e.g., allergy, anaphylaxis)
Extrinsic factors	Mis-identification, lack of standardisation, contamination, substitution, incorrect preparation and/or dosage, and inappropriate labelling

Intrinsic effects are caused by the plant itself and are characterised as type A (predictable, dose-dependent) and type B (unpredictable, idiosyncratic) reactions. *Pausinystalia yohimbe* has an alkaloid called yohimbine. This alkaloid has α_2-adrenoceptor antagonist activity, and is taken for male impotence, and can cause hypertension and anxiety in predictable, dose-related manner (type A reaction). It has been associated with serious idiosyncratic reactions of bronchospasm and increased mucus production when taken in normal doses by a patient with severe allergic dermatitis (type B reaction) (Drew and Meyers, 1997).

Extrinsic factors are related to the commercial manufacturing of the medicine. A plant can have up to four different names, the common English name, the transliterated

name, the Latinised pharmaceutical name, and the scientific name. It is important to use their binomial Latin names to avoid misidentification (Drew and Meyers, 1997).

The therapeutic uses or toxicity of the plant vary, depending on the part of the plant that is used, stage of ripeness, geographic area where the plant is grown, and storage conditions. In order to improve standardisation and reproducibility it is important to look at the mentioned variables. Storage of the plant material is important to avoid contamination, because during storage, crude plant material can be contaminated by microorganisms. Substitution of plants can be severe. In Belgium women who were on a slimming treatment were taking *Aristolochia fangchi* (containing the nephrotoxic component aristolochic acid) instead of *Stephania tetrandra* (Vanherweghem *et al*., 1993; Vanhaelen *et al*., 1994; van Ypersele and Vanherweghem, 1995). This led to terminal renal failure (Drew and Meyers, 1997).

While stressing the dangers of using traditional medicines, it should not be forgotten that a multitude of life-saving drugs have been provided by plants. Despite the increase in the use of medicinal plants, their future is being threatened by their commercialisation. Stocks of medicinal plants in developing countries are diminishing in danger of extinction as a result of growing trade demands for cheaper healthcare products and new plant-based therapeutic drugs (Drew and Meyers, 1997).

1.10.1 *Leonotis leonurus*

Leonotis leonurus is a plant that belongs to the Lamiaceae family which has wide distribution over South Africa (figure 1.19). *Leonotis leonurus* is also known in Afrikaans as 'wilde dagga', in Zulu as 'umunyane', in Sotho as 'lebake', in Xhosa as 'umfincamfincane' and in Shona as 'umhlahlampethu' (Bienvenu *et al.*, 2002).

a   b
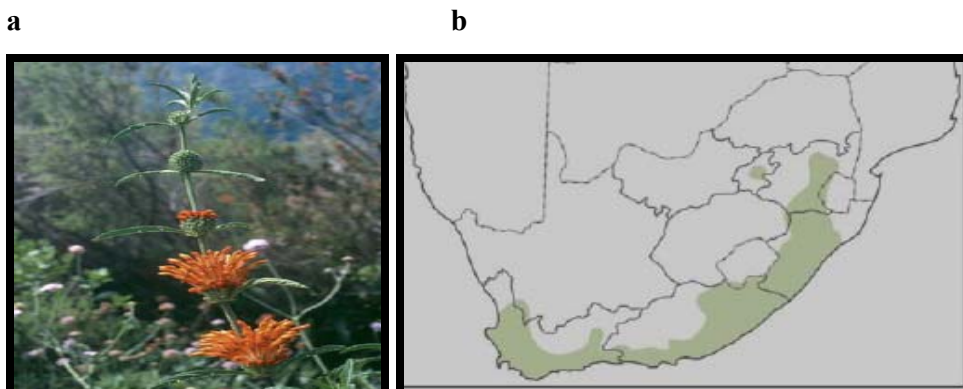
Figure 1.19: An illustration of (a) *Leonotis leonurus* and (b) geographical distribution of *Leonotis leonurus* in Southern Africa (indicated by the shaded regions) (Adams, 1999).

Lamiaceae is a family that is characterized by the presence of essential oils as well as higher terpenoids. In this family, tannins, iridoids, and saponins are common, and alkaloids are present in a few genera (Scott *et al.*, 2004). In addition to the above mentioned chemical constituents of the lamiaceae family, *Leonotis leonurus* contains the diterpenoid labdane lactones premarrubiin and marrubiin (figure 1.20) (Scott *et al.*, 2004). Marrubiin is derived from premarrubiin during extraction. Marrubiin is accumulated in large amounts in the aerial parts of the plant. The actual pharmacological effect of marrubiin is not known (Knoss *et al.*, 1997; Scott *et al.*, 2004).
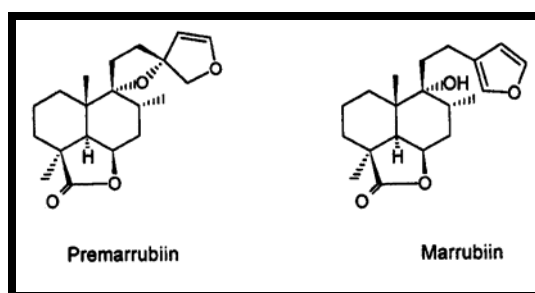
Figure 1.20: The chemical structures of premarrubiin and marrubiin (Knoss *et al.*, 1997).

1.10.2 Biological Activity of *Leonotis leonurus* and Related Species

Premarrubiin has been found to be an effective expectorant, consistent with the traditional uses of *Leonotis leonurus* (Scott *et al.*, 2004). This plant has been

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traditionally smoked for the relief of epilepsy. An infusion and decoction of the leaf and stem have been used to treat cough, colds, influenza, bronchitis, high blood pressure and headaches (Scott *et al*., 2004). It has been used externally for relief of haemorrhoids, eczema, skin rashes and itching, muscular cramps and boils (Bienvenu *et al*., 2002).

Water extracts of *Leonotis leonurus* have been shown to have anticonvulsant activity against chemically induced seizures in mice (Bienvenu *et al*, 2002). The anticonvulsant activity of *Leonotis leonurus* is attributed to saponin triterpenoids detected in aqueous (AL) extracts of the leaves of *Leonotis leonurus*. Water extracts of *Leonotis leonurus* exhibit hypotensive effects in rats. *Leonurus sibiricus* which contains diterpene labdane lactones of similar structure to those found in *Leonotis leonurus*, exhibited moderate cytotoxic activity (inhibitor concentration required to yield 50% inhibition (IC_{50}) = 50-60 µg/ml) against leukaemia cells (L 1210) in tissue culture (Ojewole, 2003). AL extracts of *ballota africana* which also has the diterpene labdane lactones of similar structure to those found in *Leonotis leonurus,* were not markedly cytotoxic at any concentration when used in the assay on Hela, Vero, Jurkat E6.2 AA – 2, and CEM – SS cells (Bienvenu *et al.*, 2002). The same extract was found to reduce infectivity of both Coxasckie B2 virus and HSV–1, but only at higher concentrations of the plant extract (Bienvenu *et al*, 2002). In a direct *in vitro* cell culture antiviral assay, however, the replication of neither virus was inhibited (Bienvenu *et al*, 2002; Scott *et al*., 2004). *In vivo* studies by Kenechukwu (2004) displayed that a crude AL extract of *Leonotis leonurus* has cardioactive properties. It did so by increasing systolic pressure of Wistar rats. The methanol extract was also found to have cardioactive properties, because it increased systolic pressure, diastolic pressure, mean arterial pressure, and heart rate in a manner similar to epinephrine. An *in vitro* system was also investigated, and the results confirmed those obtained from the *in vivo* system (Kenechukwu, 2004). Factor VII and fibrinogen are two of the intrinsic coagulation factors that are predictors of coronary heart disease (Colwell, 2001). Antithrombotic activities of *Leonotis leonurus* have been found by Miss N. Low-Ah-Kee (2005) in a previous Masters study. The methanol extract of the plant had antithrombotic activity before and after tannin extraction, although the activity decreased after tannin extraction. The leaf AL extract and the methanol extract of *Leonotis leonurus* had IC_{50} values of 3mg/ml and 9.69mg/ml, respectively. The effect of the plant extracts on clot formation was also investigated.

Two types of thrombin-induced clotting time assays were used: (1) a waterbath clotting assay, and (2) a microtitre plate-based assay. The IC_{200} value (concentration of the inhibitor required to double the clotting time, the time taken for the appearance of a fibrin thread) could be determined from the waterbath clotting assay. The IC_{200} values of the root AL extract was 21mg/ml, while that of the leaf AL extract was 2.49mg/ml. The methanol extracts and the root AL extract did not show any inhibitory effects on clotting. The microtitre plate-based assay was found to be more sensitive than the waterbath method. The methanol extracts inhibited thrombin-induced clot formation, with IC_{50} values of 8.26mg/ml and 10.4mg/ml for the leaves and stems respectively. The AL extract did not show inhibition of thrombin-induced clot formation in this assay. The extracts were also screened for calcium-induced clotting time. The extracts decreased fibrin formation at 1mg/ml and 10mg/ml. The concentrations that were required to halve (50%) clotting time were between 9mg/ml and 10.78mg/ml for both the AL extract and methanol extract.

A decoction of *Marrubium vulgare* displayed a hypoglycemic effect and antidiabetic effects on rats. The hyperglycemic state was induced on the rats by subcutaneous injection of dextrose solution (Ramos *et al.*, 1992).

1.11 Objective and Aims of the study
The marrubiin content of both the AL extract and the OL extract will be determined using high performance liquid chromatography (HPLC) and thin layer chromatography (TLC), before determining if the anticoagulant or antidiabetic activity shown by the extracts can be attributed to marrubiin.

The aim of this project was to find a potential multitherapeutic drug that can help in both coagulation and diabetes, therefore, the extract that showed anticoagulant activity was further studied for antidiabetic activity.

The relationship between coagulation and diabetes has been clearly described. In this study, organic (OL) and AL extracts of *Leonotis leonurus* was screened for anticoagulant/antithrombotic activity. To screen for anticoagulant activity, three aspects that are vital in the coagulation cascade will be targeted:

(a) Thrombin assay: this assay determines the effect of the extracts and marrubiin on thrombin. Thrombin is an enzyme that plays a central role in the coagulation cascade. It activates the platelets and acts on the cell of the vessel wall. In turn, the activated platelets, foster thrombin formation. Thrombin is involved in a whole set of positive and negative feedback reactions that first increase its own production enormously but inhibit it in a later stage. Therefore the way in which thrombin is formed and decreased will be investigated for the various plant extracts and standard marrubiin.

(b) Fibrinolysis assay: this assay looks at the rate at which the clot is lysed. In a diabetic state, fibrinolysis is impaired and this can lead to heart complications. The effect of the extracts and standard marrubiin on fibrinolysis and fibrin formation will be determined using the fibrinolysis assay.

(c) Platelet aggragation assay: hyperactivity of platelets is observed in a diabetic state. The effect of the extracts and standard marrubiin will tested using a spectrophotometric and flow cytometric method.

To screen for antidiabetic activity, three methods will be used:

(a) glucose-stimulated insulin secretion (GSIS): this assay will be used to investigate the response of the islets to various glucose levels in the presence of the extracts and standard marrubiin. This assay will determine if insulin is release is increased or decreased by the presence of the extracts and standard marrubiin.

(b) The expression levels of insulin in the presence of the plant extracts and standard marrubiin will be determined using Real-Time Polymerase Chain Reaction (RT-PCR). This method will be used to validate the results that will be obtained from GSIS.

(c) Proliferation and apoptotic studies will also be conducted on rat islets, to determine whether exposure to the extract and marrubiin have an effect on β-cell turnover.

CHAPTER 2: Materials and Methods

2.1 Extraction Procedures for *Leonotis leonurus*

Leonotis leonurus was collected at Nelson Mandela Metropolitan University (South campus) and identified by the Curator of the herbarium of the Department of Botany. Running water was used to remove soil and dust from the plant material. The plant was then separated into leaves, stems, roots, and flowers. Two types of extracts were prepared, an AL extract and an OL extract. The extraction procedure was described by Light *et al.* (2002) and it is outlined in figure 2.1.

Plants were divided into leaves, stems, and roots

Leaves were dried at 50˚C in an oven

AL extraction

Extractions with water (20ml/g ground plant material) were performed in a sonicator for 1 hour

Extracts were filtered through Whatman No.1 filter paper

Samples were freeze-dried

Dried extracts were stored at 4˚C in the dark

OL extraction

Extractions with acetone (10ml/g ground plant material) were performed in a sonicator for 1 hour

Extracts were filtered through Whatman No.1 filter paper

The solvent was removed using a rotary evaporator at 60˚C

Distilled water was added to the concentrated extracts and samples were freeze-dried

Dried extracts were stored at 4˚C in the dark

Figure 2.1: A flowchart showing the preparation of the AL and the OL extracts of *Leonotis leonurus*.

For subsequent assays, the appropriate weight of the extracts was dissolved in the relevant buffer system or media. In the extraction procedure in figure 2.1, acetone was used instead of methanol for the OL extract because in literature acetone was used for the isolation of marrubiin, since the polarity of methanol and acetone is similar (Houghton and Raman, 1998).

2.2.1 Thin Layer Chromatography (TLC)

TLC is a simple, quick, and inexpensive procedure that provides data on the variety of components found in a mixture. TLC is used to support the identity of a compound in a mixture when the retardation factor (R_f) of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate). A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of the plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. As the solvent moves past the spot that was applied, equilibrium is established between the molecules of each component of the mixture which is adsorbed on the solid adsorbent and the molecules in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are coloured, visualization is straightforward. Usually the compounds are not coloured, so a UV lamp is used to visualize the plates. The plate itself contains a fluor which fluoresces everywhere except where an organic compound is on the plate (Boyer, 1993).

The method that was used was adapted from Scott *et al*., 2004. Ten milligrams of the OL extract was dissolved in 1ml of toluene and 5µl of this solution was applied on a pre-coated silica plate. Standards of commercial marrubiin (LGC Promochem, UK) were also prepared, ranging from 0.5- 5µg, and spotted on the plate. A solution of toluene: diethyl ether: 1.75M acetic acid (1:1:1) was used to develop the plate. The plate was developed by dipping it in a 5% solution of sulphuric acid in 96% ethanol for

20 seconds and immediately into a 1% solution of vanillin for another 20 seconds. The plate was then heated in an oven at 105°C for 10 minutes and viewed in daylight. Analysis was performed using densitometry (Alphalmager[TM] 3400). Figure 2.2 is the marrubiin standard curve that was generated.
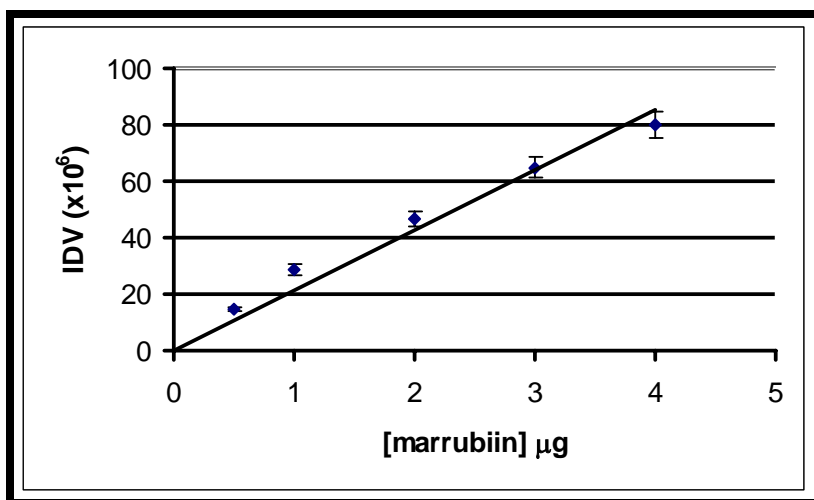
Figure 2.2: A densitometry calibration curve using marrubiin (R^2= 0.9937). The error bars represent a standard deviation of three independent experiments, where IDV= integrated density value.

2.2.2 High Performance Liquid Chromatography

HPLC depends on the interaction of sample analytes with the stationary phase (packing) and the mobile phase to effect a separation. In adsorption chromatography the stationary phase is the liquid-solid interface. Molecules are reversibly bound to this surface by dipole-dipole interactions. Since the strength of interaction with the surface vary for different compounds, residence time at the stationary phase varies for different substances, thus achieving separation. Liquid-solid adsorption chromatography is most often used for polar, non-ionic organic compounds. Partition chromatography is the fundamental distribution mechanism in liquid-liquid chromatography, i.e. when both mobile phase and stationary phase are liquids. Separation by distribution is based on the relative solubility of the sample in the two phases. In normal phase partition chromatography the stationary phase is more polar than the mobile phase, in reversed phase (RP) chromatography the mobile phase is more polar than the stationary phase. Stationary phases may be either coated onto a support, or they may be chemically bonded to the surface. Normal phase partition chromatography is used for very polar

organic compounds, while RP chromatography is commonly used for nonpolar or weakly polar substances (Boyer, 1993; Sheehan, 2000).

Ionic compounds are often better separated by ion exchange chromatography (IEC). In this case, the stationary phase consists of acidic or basic functional groups bonded to the surface of a polymer matrix (resin or silica gel). Charged species in the mobile phase are attracted to appropriate functional groups on the ion exchanger and thereby separated. Ion pairing chromatography is an alternative to ion exchange chromatography. Mixtures of acids, bases and neutral substances are often difficult to separate by ion exchange techniques. In these cases ion pairing chromatography is applied. The stationary phases used are the same reversed phases as developed for reversed phase chromatography. An ionic organic compound, which forms an ion-pair with a sample component of opposite charge, is added to the mobile phase. This ion-pair is, chemically speaking, a salt which behaves chromatographically like a non-ionic organic molecule that can be separated by RP chromatography (Boyer, 1993).

Before the marrubiin standard was quantified, 1ml of a 100x dilution of a commercial marrubiin stock (0.1μg/μl) in water was scanned to determine the maximum absorbance that could be used in the analysis, and this was found to be 225nm (Spectronic Genesys 5) (figure 2.3).
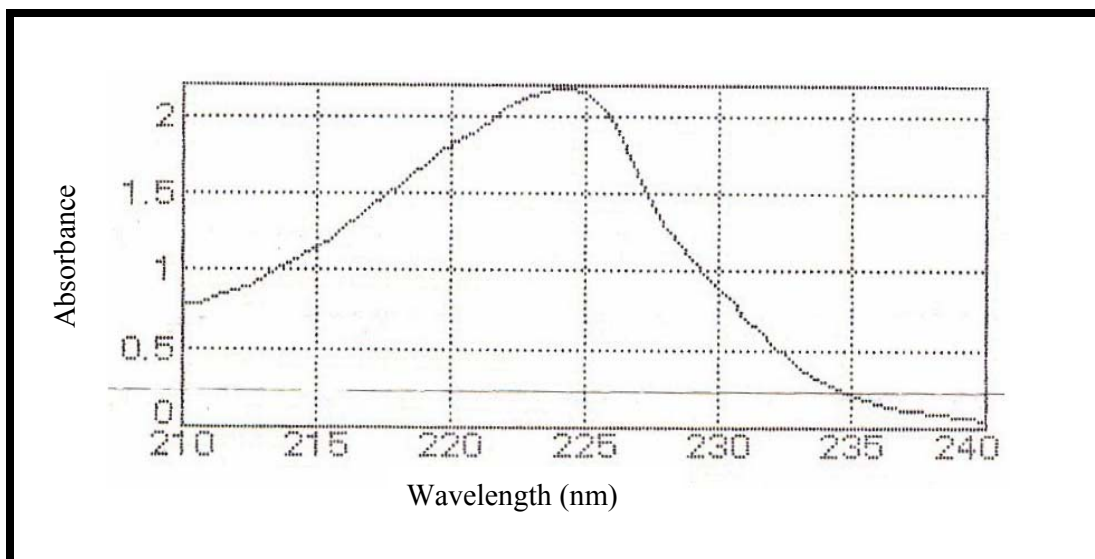
Figure 2.3: A characteristic absorption spectra of commercial marrubiin (0.1 μg/μl).

Ion-pair RP-HPLC for analysis was performed on a Beckman system consisting of a double pump Programmable Solvent Module model 126, Diode Array detector Module model 168, and a computer with System Gold software. The modified method that was used was adapted from Marchand *et al*., (1983). The chromatographic column used was a VYDAC C18 column with 5µm particle size (4.6 x 250mm).   Separation was achieved by using a gradient system consisting of acetonitrile and 10mM ammonium dihydrogen phosphate/5mM tetrabutylammonium phosphate (TBA), pH 3.5. The gradient separation was from 0% acetonitrile for 5min, thereafter a 15min gradient to 100% acetonitrile at a flow rate of 1ml/min was completed. The retention time of commercial marrubiin was used to identify marrubiin in the OL extract.

2.3 Determination of Anticoagulant Activity

The thrombin generation curve is an old and established tool in blood coagulation research (Macfarlane and Biggs, 1953; Pitney and Dacie, 1953). The functions of some factors if not all factors involved in the blood clotting system are reflected by this curve. Platelet-poor plasma (PPP) contains all clotting factors with the exception of factor XIII, and it is sensitive to the action of oral anticoagulation, heparins of all types, direct thrombin inhibitors and in fact all anticoagulant drugs tested. The generation of thrombin can be increased by hyperprothrombinemia, lack of antithrombin, protein S, as well as activated protein C. The part played by platelets, and the effect of vWF, anti-platelet drugs, and GPIIb/IIIa antagonists can be observed in a thrombin generation curve when using platelet rich plasma (PRP). Agents that increase platelet reactivity (e.g. ADP, arachidonic acid, epinephrine and collagen) accelerate thrombin generation in PRP. The change in activity of a thrombin generating curve can be calculated as a function of time by comparing a measurable signal to that from a test sample measured simultaneously (Hemker *et al*., 2003).

2.3.1 Thrombin Assay

The thrombin assay was used to determine the effect of the extracts and marrubiin on the enzymatic action of thrombin. This modified method was adapted from Rob *et al*. (1997). The method involves the action of thrombin (Sigma) on a chromogenic thrombin specific substrate known as S2238 (α-Phe-L-Pipecolyl-L-Arg-p-nitroanilide) (Chromogenix). A volume of 10µl of bovine thrombin stock concentrations ranging from 10 to 50 U/ml were added to a 96-well microtiter plate. To this, 50µl of either

buffer (50mM Tris-HCl, 7.4 mM ethylene diamine tetra-acid (EDTA), 175mM NaCl, pH adjusted to 8.4), extract, or marrubiin was added to the wells. The plate was then incubated for 5 minutes at room temperature (RT). Thereafter 190µl of 0.75mM substrate S2238 was added to each well to give a total volume of 250µl in each well. Thrombin cleaves S2238 at the C-terminal to release p-nitroanilide to yield a yellow colour. The absorbance of p-nitroanilide was measured at 410nm every 10 seconds for 190 seconds (Labsystems Multiskan MS). A standard curve was constructed using a range of thrombin concentrations (figure 2.4). For screening the inhibition potential of plant extracts and marrubiin, 30U/ml of thrombin was used because at this thrombin concentration there was at least change a 0.1 change in rate. The IC_{50} values were determined from the resulting curves, where 30U/ml was considered to have 100% activity. Concentrations between 2mg/ml and 10mgl; 0.5mg/ml and 6mg/ml; and 20µg/ml and 100µg/ml of OL, AL, and marrubiin, respectively, were tested for the inhibition of thrombin. Various concentrations for each extract and marrubiin were tested before dose-dependent curves could be obtained, concentration below the lowest concentrations or above the highest concentrations did not have an effect on thrombin. The results were expressed as percentage inhibition with respect to controls:

$$\text{Percentage inhibition} = \left[\frac{\Delta A/min \text{ [control]}}{\Delta A/min \text{ [test sample]}} \quad X \quad 100 \right] - 100$$

were ΔA/min is the rate change.
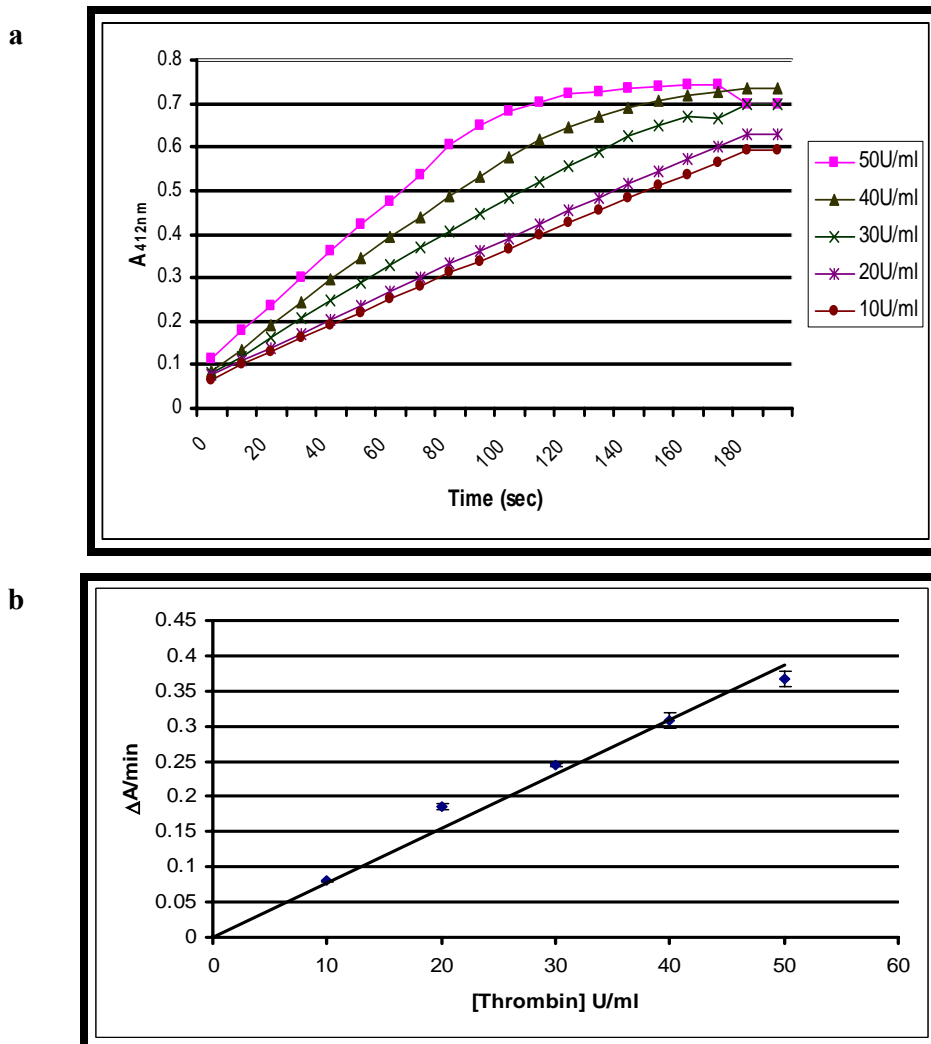
Figure 2.4: (a) An example of a typical set of thrombin assay progress curves and (b) a standard curve obtained for varying concentrations of thrombin (R^2 = 0.9679).

2.3.2 Fibrinolysis Assay

It is well known that the balance between fibrinolysis inhibitors (PAI-1 and α_2-plasmin) and activators (tPA and uPA) is important in bringing about balance in this process. Plasmin is the enzyme responsible for lysis of a plasma clot (Nagaoka *et al.*, 2000). A synthetic substrate S2251 (H-D-Val-Leu-Lys-p-nitroanilide) has been widely used to determine the activity of plasmin, but the use of this substrate determines the specificity of plasmin for its binding site rather than its catalytic activity. For example, α_2-plasmin inhibits the fibrinolytic activity of plasmin, but it does not effectively inhibit the hydrolysis of chromogenic substrates. In addition, plasminogen activators such as streptokinase, tPA, uPA all have different binding affinities and modes of action for

clot lysis. Therefore, a more direct assay for clot lysis mediated by plasmin is often required rather than using a chromogenic substrate (Mao and Tucci, 1991).

The assay used to investigate the effect of the extracts and marrubiin on fibrinolysis, i.e., whether they prolong or accelerate the rate of clot lysis. A modified turbidimetric assay adapted from Schatteman et al. (1999) was used.

A volume of 100μl of plasma from healthy individuals, who have not taken aspirin or any other medication 2 weeks prior to the assay, was added to a 96-well microtiter plate. This was subsequently followed by an addition of 20μl of either saline, OL, AL, or marrubiin; 10μl streptokinase, 10μl calcium chloride (160mM), and 10μl thrombin (10U/ml). The solution of calcium chloride and thrombin were used to initiate clotting of the plasma, and streptokinase (Sigma) was used to initiate fibrinolysis. The change in absorbance was measured at 412nm every minute for an hour (Labsystems Multiskan MS). A range of streptokinase concentrations between 125-1000U/ml was used to obtain a set of progress curves (figure 2.5). The screening of plant extracts and marrubiin was completed using 750U/ml as this had been determined from variety of concentrations of streptokinase tested (figure 2.5). For all assays completed, IC_{50} for the lowest possible extract concentration was determined. To ensure physiological relevance first nanogram, then microgram, and finally milligram concentrations were tested to obtain IC_{50} values. Concentrations between 6.25μg/ml and 100μg/ml of the OL and AL were tested for their anti-fibrinolytic activity. Similar to the thrombin assay, various concentrations of the extracts and marrubiin were tested before dose-dependent curves were obtained.
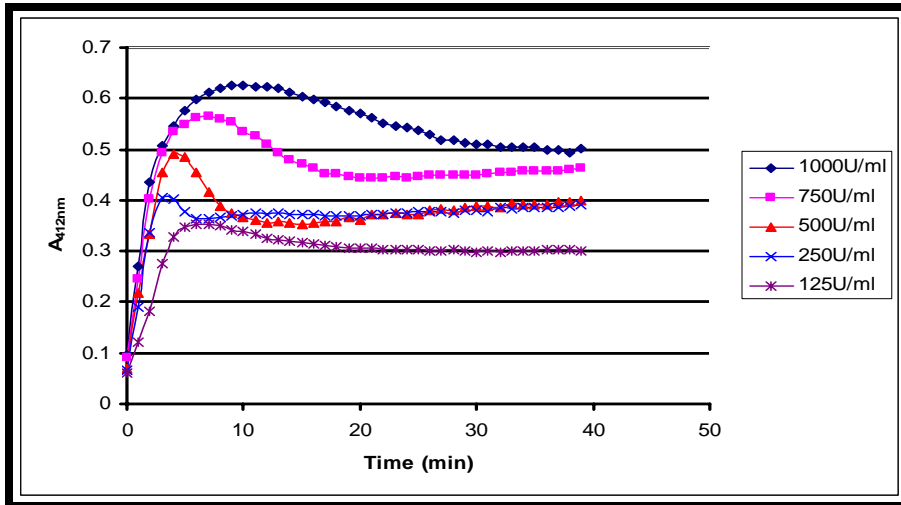
Figure 2.5: Typical fibrinolysis curves using varying concentrations of streptokinase.

A typical progress curve for the fibrinolysis assay has a bell-shaped curve, showing an increased absorbance during clot formation and the decrease down to baseline represents fibrinolysis. The 50% clotting time can be obtained at the point at which half-maximal turbidity is reached on the ascending leg of the curve and 50% lysis time can be obtained at the point when half-maximal turbidity on the descending leg of the curve is reached (Nagashima, 2000). From the fibrinolysis curves; fibrin formation, 50% clotting time, and 50% lysis time are obtained. Fibrin indices (FI) were also calculated using the following equation:

$$FI = \left[\frac{(T2/T1)\ sample}{(T2/T1)\ standard} \right] \times \left[\frac{MA\ sample}{MA\ standard} \right] \times 100$$

FI may be calculated by relating the ratio of the time to completion of the first phase of decline in absorbance (T2) to the time to maximum absorbance (T1) for the sample as compared to the standard, with a correction factor for differences in maximum absorbance [MAstandard/MAsample] (Goldernberg *et al.*, 2005).

For all subsequent coagulation assays, healthy individuals who are not on medication donated blood. Ethical clearance in line with the guidelines set by the Human Ethical Committee was obtained.

2.3.3 Platelet Aggregation Studies

Spectrophotometric and flow cytometric methods were used to determine the effect of the OL and AL extracts and marrubiin on platelet aggregation.

2.3.3.1 Platelet Aggregation Studies using Spectrophotometry

2.3.3.1.1 Isolation of Platelets

Platelets were harvested from various healthy human blood by centrifugation. Blood was drawn by venipuncture in an anticoagulant solution consisting of sodium citrate. PRP was obtained by centrifugation at 300xg for 10 minutes, PPP was obtained by centrifugation at 900xg for 15 minutes. The platelets were gently suspended in a buffer composed of 145mmol/l NaCl, 5mmol/l KCl, 10mmol/l Hepes, 0.5mmol/l Na_2HPO_4, 6mmol/l glucose, and 0.2% bovine serum albumin (BSA), pH7.4 (buffer A). The platelet suspension was kept at RT and utilized within 1 hour. Ten minutes before use, the platelets were warmed up at 37˚C (Bellavite *et al*., 1993).

2.3.3.1.2 Platelet Count

A rapid spectrophotometric method for determining the number of platelets in a platelet suspension and in blood plasma developed by Walkowiak *et al*. (1997) was used. Specific dilutions were made both for PRP and platelet suspension in buffer A. A sample (200µl) of each dilution was placed in a 96-well microtiter plate for measurement of turbidity (Labsystems Multiskan MS) (600nm). Each sample was also used to perform triplicate platelet counts using a haemocytometer chamber. A standard curve was constructed from the platelet counts (figure 2.6).
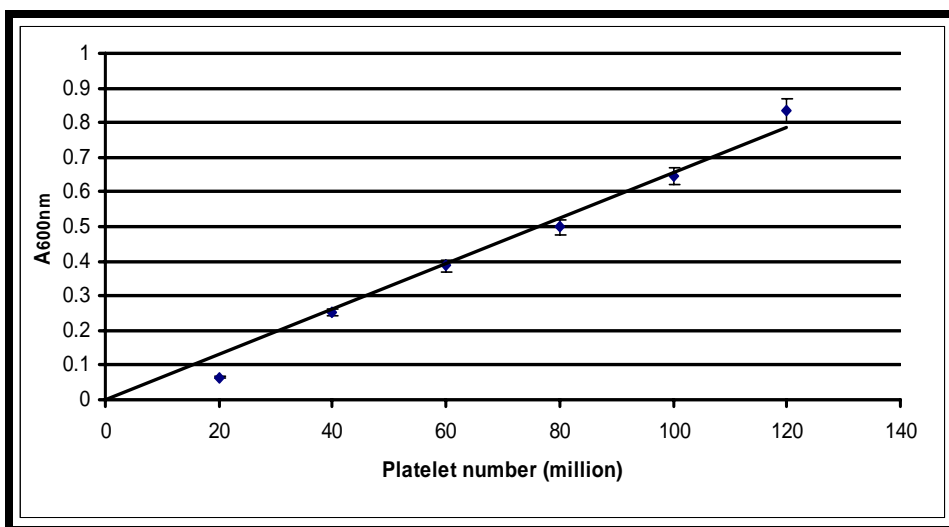
Figure 2.6: A standard curve showing platelet count using a haemocytometer chamber and a microtiter plate reader, the error bars represent standard deviation of three independent experiments ($R^2 = 0.9812$).

Platelet aggregation plays a vital role in vessel constriction, blood coagulation, haemostasis, and thrombosis. To investigate the effect of plant extracts and marrubiin on platelet aggregation, a turbimetric method was used to measure platelet aggregation in PRP. The method that was used to measure platelet aggregation was adapted from Lũthje and Ogilvie (1999). It is a very simple method, inexpensive, and does not require sophisticated laboratory equipment.

The experiment is based on the fact that platelet aggregates can be preserved in a blood sample with a buffered EDTA/formalin solution. Various healthy individuals donated blood, for both the spectrophotometric and flow cytometry experiments. Blood (3ml) in a glass beaker was continuously stirred for 2 minutes. A sample (200µl) was transferred into an Eppendorf tube with 800µl of a fixing solution, to represent a time zero sample. A 100µl aliquiot of a 200µM stock solution of ADP, was added 40 seconds after sample zero was taken. Two hundred microlitres of blood was transferred from the beaker to an Eppendorf tube containing a fixing solution every 30 seconds for 2 minutes. The negative control replaced ADP with an equal volume of saline. Hundred microliters of the OL extract or AL extract or marrubiin was added 1 minute before the addition of the agonist. All samples were then centrifuged at 300xg for 3 minutes, and 200µl of the PRP supernatants were transferred to a 96-well plate. The absorption of

PRP was measured at 540nm. Blood was centrifuged at 900xg for 5 minutes to obtain PPP, and the absorption of PPP was also measured at 540nm. The difference between PRP and PPP was calculated and was used as a measure of platelet aggregation. Since this assay is conducted under physiological conditions, low concentrations of the extracts and marrubiin were used. The concentrations that were used in the fibrinolysis assay had no effect on platelet aggregation, hence, various concentrations were tested to obtain an optimal concentration for each extract and marrubiin. Concentrations between 0.25mg/ml and 1mg/ml of the OL and AL extract were tested, and concentrations between 1250ng/ml and 5000ng/ml of marrubiin were tested.

2.3.3.2 Platelet Aggregation Studies using Flow Cytometry

Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5μm-40μm in diameter. One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of a specific wavelength of light is measured for a bulk volume of sample. Platelet activity, circulating activated platelets, platelet-platelet aggregates, leukocyte-platelet aggregates, procoagulant platelet-derived microparticles, and calcium flux can be measured by aggregometry but cannot determine whether a particular condition directly activates platelets, since it only shows results in changes in platelet reactivity. Activation of platelets by a clinical condition can be indirectly determined in plasma assays, but these assays cannot measure changes in platelet reactivity associated with the condition. Measurement of platelet aggregation by such assays is semi-quantitative and it has standardization problems. Previous aggregation studies only investigated platelet-platelet interaction for the development of thrombosis, whereas recent developments in flow cytometric analysis have made it possible to detect specific markers of platelet activation. The most studied markers are P-selectin, a marker for platelet degranulation, and PAC-1, a marker for GPIIb/IIIa activation. Flow cytometry is an answer to all of these problems. Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined (Kasuya *et al*., 2006; Michelson *et al.*, 2000; Shattil *et al.*, 1987; Karlheinz *et al.*, 1998; Heijnen *et al.*, 1999; Michelson *et al.*, 1991).

Platelets prevent blood loss by constantly patrolling the inner surface of blood vessels searching for leaks to be sealed. Once the injury has been detected the response of platelets can be divided into three phases, (1) adhesion; where platelets adhere to the injured vessel via their receptors GP1b and GPIIb/IIIa mediated by the ligands vWF, (2) aggregation; where the platelets adhere to each other via fibrinogen binding GPIIb/IIIa, and (3) secretion; where granules are released by exocytosis when the platelets are activated, forming a haemostatic plug. Flow cytometry allows reseachers to mimic one or several aspects of these three phases to obtain reliable data (Reininger, 2006).

The test monoclonal antibody used was PAC1. PAC1 is an antibody that binds to a fibrinogen-binding site exposed by conformational change that occurs in the GPIIb-IIIa complex when it is activated (figure 2.7) (Michelson, 2000).
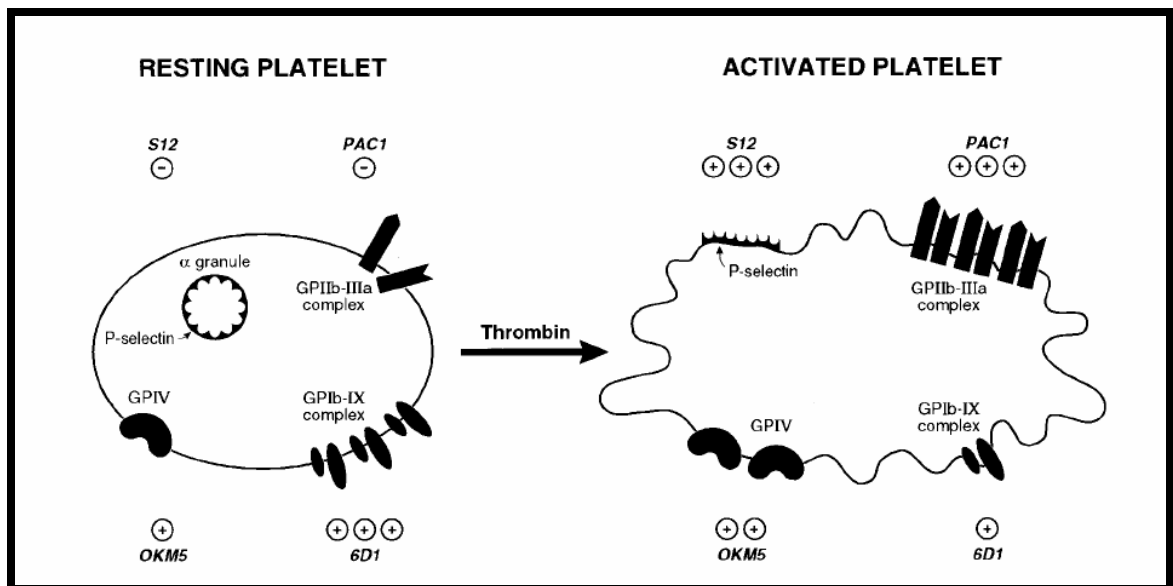
Figure 2.7: Effect of platelet activation on monoclonal antibody binding (Michelson *et al*., 2000).

The method that was used was adapted from Michelson *et al.* (1991). At first, the effect of the plant extracts and marrubiin on platelets was determined to observe if the plant extracts and marrubiin induced platelet aggregation. A PRP sample containing a platelet count of 30 x10^6 platelets/ml was used for all flow cytometry experiments.

Within 15 minutes of drawing blood, whole blood was centrifuged at 300x g to obtain PRP. PRP was diluted with Tyrode's buffer (137mmol/L NaCl, 2.8mmol/L KCl, 1mmol/L MgCl$_2$, 12mmo/L NaHCO$_3$, 0.4mmol/L Na$_2$HPO$_4$, 0.35% BSA, 10mmol/L HEPES, 5.5mmol/L glucose, pH 7.4) resulting in a final pH of 7.3 to obtain 30×10^6 platelets/ml. A volume of 40μl of PRP was aliquoted into tubes containing 10μl saturated concentration (1μg/ml) (to ensure that PAC-1 binds to most of GPIIb/IIIa binding sites, so that a better yield for the activation of this complex can be obtained) of fluorescein isothiocyanate (FITC)-PAC-1 (BD Biosciences). To inhibit fibrin polymerization, 2μl of 2.5mmol/L (final concentration) of peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (Sigma) was added. For a positive control, 100μl of thrombin stock (50U/ml) was added to make a final concentration of 10U/ml, the same volume of water was added for a negative control, and 10μl of the OL or AL extract was added, or the same volume of marrubiin was added. The tubes were then incubated at RT for 15 minutes. The platelets were subsequently fixed after the incubation period with 2:1 ratio of 1% formaldehyde for 30 minutes at RT. A threefold volume of Tyrode's buffer was added and the cells were immediately analyzed with a flow cytometer (Beckman Coulter Cytomics FC 500). Twenty thousand events were counted. The excitation and emission wavelengths were 488nm and 530nm, respectively. Concentrations between 0.5mg/ml and 2mg/ml of the OL and AL extract were tested, and 10μg/ml and 100μg/ml of marrubiin were tested (Michelson *et al*., 1991). Concentrations that were close as possible to the concentrations used for the platelet aggregation studies conducted on the spectrophotometer were used in order to compare results. The results were expressed as percentage inhibition with respect to controls:

$$\text{Percentage inhibition} = \left[\frac{\Delta \text{ PAC-1 positive cells [control]}}{\Delta \text{ PAC-1 positive cells [test sample]}} \quad X \quad 100 \right] \quad - \quad 100$$

2.4   Rat Islet Isolation

Male Wistar rats were used for the isolation of pancreatic islets. The rats were maintained on normal chow and water *ad libitum*. Isolation of rat islets was routinely performed on young rats (4 weeks old). The method that was used was adapted from Maedler *et al*. (2001). The isolation procedure is outlined below.

❖ The rats were injected intraperitoneally with 500μl of Sodium Pentobarbital

❖ The rats were disinfected with 70% ethanol.

❖ The ventral surface was opened and the pancreas exposed.

- ❖ The common pancreatic duct was ligated where it enters the duodenum.
- ❖ A small incision was made in the pancreatic duct and thin tubing was inserted to perfused about 10ml of collagenase P (Sigma) (2 units per rat) into the common pancreatic duct.
- ❖ Fat, spleen, and connective tissue was removed from the pancreas and the pancreas (after cutting it into smaller portions to facilitate digestion) was placed into a 50ml tube containing 5ml of Collagenase P
- ❖ The pancreas was digested for 13 minutes in a 37˚C water bath.
- ❖ The pancreas was shaken twice to facilitate digestion (shaken at 6 minutes and after 13 minutes).
- ❖ Ice cold quenching buffer (refer to table 2.1) was added to the pancreas up to 50ml.
- ❖ This was then centrifuged at 400x g for 2 minutes at 4˚C.
- ❖ The liquid was aspirated off and the pellet was resuspended in 20ml quenching buffer.
- ❖ The mixture was filtered through a gauze (cheese cloth), and 30ml of quenching buffer was added to the filtrate to make up 50ml.
- ❖ The mixture was then centrifuged at 400x g for 2 minutes at 4˚C.
- ❖ The supernatant was aspirated off, and the pellet resuspended in 10ml Histopaque 1119 (Sigma).
- ❖ Islets were spun through a Histopaque gradient, to separate the endocrine islets from the exocrine portions and cell debris.
- ❖ Centrifugation speed was set at 660x g for 30 minutes with slow acceleration and zero brake.
- ❖ Islets were collected from the Histopaque interphase, and transferred to a 50ml tube.
- ❖ The islets were then washed with RPMI containing 11.1mM glucose, and centrifuged at 800xg for 15 minutes.
- ❖ Media was aspirated off and the pellet resuspended in 10ml of RPMI containing 11.1mM glucose.
- ❖ Two hundred and fifty to three hundred islets were isolated from each rat.
- ❖ The islets were pooled and plated for the subsequent assays.
- ❖ Control islets were used to measure experimental conditions

To ensure sterility the following steps were taken:

The islets were isolated using sterile conditions (i). The rats were disinfected with 70% ethanol (ii). The isolated working area was also disinfected with 70% ethanol (iii). All the solutions/buffers/media, tubes and dissecting instruments were autoclaved (iv). After the pancreas digestion step, the subsequent steps were conducted in a fume hood in the cell culture laboratory (v).

2.5   Experimental Design

The extract which showed the greatest anticoagulant activity was further investigated for anti-diabetic activity.

The following studies were included in the experimental design: GSIS, apoptosis, proliferation, and RT-PCR. For all four studies, the islets were cultured in both normoglycemic (11.1mM glucose) and hyperglycemic conditions (33.3mM glucose).

2.5.1 Glucose Stimulated Insulin Secretion (GSIS)

For each of the three experiments completed, 15 islets were seeded per plate. The concentration of the OL extract and marrubiin (M) used in the coagulation studies were considered to be very high for cell work, therefore, further dilutions were required. The islets were exposed to 2.5µg/ml (OL 1) and 10µg/ml (OL 2) OL extract and 150ng/ml (M). Each condition was done in triplicate in each of the experiments.

2.5.2 Apoptotic Studies

For each of the two experiments completed, 10 islets were seeded per plate. The islets were exposed to 2.5µg/ml (OL 1) and 10µg/ml (OL 2) extract and 150ng/ml (M). Each condition was done in duplicate in each of the experiments. The results of these studies were normalized to media-treated islets (set to 100%), with a minimum of 500 cells counted per plate. Detail discussed in section 2.9.

2.5.3 Proliferation Studies

For each of the two experiments completed, 10 islets were seeded per plate. The islets were exposed to 2.5µg/ml (OL 1) and 10µg/ml (OL 2) extract and 150ng/ml (M). The results of these studies were normalized to media-treated islets (set to 100%), with a minimum of 500 cells counted per plate. Detail discussed in section 2.10.

2.5.4 Real-Time Polymerase Chain Reaction

For each experiment completed, 75-100 islets were seeded. The islets were exposed to OL 2 extract and M.

Table 2.1: Preparation of NB 8 solution and Quenching buffer required in islet isolation.

Collagenase P (2 units/rat) Solution	For 1 rat	for 4 rats	for 6 rats	for 8 rats
*HBSS (Highveld Biological)	15ml	60ml	90ml	120ml
Dnase	1mg	6mg	9mg	12mg
1M HEPES, pH7.4	0.375ml	1.5ml	2.25ml	3ml
1M CaCl$_2$	131µl	524µl	786µl	1048µl
Quenching buffer				
HBSS		200ml	300ml	400ml
BSA (Sigma)		1.15g	1.75g	2.5g
1M Hepes		5ml	7.5ml	10ml

* HBSS = Hank's balanced salt solution

Table 2.1 displays the preparation of collagenase solution and the quenching buffer that are used in the islet isolation process.

2.6 Islet Exposure to Organic Extract of *Leonotis leonurus* and Marrubiin

The islets were seeded into 35mm plates coated with bovine corneal cells which served as ECM (Novamed, Israel), to which the islets attached (Maedler *et al*., 2001). It took 48 hours for the islets to attach and flatten in the plates. After 48 hours, media (preparation of RPMI media is shown on table 2.2) was aspirated off. For control cells fresh media was added, no extract or marrubiin was added. The experimental islets were exposed to OL 1 or OL 2 extracts, or 150ng/ml of marrubiin. The control islets were cultured in either 11.1mM glucose RPMI or 33.3mM glucose RPMI media. The islets were exposed to the OL extracts or M for 48 hours (standard time of exposure used in islet studies), after which a GSIS assay and RNA isolation were immediately completed on respective dishes (section 2.7 and 2.11). Some cells were fixed with 4% paraformaldehyde (pH 7.3) and stored at 4˚C, and were later used for immunohistochemical studies; viz terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) and Ki-67 (discussion in section 2.9 and 2.10, respectively).

The paraformaldehyde solution was prepared as follows:

Paraformaldehyde	4g
Distilled water	40ml
*10x Phosphate Buffered Saline (PBS)	10ml
1N NaOH	0.1ml

While stirring warm at 60˚C, set pH to 7.3 and warm again to 60˚C. Aliquot into 50ml tubes and store at -20˚C.

* 10x PBS was prepared as follows:

NaCl	80g
KH_2PO_4	2g
$Na_2HPO_4.12\,H_2O$	29g
KCl	2g
H_2O	1L

pH was adjusted to 7.4 using NaOH and HCl.

Table 2.2: Media for treatment of rat islets – RPMI.

Reagent	11.1mM glucose	33.3mM glucose
RPMI 1640 (Highveld Biological)	437ml	437ml
Bovine Fetal serum (Sigma)	50ml	50ml
Glutamax 10mg/ml (The Scientific group)	5ml	5ml
Streptomycin/Penicillin 10mg/ml (The Scientific group)	5ml	5ml
Fungison 10mg/ml (The Scientific group)	0.5ml	0.5ml
Gentamycin 10mg/ml (Highveld biological)	2.5ml	2.5ml
Glucose powder (Sigma)	1g	3g
Total volume	500ml	500ml

NB: RPMI that was used was without glucose and L-glutamine.

2.7 Glucose Stimulated Insulin Secretion Assay

GSIS was performed to determine if the OL extract or marrubiin had an effect on insulin secretion of the islets (Maedler, 2001). After 48 hours of exposure of the islets to the OL extract or M, GSIS was performed as follows:

- ❖ 1ml of media was transferred to an Eppendorf tube (chronic sample, equivalent to 48 hour insulin secretion).
- ❖ RPMI media was removed from 35mm culture plates.
- ❖ 1ml of 1x Krebs 3.3mM glucose solution was added to the plates.
- ❖ The islets were incubated at 37˚C for 30 minutes.
- ❖ Subsequent to the incubation period, the solution was aspirated off.
- ❖ 1ml of 1x Krebs 3.3mM glucose solution was added to each culture plate.
- ❖ The islets were incubated at 37˚C for 1 hour.
- ❖ This solution was transferred to an Eppendorf (basal sample, insulin secretion after stimulation with low glucose concentration).
- ❖ 1ml of 1x Krebs 16.7mM glucose solution was added to each plate.
- ❖ The islets were incubated at 37˚C for 1 hour.
- ❖ This solution was transferred to an Eppendorf tube (stimulated sample, insulin secretion after stimulation with a high glucose concentration).

- ❖ The samples were stored at -20˚C.
- ❖ 1ml of 0.18M HCl in 70% was added to the dishes.
- ❖ The islets were incubated at 4˚C for 1 hour.
- ❖ This solution was transferred to an Eppendorf tube (insulin content sample, insulin content of the islets).
- ❖ The samples were stored at -20˚C, until analyzed using radioimmunoassay (RIA) to assay for insulin.
- ❖ The stimulatory index was determined using the following equation: Stimulatory index = stimulating insulin secretion ÷ basal insulin secretion.

The recipe for Krebs Ringer buffer is given below:

10x Krebs Ringer buffer (buffer required in GSIS)

NaCl	33.6g
KCl	1.76g
$CaCl_2.2H_2O$	1.88g
$MgSO_4.7H_2O$	1.48g

Add 500ml of H_2O, autoclave and store at 4˚C

2.8 Rat Insulin Radioimmunoassay

A Linco rat insulin RIA kit was used according to the manufacture's instructions. In a radioimmunoassay, a fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum. In such a system, the antigen has limited binding sites on the antibody. The addition of an unlabelled antigen brings about competition for the binding sites on the antibody. Thus the amount of labelled antigen bound will decrease as the concentration of the unlabelled antigen increases. The tracer bound can be measured by separating antibody-bound from free tracer, followed by a count of free tracer, bound tracer, or both fractions. A standard curve with increasing concentrations of unlabeled antigen can be generated, and the concentration of the antigen in the unknown sample can be determined using the standard curve (figure 2.8). Therefore the most important entities in a radioimmunoassay are: a specific antiserum to the antigen to be measured, the availability of a radioactive labelled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and an instrument to count radioactivity (Derberg *et al*., 1998).
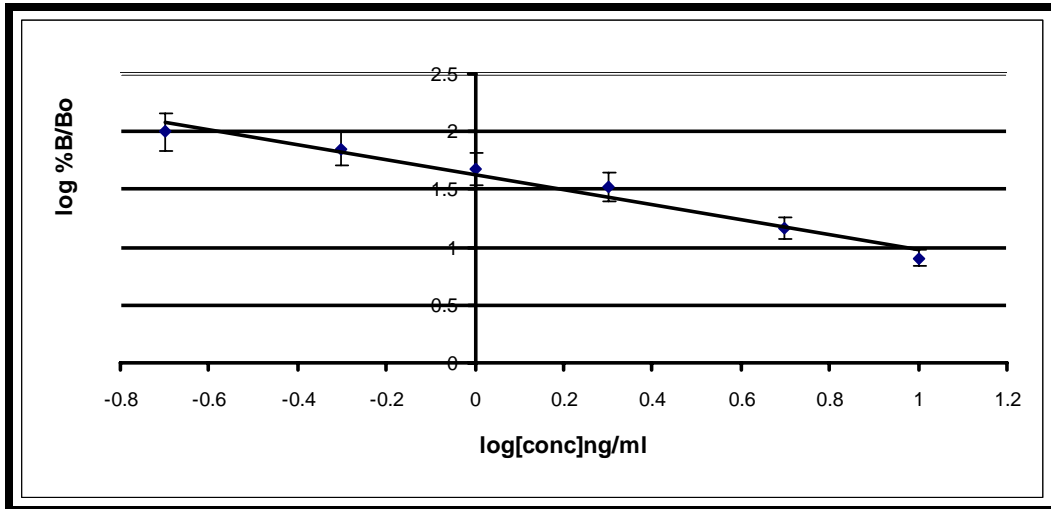
Figure 2.8: An example of a radioimmunoassay standard curve of rat insulin standards (0.2-10ng/ml) used during experimentation. The error bars represent a standard deviation of duplicate standards per experiment, R^2 = 0.9822.

The samples from GSIS (section 2.7) were incubated with ^{125}I insulin sensitive tracer to quantify the amount of insulin secreted. A liquid scintillation analyser (TRI-CARB 2300TR) was used to count the radioactivity of the precipitated samples.

2.9 Immunochemistry Studies

Ten islets per dish were plated for TUNEL and Ki-67 staining respectively. All immunohistochemical studies were conducted in duplicate, with duplicate plates per condition per experiment being analyzed. The islets that were fixed in section 2.6 were used for the immunohistochemical studies.

2.9.1 TUNEL Staining of Rat Islets Plated on Extracellular Matrix Plates

The mechanism of the apoptotic process is generally recognized as being highly intricate, as a result its detection is very difficult. The sensitivity of a detection method is reflected by both the ability of a particular technique to label most of the cells displaying the appropriate marker and also the possibility of labelling cells in the earliest phase of the apoptotic process. There are basically three techniques that are commonly used: (1) terminal dUTP (Terminal deoxynucleotidyl transferase)-transferase-mediated nick end labelling (TUNEL), (2) apostain, and (3) lamin B, all

three of them use immunohistochemistry to visualize. TUNEL and apostain showed the most sensitivity when compared to lamin (Prochazkova *et al.*, 2003).

In this study, TUNEL was used as a technique to detect apoptosis. The principle of this technique is that DNA ends generated during apoptosis are extended by dUTP using biotin- or digoxigenin-labeled BrdUrd (bromodeoxyuridine); incorporated material will be detected by fluorescent reagents; TUNEL preferentially labels apoptotic cells to necrotic cells. TUNEL is preferred to other methods because it is simple, rapid, and provides accurate results (Omran, 2003). After staining the islets for TUNEL, the islets were viewed under a microscope and TUNEL positive islets could be viewed as they stained black. The steps for the completion of staining are outlined below.

- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 1ml of 0.5% Triton X-100 for 4 minutes at room temperature (RT).
- ❖ Wash with 3ml of PBS for 2 minutes (2x).
- ❖ Dry the area around cells with cotton buds and apply a layer of wax using PAP pen (Zymed) to restrict the stain, preventing it from flowing from periphery of the dish.
- ❖ Wash with 3ml of PBS.
- ❖ Apply 50μl of TUNEL reaction mixture (*In Situ* Cell Death Kit, AP, Roche, dilute bottle 1 into bottle 2 at dilution 1:10 in an Eppendorf tube)
- ❖ Cover and incubate for an hour at 37˚C.
- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Apply 50μl of Converter-AP (*In Situ* Cell Death Kit, AP, Roche)
- ❖ Cover and incubate for 30 minutes at 37˚C.
- ❖ Rinse with 3ml of PBS for 2 minutes (3x).
- ❖ Apply 50μl of substrate solution 5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) (Sigma).
- ❖ Incubate for 10 minutes at RT.
- ❖ Rinse with 3ml of water.
- ❖ Wash with 3ml of PBS for 1 minute (2x).
- ❖ Add 50μl of anti-insulin antibody (DAKO, 1:50 in PBS) and incubate at 37˚C for 30 minutes.
- ❖ Wash with 3ml of PBS for 2 minutes (3x).

- ❖ Add 1 drop of reagent B (biotinylated secondary antibody, Histostain-Plus Kits, Zymed) incubate for 10 minutes at RT.
- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 1 drop of reagent C (streptavidin-peroxidase conjugate, Histostain-Plus Kits, Zymed) and incubate for 10 minutes at RT.
- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 1 drop of reagent D (3-amino, 9-ethyl carbozol (AEC) single solution chromogen, Histostain-Plus Kits, Zymed) and incubate for 3-8 minutes.
- ❖ Wash with 3ml of water (2x).
- ❖ Wash with 1ml of PBS.
- ❖ Add 1-2 drops of 37-40˚ C gelatine (23ml of water, 12ml glycerol, 7g gelatin, soak for 5 minutes and melt at 40˚C) on cells, place coverslip on top.

For TUNEL and Ki-67 staining (section 2.10), five hundred insulin positive β-cells were counted per plate and were normalized to 100% for controls, thereby, measuring experimental cells relative to the control cells.

2.9.2 Ki-67 Staining of Rat Islets Plated on Extracellular Matrix Plates

Ki-67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle (G_1, S, G_2 and M phases) but absent in resting cells (G_0). Ki-67 is also related to growth potential in a variety of tumours, therefore, an increase in the expression of this antigen reflects cell proliferation (Honegger *et al*., 2003).

The following protocol was followed:

- ❖ Pipette 3ml of PBS to wash the islets for 2 minutes (3x).
- ❖ Add 1ml of 0.5% Triton X-100 for 4 minutes at RT.
- ❖ Wash with 3ml of PBS for 2 minutes (2x).
- ❖ Dry the area around cells and apply PAP pen.
- ❖ Wash with 3ml of PBS.
- ❖ Add 1-2 drops of reagent A (serum blocking solution, Histostain-Plus Kits, Zymed) for 10 minutes at RT.
- ❖ Add 50µl of anti mouse Ki-67 antibody (Santa Cruz Biotechnology) for 30 minutes at 37˚C (dilution 1:50).

- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 50μl of donkey anti-goat biotin conjugated antibody (Santa Cruz Biotechnology) for 30 minutes at 37˚C (dilution 1:50).
- ❖ Add 1-2 drops of reagent D for 10 minutes at RT.
- ❖ Wash with 2ml of water for 2 minutes (2x).
- ❖ Wash with 3ml of PBS for 2 minutes.
- ❖ Add 50μl of anti-insulin antibody (DAKO, 1:50 in PBS) and incubate at 37˚C for 30 minutes.
- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 50μl of secondary antibody (fluorescein conjugated rabbit anti-guinea pig antibody), incubate for 30 minutes at 37˚C (dilution 1:20).
- ❖ Wash with 3ml of PBS for 2 minutes (3x).
- ❖ Add 1-2 drops of 37-40˚ C gelatine on cells, and place a coverslip on top.

2.11 RNA Isolation

The islets were exposed to 10μg/ml of the OL extract or 150ng/ml M (refer to section 2.6). RNA was isolated for RT-PCR using an RNeasy Mini kit (Qiagen) to extract total RNA extraction, using the selective binding properties of a silica-gel-based membrane is combined with the speed of microspin technology. 100μg of RNA with bases longer than 200 nucleotides can be isolated with this kit because of the high salt buffer system supplied in this kit. A highly denaturing buffer containing guanidine isothiocyanate is used to lyse the cells and inactivate RNases. Appropriate binding conditions are provided by the addition of ethanol. Ethanol allows the total RNA to bind to an RNeasy column and removes any contaminants that might be present in the sample. The RNA is eluted with RNase-free water

(http://www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/RNeasyMini.aspx - 36k, 3 August 2006).

- ❖ A 100x dilution of β-mercaptoethanol was prepared in RLT buffer supplied by Qiagen to lyse the islets (350μl of the lysis buffer is required per plate).
- ❖ Cells were scraped with a cell scraper.
- ❖ 350μl of 70% ethanol was added to the homogenized lysate, mixed well with a pipette.

- ❖ 700 µl of the sample was added to an RNeasy spin column placed in a 2ml collection tube.
- ❖ The sample was centrifuged at 8000x g for 15 seconds, and the flow through was discarded.
- ❖ 700 µl of buffer RW1 was added to the RNeasy spin column, and this was centrifuged at 8000x g for 15 seconds, the flow through and the collection tube was discarded.
- ❖ The RNeasy column was transferred into a new 2ml collection tube.
- ❖ 500 µl of Buffer RPE was added to the column, and centrifuged at 8000x g for 15 seconds.
- ❖ The flow through was discarded, and 500 µl of Buffer RPE was added again, and the column was centrifuged at 13400 rpm (48x g) for 15 seconds.
- ❖ The flow through was discarded, and the column was transferred into a new 2ml collection tube, and centrifuged at full speed for 1 minute.
- ❖ To elute, the column was transferred into a new 1.5ml collection tube, and 40µl of RNase-free water was added directly to the silica-gel membrane.
- ❖ This was centrifuged at 8000x g for 1 minute.
- ❖ To concentrate the RNA, the flow through was eluted through the column again, and centrifuged at 8000x g for 1 minute.

2.12 RNA Quantification

A fifty times dilution of each sample using Tris buffer (pH 7.0) was prepared. The RNA was quantified by using a A_{260}/A_{280} ratio. Table 2.3 shows the RNA concentrations obtained from the experimental samples after RNA was isolated using the Qiagen RNeasy mini kit.

Table 2.3: RNA concetrations of the islets exposed to the OL extract and marrubiin in normoglycemic and hyperglycemic conditions

Sample	RNA concentration (µg/µl)
Normoglycemic condition	
Control	0.440
OL exposed islets	0.348
M exposed islets	0.373
Hyperglycemic condition	
Control	0.329
OL exposed islets	0.328
M exposed islets	0.322

2.13 Reverse Transcriptase Assay

Reverse trancriptase assay was performed on each sample to obtain cDNA for RT-PCR, a QuantiTect Reverse Transcription kit was used according to the manufacture's instructions. The reaction mixtures were prepared as shown in table 2.4.

Table 2.4: Genomic DNA Elimination Reaction Components.

Reagent	Volume (1 reaction)	Volume (2 reactions)
DNA Wipeout buffer, 7x	2µl	4µl
Template RNA	up to 1µg	up to 2µg
RNase-free water	Variable	Variable
Total volume	14µl	28µl

❖ The tubes were incubated for 2 minutes at 42ºC.

❖ They were immediately placed on ice.

❖ Reverse-transcription master mix was prepared on ice according to the QuantiTect Reverse Transcription kit.

- ❖ After the RNA template was added to each tube containing Reverse Transcriptase-master mix contents were vortexed and stored on ice for 2 minutes.
- ❖ The tubes were incubated for 15 minutes at 42ºC.
- ❖ The tubes were immediately incubated for 3 minutes at 95ºC to inactivate Quantiscript Reverse Transcriptase.
- ❖ Copy DNA (cDNA) obtained from Reverse Transcriptase was used in RT-PCR to determine the effect of the OL extract and M on insulin gene expression.

2.14 Real-Time Polymerase Chain Reaction

This technique is very sensitive, as a result, very low levels of cDNA can be detected. RT-PCR is frequently used for primary validation of gene expression, bacterial species identification, and single nucleotide polymorphism genotyping (Dvoràk *et al*., 2003). First a RNA dependent-DNA polymerase is used to perform reverse transcription in order to synthesize cDNA complementary to mRNA of the samples. In RT-PCR one can use total RNA (1 μg) but it is more appropriate to use mRNA because it yields a higher probability of low copy mRNA transcripts. A sufficient amount of deoxynucleotide triphosphates is mixed with the synthesized cDNA, DNA dependent DNA-polymerase, and fluorescently labelled (Sybr green-labelled) oligonucleotide primers. As in the case of classical polymerase chain reaction (PCR), one reaction of RT-PCR consists of strand separation, annealing and elongation steps. Fluorescence is monitored for each sample on each cycle of RT-PCR. The increase in number of copies of cDNA of interest is observed by an increase in fluorescence intensity, the fluorescence linearly increases until a plateau is reached (Dvoràk *et al*., 2003). Figure 2.9 shows a schematic diagram of reverse transcription and RT-PCR. Table 2.5 shows a typical protocol for RT-PCR.

RNA isolation and characterization

↓

cDNA synthesis

↓

Real-time PCR data acquisition

↓

Generation of normalization factors relative to the standard curves generated for each gene

↓

Normalized data

↓

Data analysis
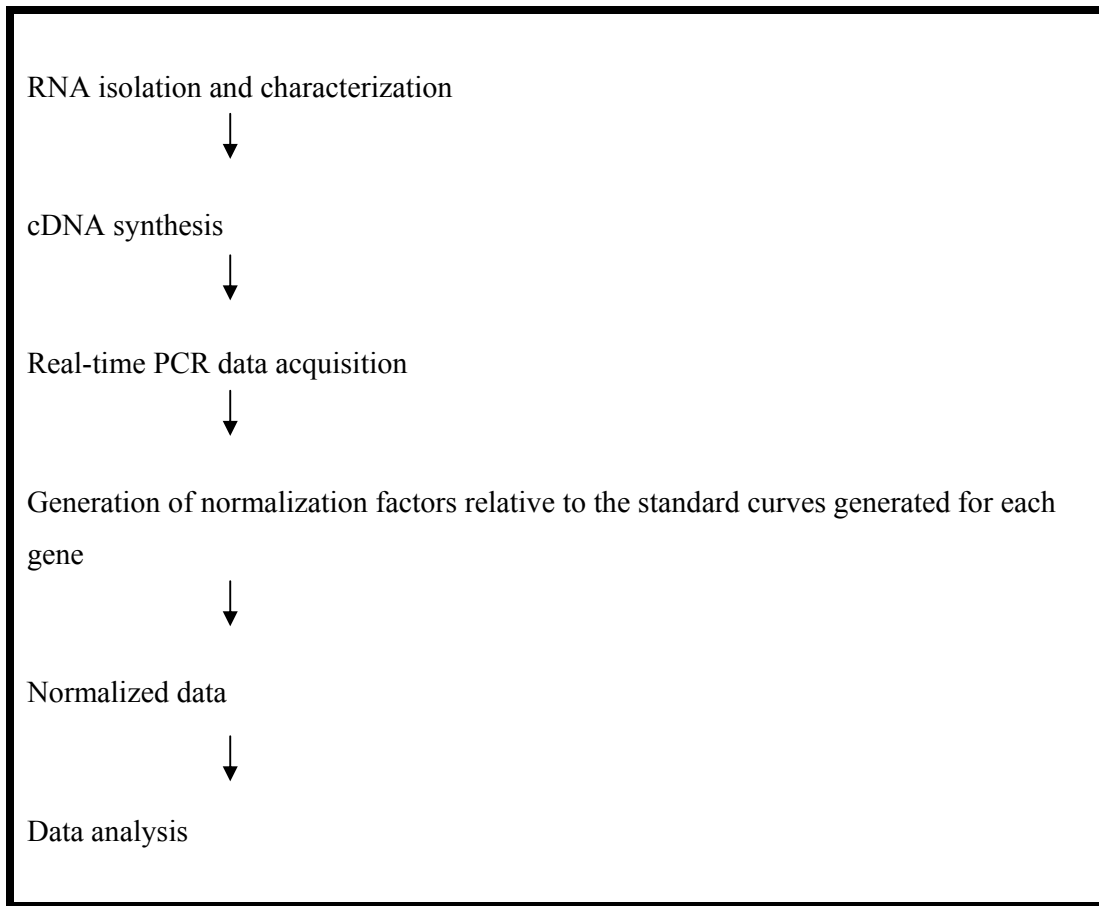
Figure 2.9: Steps performed during RT-PCR , (Wong and Medrano, 2005).

Table 2.5: A typical protocol for a RT-PCR reaction

Cycle number	Temperature (˚C)	Time (seconds)
1 (1x)		
Step 1	95	30
2 (40x)		
Step 1	95	30
Step 2	X	30
Step 3	72	30
3 (1x)		
Step 1	95	30
4 (70x)	X	30
5 (1x)		
Step 1	22	Hold

x: the annealing temperature varies, it depends on the gene that is used.

Mathematical models are needed for a reliable semi-quantitative RT-PCR (Nedelman *et al*., 1992; Peccoud and Jacob, 1996; Peccoud and Jacob, 1998). In this regard, the RT and PCR are completely different in respect to their enzymatic reactions and thus, they should be considered separately. Mathematically, the reverse transcription step in RT-PCR is very basic, it has no amplification. The sole variable in this step is the convertion of mRNA into cDNA. This can be stated in equation 1:

[cDNA] = [RNA] ×Efficiency….. [Equation 1]

Efficiency is measured as the percentage of RNA transcribed into cDNA (0 = no RT, 1 = total RT of all RNA is converted into cDNA. Efficiency can fluctuate from 5% to 90% because this step is susceptible to contaminants (Ferré *et al*., 1994**).** The mathematical description of the PCR step of RT-PCR is more complex than that of the RT step. The amplification of cDNA is not constant, it differs as the reaction progresses and for each gene or tube. The amount of DNA is theoretically doubled for each cycle of temperatures. This can be expressed in equation 2:

P = T (1+E)n……. [Equation 2]

where P is product (amount measured after n cycles); T is template or target (amount of cDNA from RT reaction); E is efficiency (percentage of cDNAs copied in a PCR cycle [from 0 to 1]); and n is cycle number (number of cycles through which the reaction proceeds). From Equation 2, it can be seen how small differences in amplification efficiency are compounded exponentially. Most reactions in the exponential phase have an efficiency of approximately 0.8, but this varies between reactions and for different experiments. The efficiency can also vary with the stage of reaction (Ferré *et al*., 1994). Semi-quantitative RT-PCR is a variable technique, hence, there is a need for standards (Gilliland *et al*., 1990; Wang *et al*., 1989). Endogenous RNA standards or internal standards (e.g. actin and tubulin) have been used as amplification standards.

Table 2.6 has a list of gene primers identified from rat-specific database with their amplification temperature. The housekeeping genes were used to normalize data obtained from RT-PCR. Nine various housekeeping genes, viz alpha (α) albumin, beta-2 microglobulin (B2M), cyclophilin A (cyclo A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethylbilane synthase (HMBS), thymine adenine thymine adenine (TATA) protein binding box, transferrin receptor (TR), beta- tubulin, and tyrosine-3-monooxygenase/tryptophan-5-monooxygenase-activation protein were tested to observe their stability across all the experimental conditions, but most of them showed variation in the threshold cycle (Ct) values, the difference between conditions within an experiment was more than one cycle. The Ct value denotes the number of cycles required before the fluorescence reaches a threshold. The more the target DNA is present in a sample, the lower is the Ct value (Fujikawa *et al*., 2006). Two housekeeping genes, β-tubulin and cyclo A were stable in one experimental condition, viz M under normoglycemic conditions, therefore, they were used to normalize data in normoglycemic conditions for marrubiin only, the other conditions could not be normalized because of the variations in Ct values of the internal standards. Insulin was the gene of interest. Due to time constraints, the search for appropriate housekeeping genes was halted. Therefore, the results will be expressed as insulin expression per condition.

Table 2.6: The primers associated with genes used for RT-PCR on cDNA of isolated rat islet cells

Gene	Accession no.	5´-3´	3´-5´	Amplification T°C
A-Albumin	NM_172320	AGCCCAGAGCCAGGTTATG	TCCTTGTTTGCGTTGATTATGC	58
B2M	NM_012512	CGTGATCTTTCTGGTGCTTGTC	TCTATCTGAGGTGGGTGGAAC	58
Cyclo A	XM341363	ACGTGGTCAAGACTGAGTGG	CGTGCTTCCCACCAGACC	58
GAPDH	NM_017008	TCCTTGCCTCTCAGACAATG	ACTTGGATGTTCCTTCTCTTAC	60
HMBS	NM_013168	ACCTCAACACCCGCCTAC	TCCCTGACCCACAGCATAC	58
Insulin	NM_019129	CTGCCCAGGCTTTTGTCAAA	TCCACTTCACGACGGGACTT	60
TATA	XM_217785.2	CAACAGCCTTCCACCTTATGC	AGTAAGCCCTGTGCCGTAAG	58
TR	XM_340999	AGGAACCAGACCGCTACATTG	GGGCAAGTTTCAACAGAAGACC	58
ß-Tubulin	NM_173102	AACCCAGCCCAGTTCTAAG	GCGATTCCCGTGTCTAAATG	58
Ywhaz	NM_013011	GAGTGTAGTCTGTGTGGGTAC	GCTGTGGTCAAGGGTGTG	58

Data were analyzed by using a semi-quantitative comparative Ct method, where the amount of the gene of interest was normalized to the housekeeping gene and expressed relative to the mean values of control samples using $2^{-\Delta\Delta Ct}$. The number of amplification cycles needed to reach the Ct and result in fluorescence was determined for both the gene of interest and the housekeeper gene. Each sample was run in duplicate. A ΔCt was then calculated for each sample, as the difference between the mean Ct value for the gene of interest and the mean Ct value for the housekeeper gene for that sample. In addition, a reference Ct value was calculated as the mean of the ΔCt values for the control sample. The reference Ct value obtained from the average of controls was then subtracted from each sample's ΔCt value to generate a variable, $\Delta\Delta Ct$, reflective of the activity of the gene of interest for each sample relative to the housekeeping gene. The mean $\Delta\Delta Ct$ value for a sample reflects the differences in gene expression relative to the housekeeping gene compared to controls. The mean $\Delta\Delta Ct$ value for the controls must mathematically equal zero. The fold-change in target quantity for the gene of interest relative to the housekeeper gene is then given by the equation $2^{-\Delta\Delta Ct}$, based on primer efficiencies being similar, hence the value of 2 (Hundley *et al*., 2006). Also, efficiency was determined for Tubulin, cyclo A, and insulin by setting up standard curves for each

of theses genes. IQ software calculated the gene efficiency of each gene as stated in figure 2.10, 2.11, and 2.12.

Tubulin and cyclo A standard curves were generated (figures 2.10 and 2.11). These two housekeeping genes were used to normalize RT-PCR data
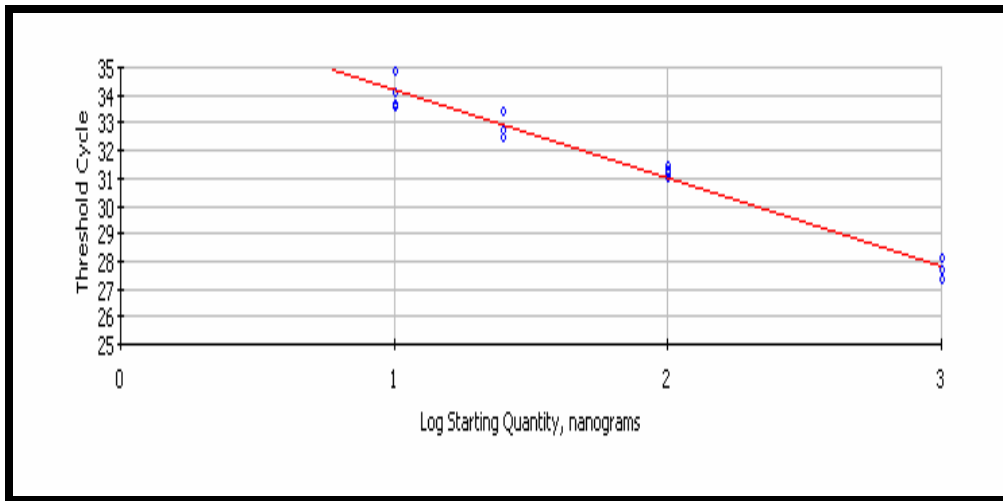
Figure 2.10: A typical standard curve for tubulin (10-30 ng/ml), the PCR amplification efficiency was 104.8%
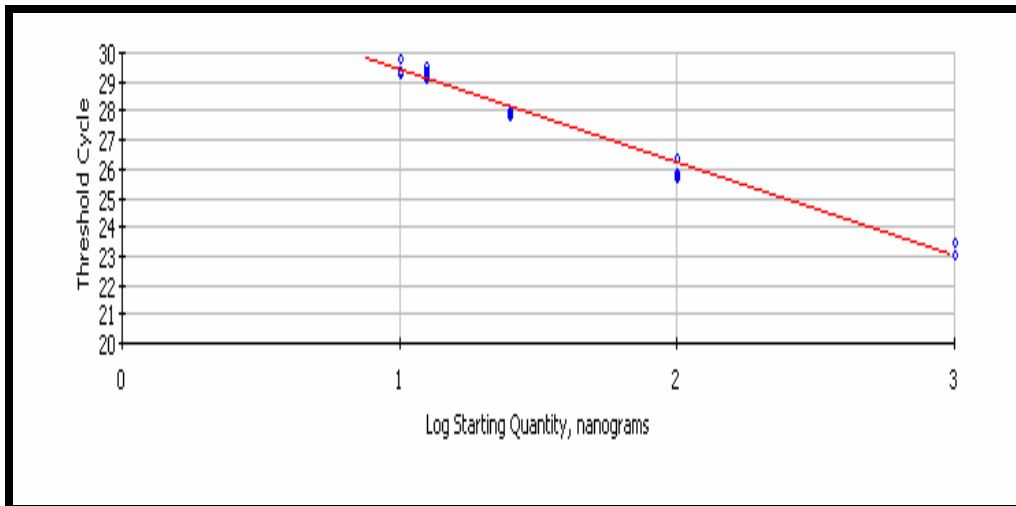
Figure 2.11: A typical standard curve for cyclophilin A (10-30 ng/ml), the PCR amplification efficiency was 104.8%
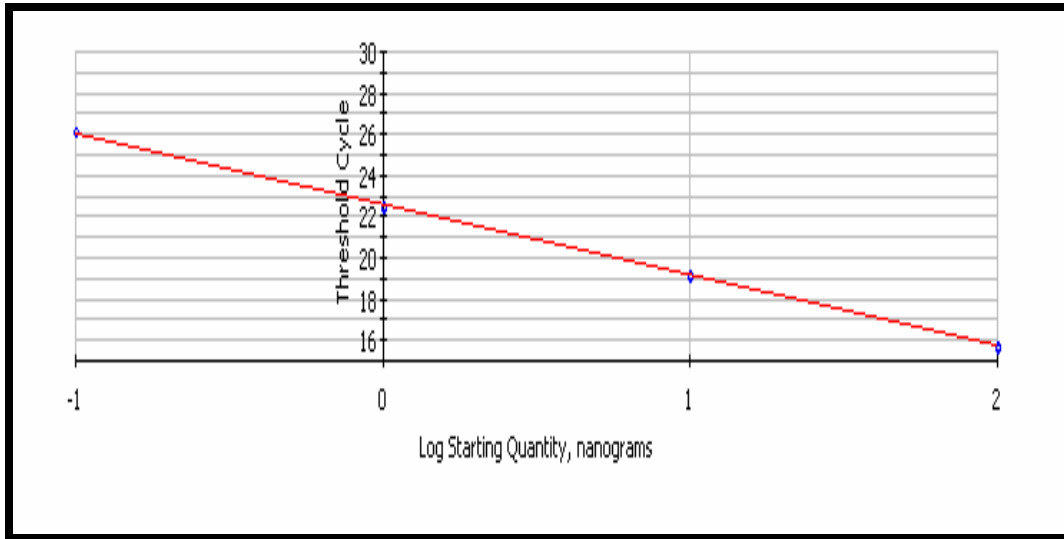
Figure 2.12: A typical standard curve for insulin (0.1-20 ng/ml), the PCR amplification efficiency was 95.6%

2.15 Ethical Clearance

Ethical clearance for this study was approved by the NMMU Human ethic committee and the NMMU Animal ethic committee (see appendix for a copy).

2.16 Statistical Analysis

Results were obtained as triplicate values and represented as mean ± standard error of the mean (sem), unless otherwise indicated. Paired student t-tests were performed to determine whether the observed results were statistically significant, where values of $P<0.05$ were considered to be statistically significant.

CHAPTER 3: The *in vitro* Effects of *Leonotis leonurus* and Marrubiin on Blood coagulation, Fibrinolysis, and Platelet aggregation

3.1 Quantification of Marrubiin

3.1.1 Quantification of Marrubiin Content with TLC

The quantification of the marrubiin content of *Leonotis leonurus* was completed using TLC and RP-HPLC. The thin-layer chromatogram (figure 3.1) identified the presence of marrubiin in the OL extract. The R_f value for the experimental conditions of the commercial marrubiin was found to be 0.55 as was that of the spot which corresponded with marrubiin in the OL extract. A TLC fingerprint of *Leonotis leonurus* has been determined by Scott *et al*. (2004). On this TLC fingerprint by Scott *et al*. (2004), one of the R_f values of the major compounds found in the methanol extract of *Leonotis leonurus* was 0.66, which yielded a purple colour similar to the marrubiin in the TLC chromatogram in figure 3.1. The picture had to be taken immediately because the spots fade away very quickly.

Figure 3.1: A typical TLC chromatogram depiciting the identification of the marrubiin content from the OL and AL extracts. Lanes 1-6 have decreasing concentrations of the marrubiin standard (5000, 4000, 3000, 2000, 1000 and 500ng, respectively). Lane 7-8, the OL extract (10mg/ml) and lane 9-10 the AL extract (10mg/ml). Five microlitres of each extract was loaded. The arrow indicates the solvent front.

From the thin-layer chromatogram, a standard curve was constructed (chapter 2, section 2.2.1), and used to determine the concentration of marrubiin from OL. This was found to be 2600ng per 50μg loaded on the TLC plate, therefore, the percentage of marrubiin in the OL extract was 5.2%. The AL extract had no marrubiin. In literature only the percentage of premarrubiin, a precursor of marrubiin, is known, viz. 0.01567% (Scott *et al*., 2004). Table 3.1 shows the marrubiin content of the OL extract concentrations used in the coagulation and diabetic studies.

Table 3.1: Marrubiin content of various OL extract concentrations

[OL extract] mg/ml	[Marrubiin] μg /ml	[OL extract] μg/ml	[Marrubiin] μg /ml
10	520	100	5200
8	416	50	2600
6	312	25	1300
4	208	12.5	605
2	104	6.25	325
1	52	10	502
0.5	26	2.5	125

3.1.2 Quantification of Marrubiin Content with RP-HPLC

No known method for quantifying marrubiin using RP-HPLC could be found in literature. HPLC studies completed have only been completed to obtain *Leonotis leonurus* fingerprints (Bienvenu *et al*., 2002; and Scott *et al*., 2004). The chromatographic conditions that had been used to obtain an HPLC fingerprint for *Leonotis leonurus* does not allow for quantification of marrubiin in plant extracts. A possible explanation for these findings was that the detection wavelength that was used was too high (360nm), hence an absorption spectra (chapter 2, section 2.2.2) had to be obtained as an initial step in quantifying marrubiin. Most studies had used either an isocratic system or gradient system with 1% acetic acid and methanol as a solvent system, but marrubiin was not retained on a C18 column under these experimental conditions (figure 3.2). A retention time of 2.99 minutes was obtained at a flow rate of 1ml/min.

In this study, ion-pair RP-HPLC was used as an alternative to try and solve this problem. TBA was the ion-pairing reagent that was used. This reagent improved the binding of marrubiin to the column because TBA is a cation, which bound to the negatively charged marrubiin (due to hydroxyl group and carbonyl group) to enhance its retention on the column. The pH of the solvent system used was at a pH of 3.5 but pH ranges (2-5) were tested on TLC to observe the stability of marrubiin (the results are not shown). These results indicated that marrubiin was stable at the tested pH values.
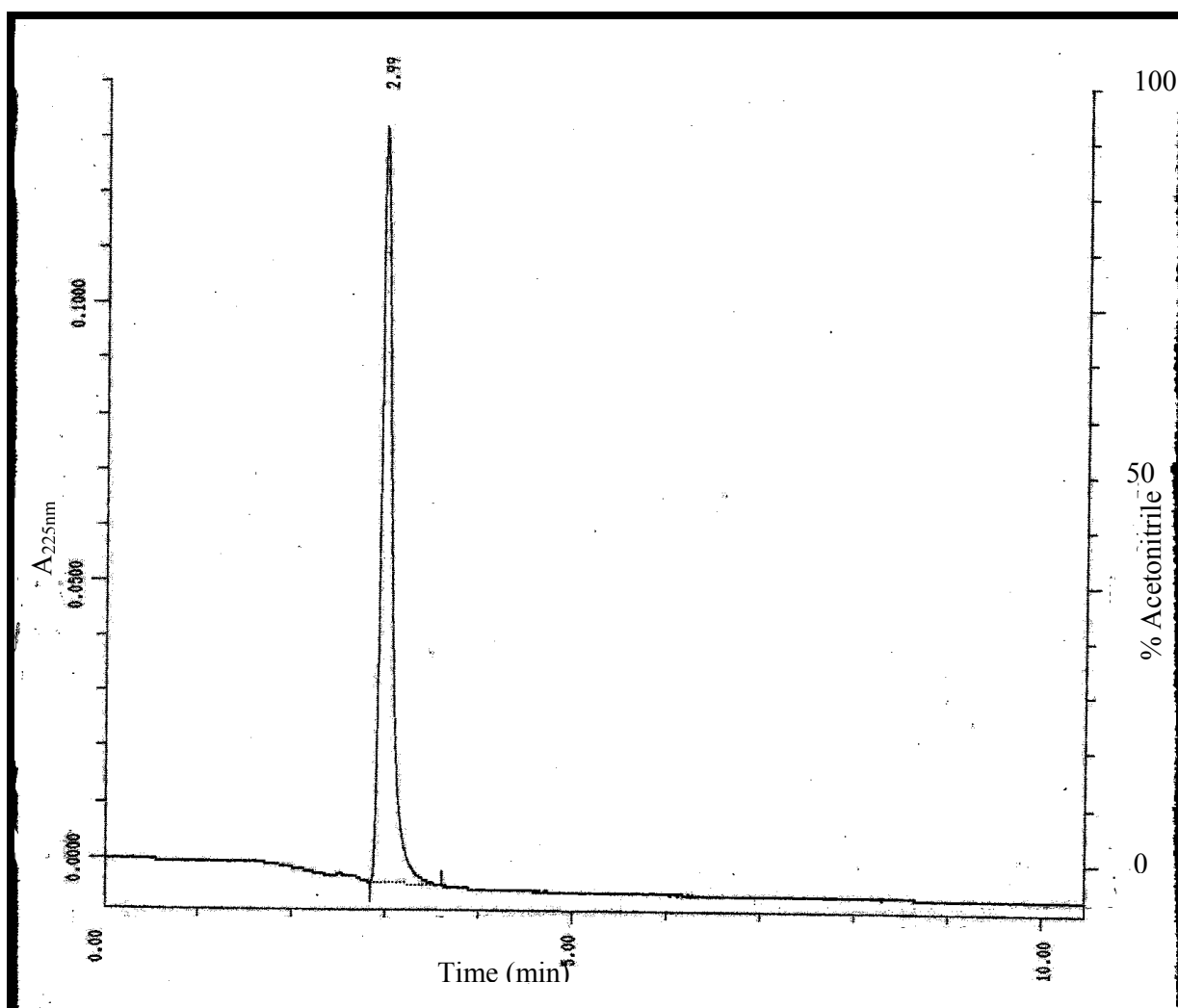
Figure 3.2: A chromatogram of a RP-HPLC elution profile of commercial marrubiin (0.05µg/µl). Samples were eluted from the column using an isocratic solvent system (10 minutes) consisting of solvent A: 20 and B: 80% and at a flow rate of 1ml/min, where A = 1% Acetic acid and B = methanol.

Under the chromatographic conditions used, as explained in chapter 2 (section 2.2.2), the chromatogram in figure 3.3 was obtained. RP-HPLC, using a hydrophobic stationary phase and a polar solvent system, ensures that samples elute from the column in increasing hydrophobicity. From the chromatogram in figure 3.3, the retention time of marrubiin was 13.56 minutes, and the amount of marrubiin bound to the column was 80%.
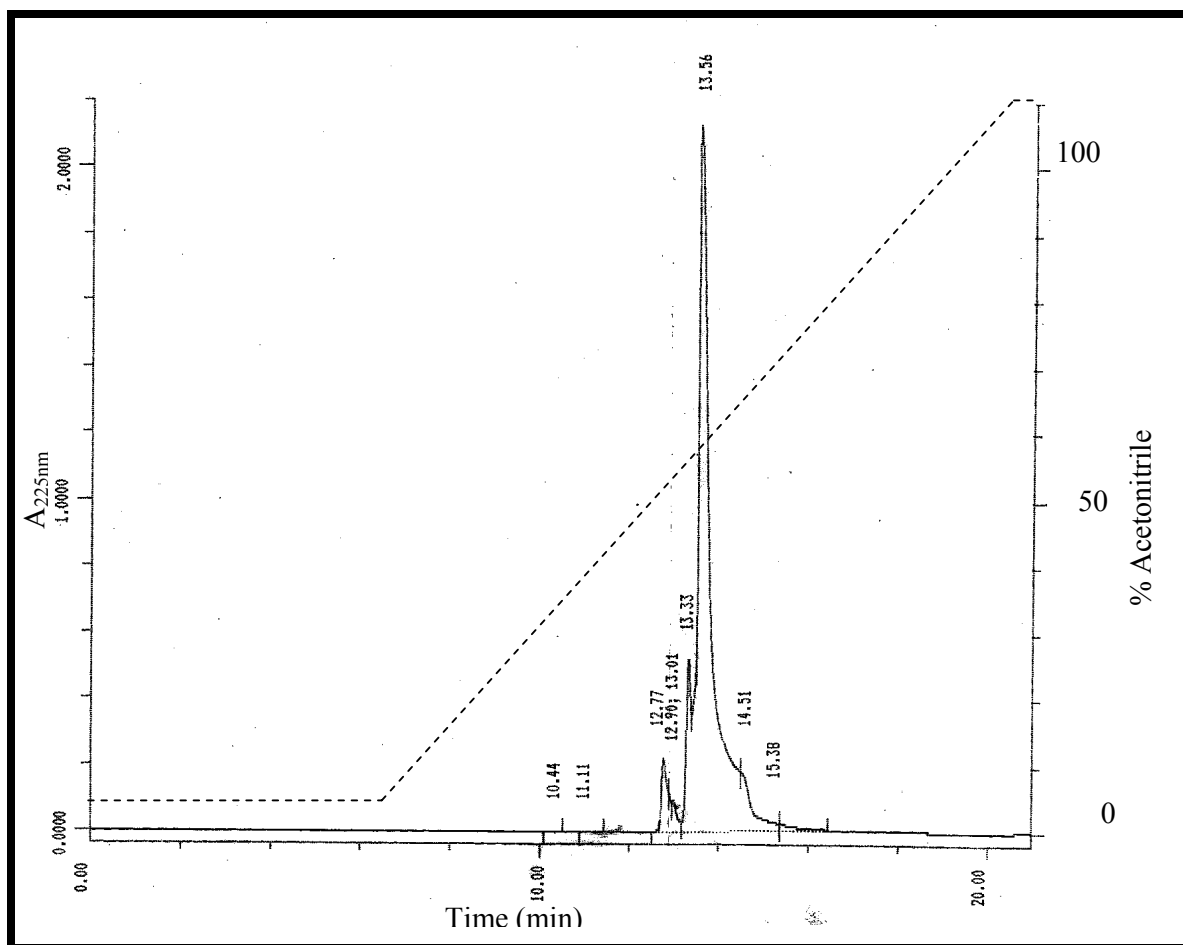
Figure 3.3: A chromatogram of an ion-pair RP-HPLC elution profile of commercial marrubiin (0.05μg/μl). Samples were eluted from the column using a gradient solvent system (15 minutes) consisting of solvent A, 10mM ammonium dihydrogen phosphate/5mM TBA (pH 3.5) and solvent B, acetonitrile at a flow rate of 1ml/min. The absorbance was monitored at 225nm.

Various concentrations of TBA were tested in order to improve the binding efficiency of marrubiin. When the same conditions as in the above chromatogram were used, and the pH was changed to 2.0, the chromatogram in figure 3.4 was obtained. The retention

time of marrubiin was found to be 10.78 minutes, and 85% of the marrubiin loaded was recovered in a sharp symmetrical peak, however a slight shoulder was obtained.
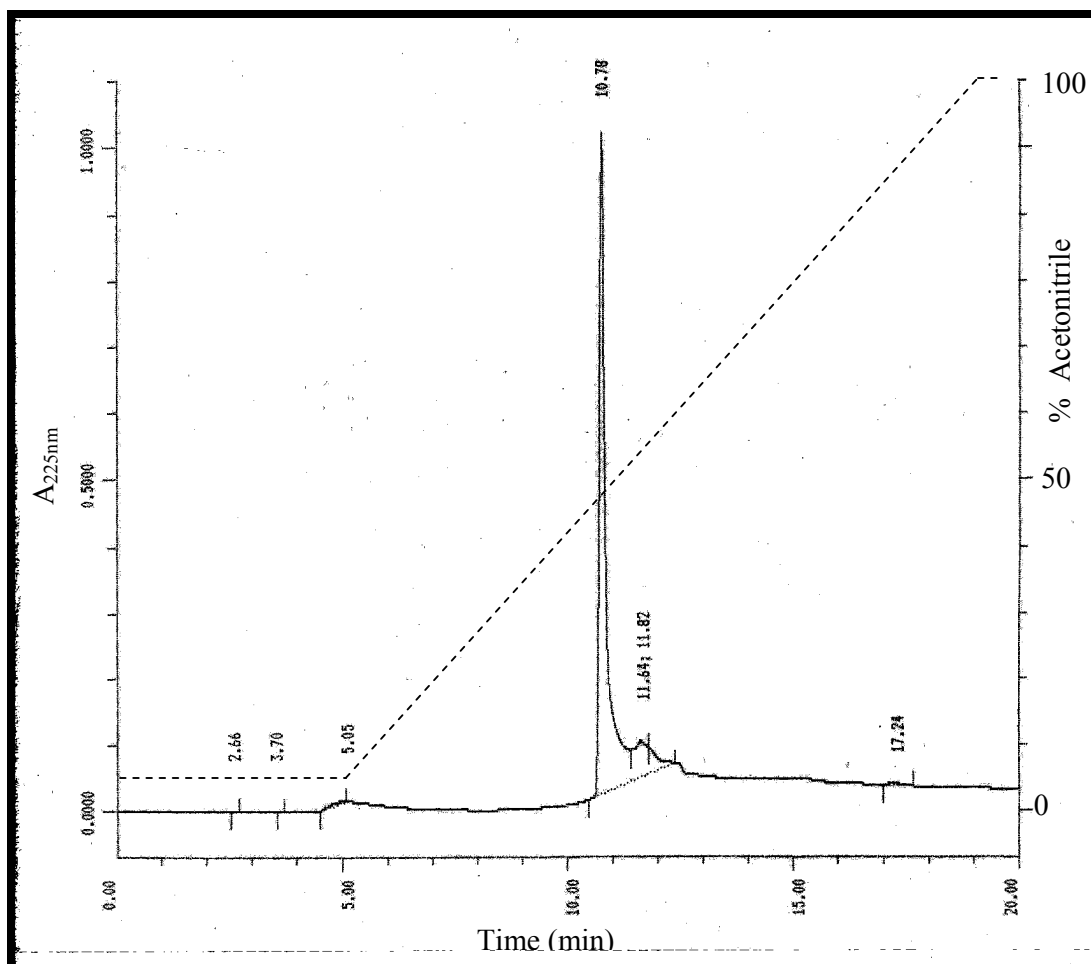
Figure 3.4: A chromatogram of an ion-pair RP-HPLC elution profile of commercial marrubiin (0.05µg/µl). Samples were eluted from the column using a gradient solvent system (15 min) consisting of 10mM ammonium dihydrogen phosphate/ 5mM TBA (pH 2.0) at a flow rate of 1ml/min. The absorbance was monitored at 225nm.

A 1 unit change in the pH greatly influenced the retention time of marrubiin. An increase in the concentration of TBA to 7.5mM was considered to determine its effect on the retention time. The chromatogram in figure 3.5 was obtained. When the TBA concentration was increased, the marrubiin was retained longer and peak broadening was noted. The highest peak area had a retention time of 19.135 minutes. Figure 3.2

and 3.5 had a low absorbance, this occurred when marrubiin was left for more than an hour on ice, a fresh sample should ideally be used every hour.
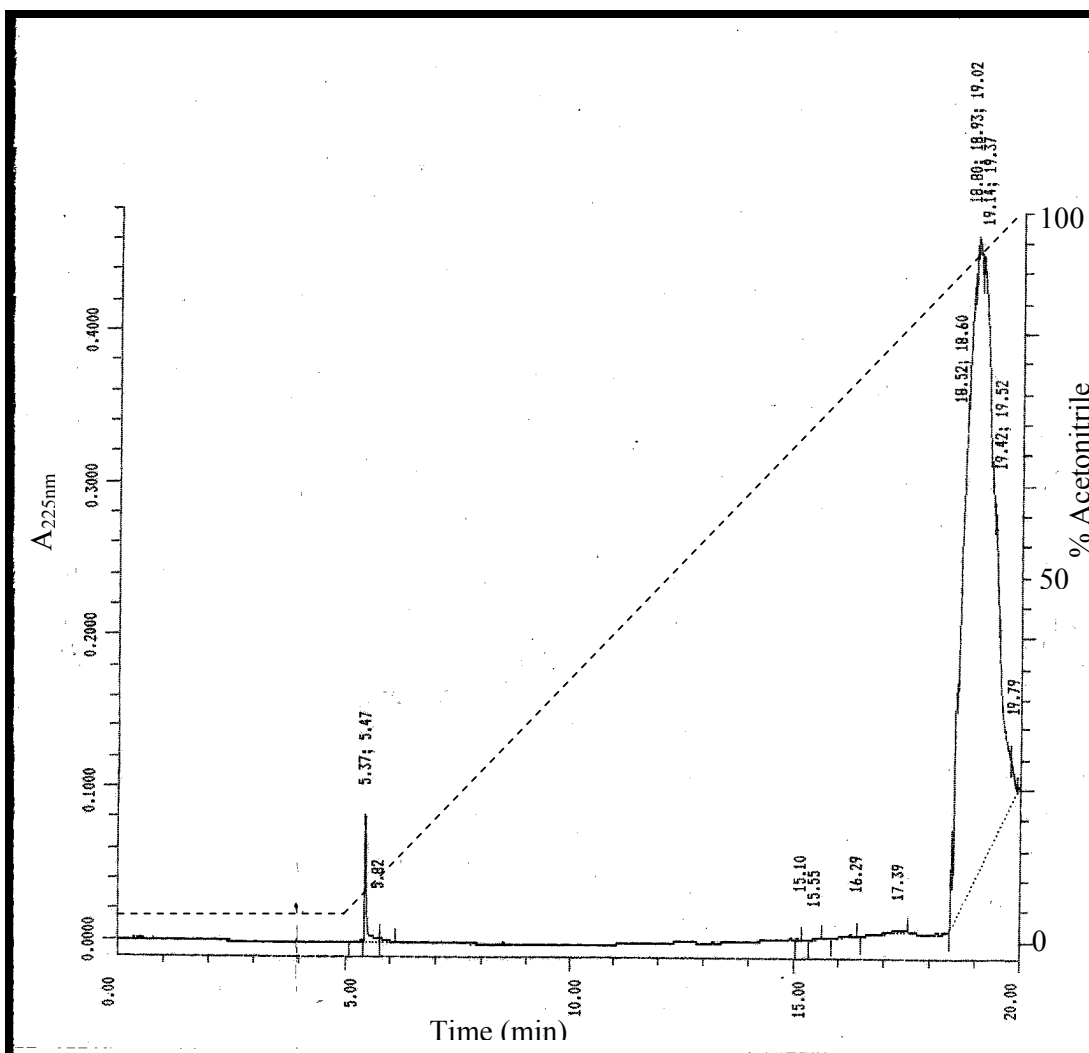
Figure 3.5: A chromatogram of an ion-pair RP-HPLC elution profile of commercial marrubiin (0.05μg/μl). Samples were eluted from the column using a gradient solvent system (15 minutes) consisting of solvent A, 10mM ammonium dihydrogen phosphate/ 7.5mM TBA (pH 3.5) and solvent B, acetonitrile at a flow rate of 1ml/min. The absorbance was monitored at 225nm.

An ideal result would have been obtained if at least 90% marrubiin was retained on the column and eluted in a single asymmetric peak. Therefore the TLC method for quantifying marrubiin was used for all subsequent experiments.

The results obtained in this section for the RP-HPLC, however, forms the basis for further development of a RP-HPLC method for quantifying marrubiin.

3.2 Thrombin Assay

A typical set of progress curves for the inhibition of thrombin by the OL extract is shown on figure 3.6 (a), and from the progress curve the percentage inhibition was determined on figure 3.6 (b). The extract inhibited thrombin, and figure 3.6 (b) displayed a linear relationship between the OL extract concentration and percentage inhibition. The higher the OL concentration, the higher the inhibitory effect on thrombin. The IC_{50} value of the OL extract was determined to be 6.20 mg/ml.

a

b
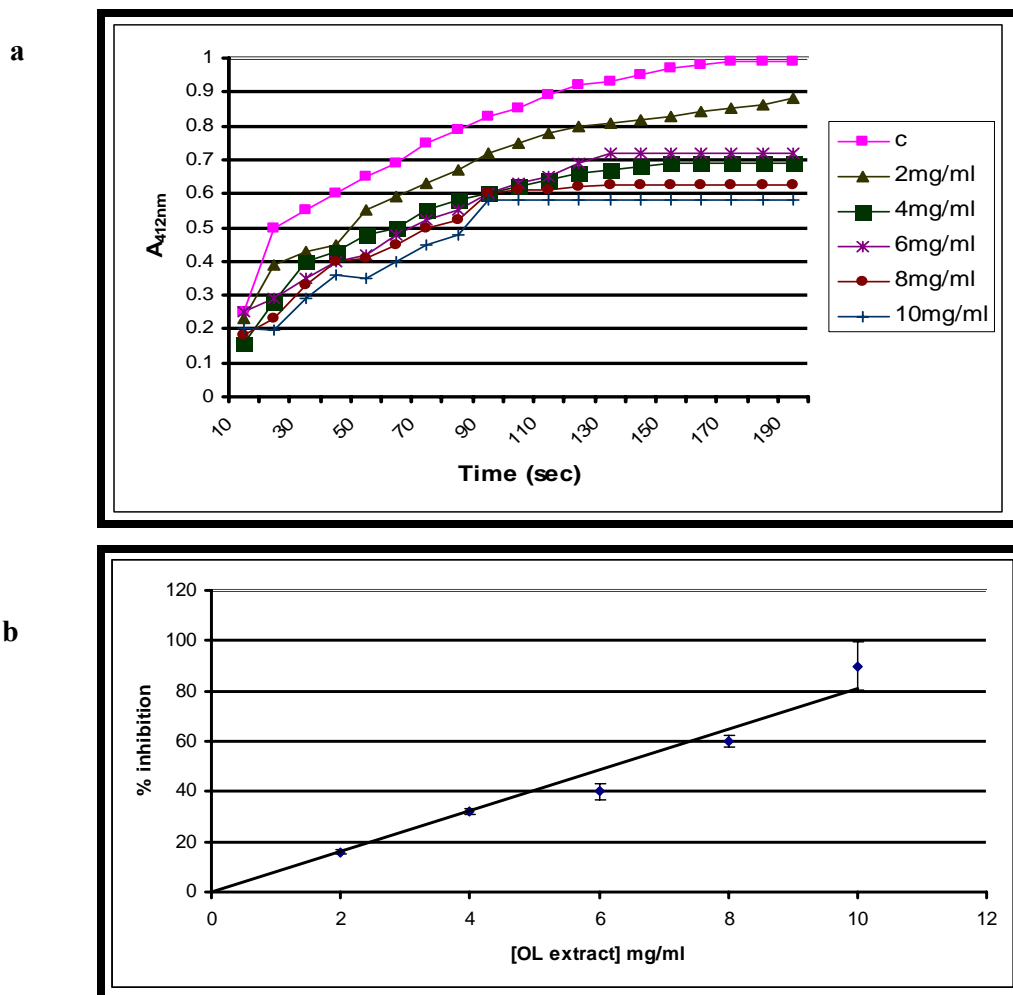
Figure 3.6: (a) Typical progress curves for the OL extract. (b) The percentage inhibition of 30U/ml bovine thrombin by the OL extract. A concentration range of 0-10mg/ml of the extract was used ($R^2 = 0.9545$). The error bars represent a standard deviation of three independent experiments.

A typical set of progress curves for the inhibition of thrombin by the AL extract is shown in figure 3.7 (a), and from the progress curve the percentage inhibition by the extract was determined in figure 3.7 (b). Similar to the results obtained for the OL extract, there was a linear relationship between the AL extract concentration and the percentage inhibition. However, note that the AL extract displayed a higher degree of thrombin inhibitory activity than the OL extract. The IC_{50} value of the AL extract was 3.2 mg/ml.

a

b
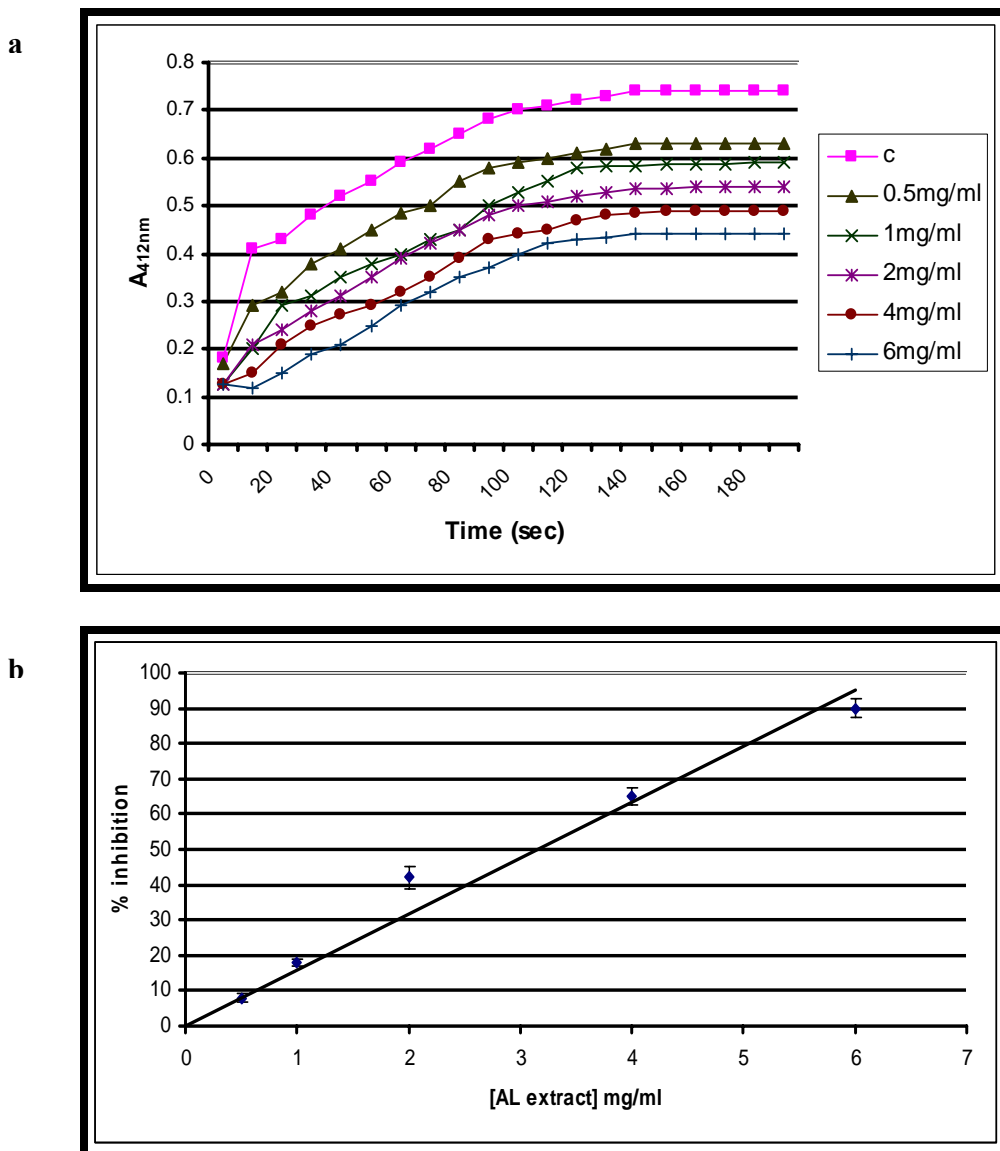
Figure 3.7: (a) Typical progress curves for the AL extract. (b) The percentage inhibition of 30U/ml bovine thrombin by the AL extract. A concentration range of 0-6mg/ml of the extract was used (R^2 = 0.9623). The error bars represent a standard deviation of three independent experiments.

The effect of marrubiin on thrombin activity was determined, where figure 3.8 (a) displays a typical set of progress curves for the inhibition of thrombin by marrubiin. Figure 3.8 (b) shows the percentage of inhibition by marrubiin. A concentration dependent inhibition of thrombin by marrubiin was not found. The inhibition of thrombin in the OL and AL extract could therefore not be attributed to marrubiin.

a

b
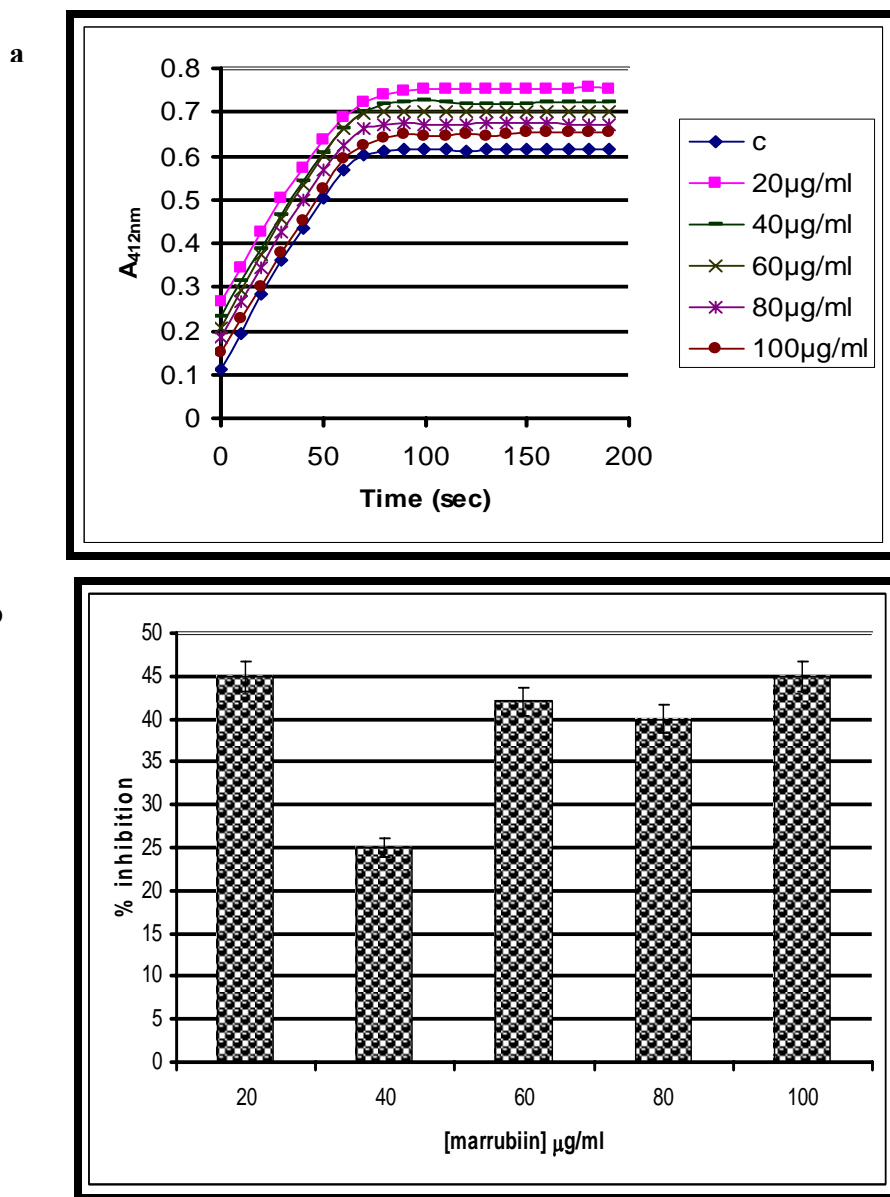
Figure 3.8: (a) Typical progress curves for marrubiin. (b) The percentage inhibition of 30U/ml bovine thrombin by marrubiin. A concentration range of 0-100µg/ml of marrubiin was used. The error bars represent a standard deviation of three independent experiments.

3.2.1 Discussion

The thrombin assay is a quantitative and sensitive method that is normally investigated in order to monitor direct thrombin inhibitors (Lange *et al.*, 2004). The cleavage of two bonds of human prothrombin by the prothrombinase complex leads to the activation of the serine protease α-thrombin. The first bond cleaved occurs at Arg320-Ile321, generating the activated intermediate meizothrombin. The second bond cleaved occurs at Arg271-Thr272, releasing the catalytic active α-thrombin. A subsequent cleavage at Arg284-Thr285 generates a stable form of α-thrombin (Senis *et al.*, 2006). Thrombin has a high specificity, determined by the selectivity of its deep active site and by a highly positively charged region in its surface called exosite I. The interaction between the enzyme and its substrate occurs at this region. Some molecules inhibit thrombin action by binding to this site (Ciprandi, 2006). The effect of the OL and AL extracts and marrubiin on thrombin amidolytic activity using a commercial substrate S2238 was tested.

The present study was the first attempt to investigate the effect of *Leonotis leonurus* on blood coagulation and platelet aggregation. The intrinsic and extrinsic blood coagulation pathway can be studied using various *in vitro* assays, including prothrombin time, thrombin time, and partial thromboplastin time (Gou *et al.*, 2003). In this study the effect of *Leonotis leonurus* on blood coagulation was evaluated with the thrombin assay. Both the OL and the AL extracts displayed a dose-dependent antithrombin activity. The AL extract yielded the highest inhibition of thrombin. Table 3.1 shows the marrubiin content of the OL extract, from the table it is clear that the marrubiin content in the OL extract was higher than the standard marrubiin concentrations used in the thrombin assay. Therefore, it is possible that the concentrations of standard marrubiin that were used in the thrombin assay, were too low to induce an effect on thrombin. However, using a concentration above 100µg for a pure compound of marrubiin would have very little physiological relevance. The TLC chromatogram shows the complexicity of both the OL and the AL extract, therefore, some compounds that are present in these extracts can be responsible for the effect shown by these extracts. The synergestic effects of a mixture of compounds could have also contributed to this effect. The IC_{50} values obtained for the OL and the AL extracts are comparable with those obtained by N. Low-Ah-Kee (2005). The IC_{50} values for the methanol extract and the AL extract were found to be 9.69mg/ml and 3mg/ml

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respectively in her study, while in this study they were 6.20mg/ml and 3.20 respectively, even though there is a 3 unit difference in respect to the concentrations for the OL extracts. This is probably due to the fact that acetone was used in this study in place of methanol, even though these solvents have the same polarity, the compounds that are extracted by them slightly differ from each other (Houghton and Raman, 1998). Many plants have showed antithrombotic effect, including tomatoes, *Hemidesmus indicus, Allium sativum* and *Allium cepa*. The antithrombotic effect of these plants has been attributed to their antioxidant activity (Yamada *et al.*, 2004). It is difficult to compare the activity of these plants with *Leonotis leonurus* on blood coagulation because different assays have been used, but in a study conducted by Goun *et al.* (2002), the effect of various Russian plants on thrombin was evaluated. The thrombin assay was used to evaluate the effectiveness of these plants on blood coagulation. Two of the plants which were tested, viz *Origanum vulgare* (1mg/ml) and *Tanacetum serpyllum* (1mg/ml) tested belong to the Lamiaceae family similar to *Leonotis leonurus*. These two plants showed highest antithrombotic activity, the percentage of inhibition of thrombin was 99% and 100% for *Origanum vulgare* and *Tanacetum serpyllum*, respectively. There was no range of concentrations tested to determine IC_{50} values for *Origanum vulgare* and *Tanacetum serpyllum*. The antithrombotic effect of *Origanum vulgare* could be attributed to its antioxidant activity. No biological activity has been conducted on *Tanacetum serpyllum*. One of its major chemical compounds is a sesquiterpene lactone, whereas marrubiin is a diterpenoid labdane lactone. The percentage of inhibition of thrombin was about 90% for both the OL (10mg/ml) and the AL (6mg/ml) extract, this could mean that there could be a compound in the Lamiaceae family that is responsible for the antithrombotic effects exhibited by these plants. The IC_{50} values for the OL and the AL extract were 6.2 and 3mg/ml respectively. Therefore, the AL extract had the strongest inhibition of thrombin.

3.3 Fibrinolysis Assay

Figure 3.9 (a) displays the typical results of fibrinolysis assay for the OL extract. From these curves the 50% clotting time, 50% lysis time, and percentage of fibrin formation were obtained. The OL extract did not have an effect on clotting time (figure 3.9 b), however the lysis time was decreased (figure 3.9 b). The 25μg/ml concentration significantly decreased (P<0.01) the lysis rate by 230% (2.3-fold). The extract significantly decreased the fibrin formation, figure 3.9 c.

The fibrinolytic effect of the AL extract was determined (figure 3.10). Figure 3.10 (b) shows that the extract did not have an effect on clotting time, however, similar to the OL extract decreased the lysis time. The 100μg/ml concentration significantly (P<0.03) decreased the rate of lysis by 200% (2-fold). The AL extract also decreased the fibrin formation.

 The fibrinolytic effect of marrubiin is given in figure 3.11. Once again, marrubiin had no effect on clotting time and significantly decreased fibrin formation, but it had a significant effect on lysis time. The 100μg/ml concentration significantly decreased the rate of lysis by 330% (3.3-fold) (P<0.001).

The concentration required to halve the lysis time for the OL extract, AL extract and marrubiin was found to be 25, 50 and 40μg/ml respectively.
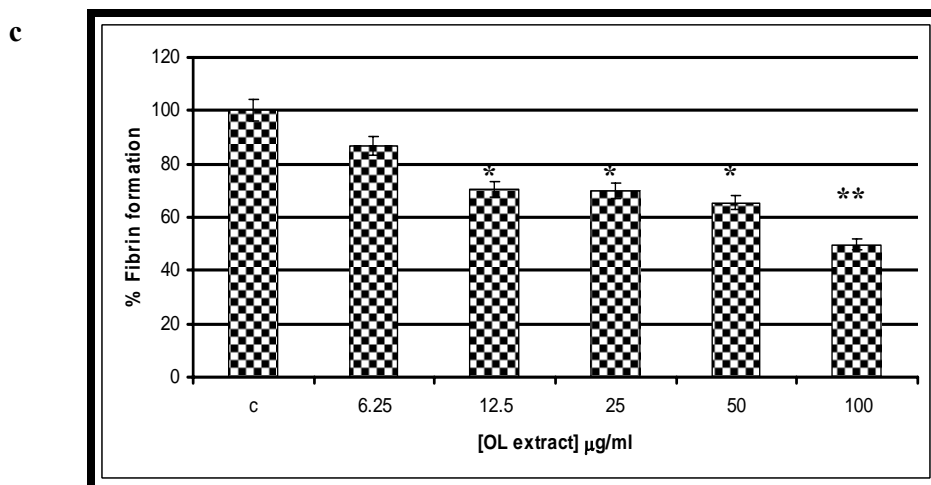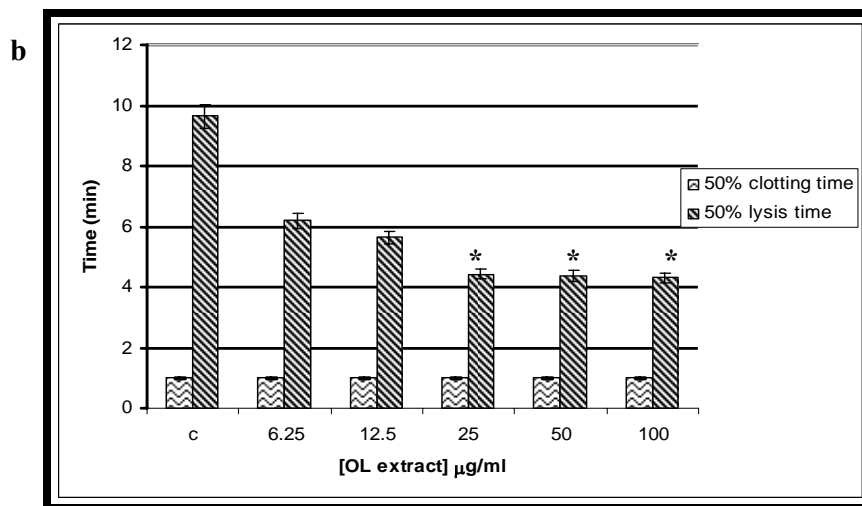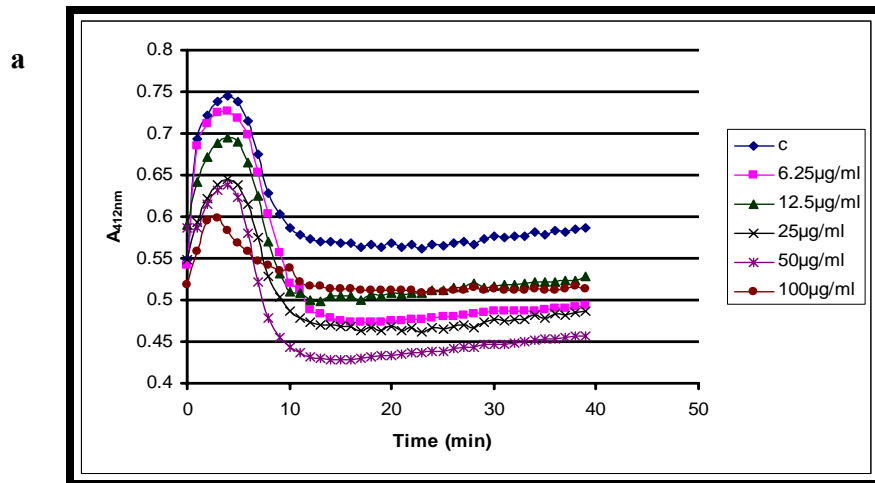
Figure 3.9: (a) Typical fibrinolytic curves for varying OL extract concentrations (6.25-100µg/ml) were c= control, (b) 50% clotting time and 50% lysis time of the OL extract, and (c) percent fibrin formation associated with the OL extract. The error bars represent the standard deviation of three independant experiments, *P < 0.05 and **P< 0.01 indicated significance relative to the control.
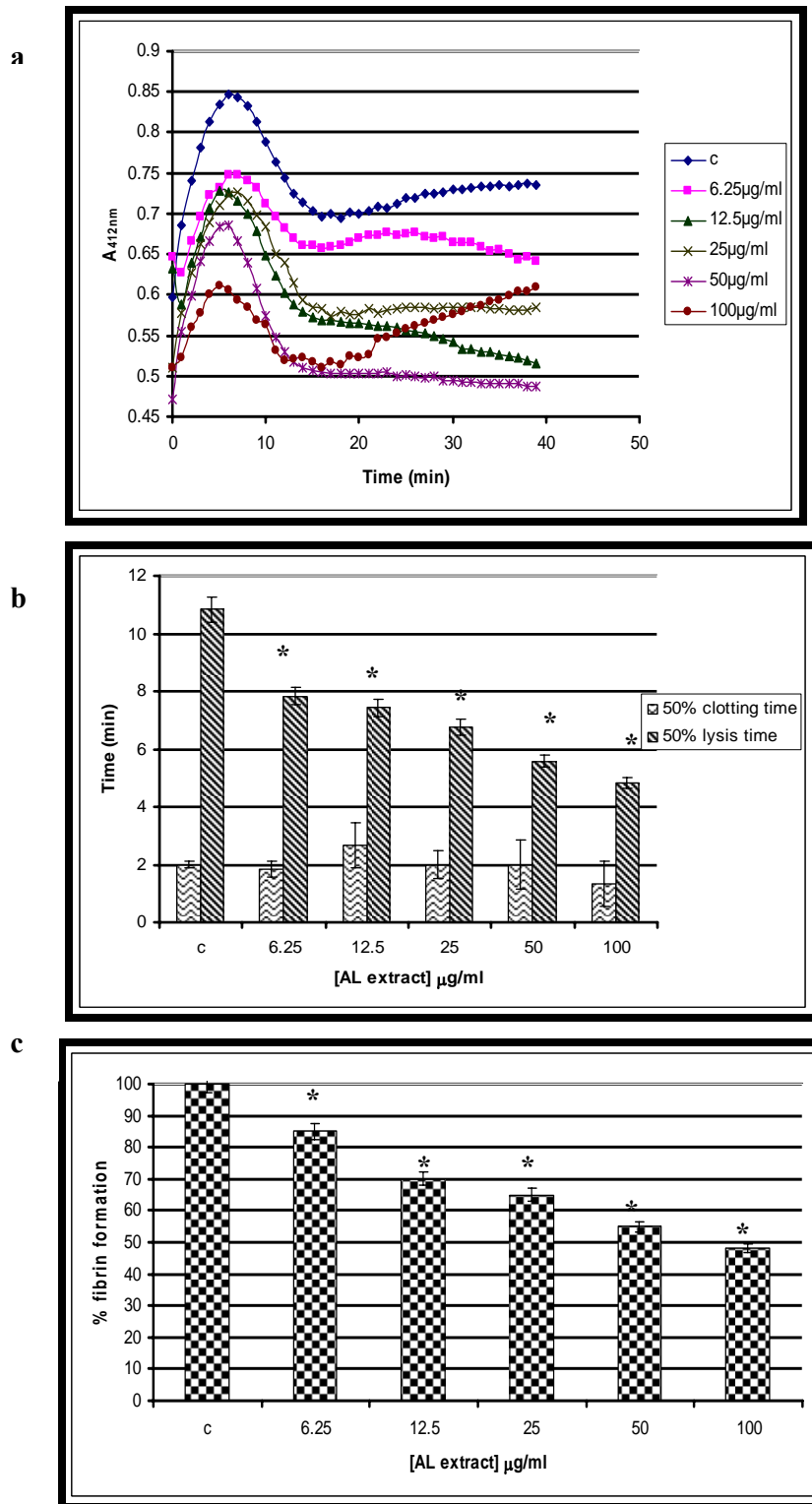
Figure 3.10: (a) Typical fibrinolytic curves for varying AL extract concentrations (6.25-100μg/ml) were c= control, (b) 50% clotting time and 50% lysis time of the AL extract, and (c) percent fibrin formation associated with the AL extract. The error bars represent the standard deviation of three independant experiments, *P< 0.05, indicated significance relative to the control.
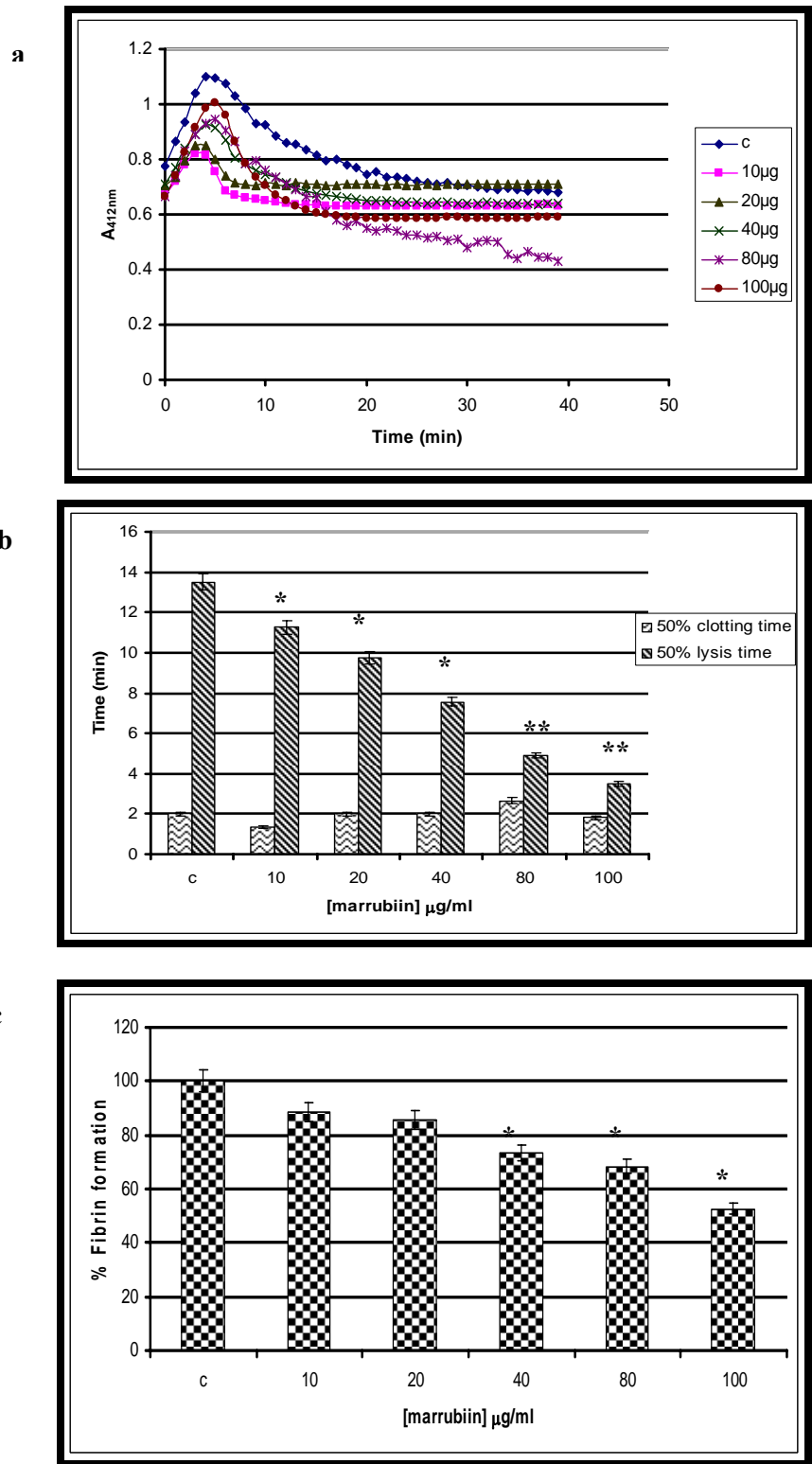
Figure 3.11: (a) Typical fibrinolytic curves for varying marrubiin concentrations (10-100μg/ml) were c= control, (b) 50% clotting time and 50% lysis time of marrubiin, and (c) percent fibrin formation associated with marrubiin. The error bars represent the standard deviation of three independent experiments, *P < 0.05, **P < 0.001 indicated significance relative to the control.

Table 3.2: A detailed summary of the results obtained from the fibrinolysis assay

Compound	Concentration required to halve clotting time (µg/ml)	Decrease of lysis time (%)	FI (%)
OL (µg/ml)			
6.25	-	153	65
12.5	-	168	87
25	-	210	93
50	-	234	106
100	-	230	117
AL (µg/ml)			
6.25	-	140	54
12.5	-	144	62
25	-	171	71
50	-	196	79
100	-	200	93
Marrubiin (µg/ml)			
10	-	127	45
20	-	140	56
40	-	170	77
80	-	230	98
100	-	330	131

- indicates no effect

Table 3.2 gives a summary of the results from the information obtained from figure 3.9 to 3.11. It was seen that the OL extract concentration required to give the same % decrease in lysis time as standard marrubiin was 100µg/ml, and only 5.2µg/ml marrubiin was present in this OL extract. This indicates that there are possibly other compounds in the OL extract causing the fibrinolytic effect. All three compounds had no effect on the clotting time. The fibrinolytic index is a measure of the fibrinolytic activity. The higher the fibrinolytic index, the higher is the rate of fibrinolysis. The OL extract displayed a concentration dependent increase in the fibrinolytic index. This effect was also observed for marrubiin and the AL extract.

3.3.1 Discussion

Although thrombin generation assays provide important information on coagulability, they do not assess fibrinolysis (Goldenberg *et al*., 2005). Clinical intervention with fibrinolytic agents such as streptokinase, urokinase, and tPA are required in order to dissolve blood clots. Streptokinase has been found to be effective in treating acute

myocardial infarction and it is cost effective. Two domains are involved in the interaction between streptokinase and plasminogen. The C-terminal of streptokinase is involved in activation and plasminogen substrate recognition, and the Asp41-His48 is important in binding to the substrate. The streptokinase assay relies on its ability to convert plasminogen to plasmin, and in turn it hydrolyzes a fibrin clot. The extent of hydrolysis is related to the concentration of streptokinase (Banerjee *et al*., 2004). The fibrinolytic activity of the plant extracts was tested to determine their effect on fibrinolysis. Thrombin was used to initiate clot formation and streptokinase was subsequently added to initiate fibrinolysis.

Thrombin can initiate both the intrinsic and the extrinsic pathways by activating a specific step in the pathway. Streptokinase was used to initiate fibrinolysis. Because of the specificity of these enzymes, a systematic approach to evaluate the inhibition by active extracts and help in identifying the complexes in the extrinsic and/or intrinsic pathways where they inhibit was used. The extracts and marrubiin did not have any effect on clotting time (table 3.1), meaning that the extracts and marrubiin directly inhibited thrombin, the key enzyme involved in blood coagulation. In the study conducted by Low-Ah-Kee (2005), the methanol extract had an IC_{50} value of 8.26mg/ml, while the AL extract did not have an effect on clotting time, but both extracts decreased fibrin formation. This could suggest that the anticoagulant effects shown by the extracts are mediated by a mechanism directly blocking thrombin during blood coagulation, as shown by the thrombin assay. This was not the case for marrubiin as it had no effect on thrombin enzymatic activity.

In vitro fibrinolysis was significantly enhanced by the OL, AL extracts and marrubiin, resulting in dissolution of clots. The OL extract had a stronger effect on fibrinolysis than the AL extract, with a fibrin index of 117. Marrubiin significantly increased the rate of fibrinolysis by 330% (3.3-fold) at 100μg/ml with a fibrin index of 131. Both extracts and marrubiin decreased the formation of fibrin as the concentration increased. Both the OL and the AL extracts and marrubiin, decreased fibrin formation, with an increase in the rate of lysis.

3.4 Platelet Aggregation Studies

Platelet aggregation studies were conducted to determine the effect of the extracts on platelets. The following section (3.4.1) shows results of a turbidometric method which was used to measure platelet aggregation.

3.4.1 Platelet Aggregation Studies using a Spectrophotometric Method

Figure 3.12 shows platelet aggregation curves obtained when PRP was exposed to the OL extract, AL extract and marrubiin. ADP was used as a positive control. In this assay, whole blood was used. After ADP was added to initiate platelet aggregation, either the OL, AL, or marrubiin was added to determine their effect on platelet aggregation. After adding the extracts, aliquots were taken every 20 seconds and the samples were centrifuged to obtain PRP.

The platelet aggregation curves that were obtained for each extract and marrubiin are given in figure 3.12.
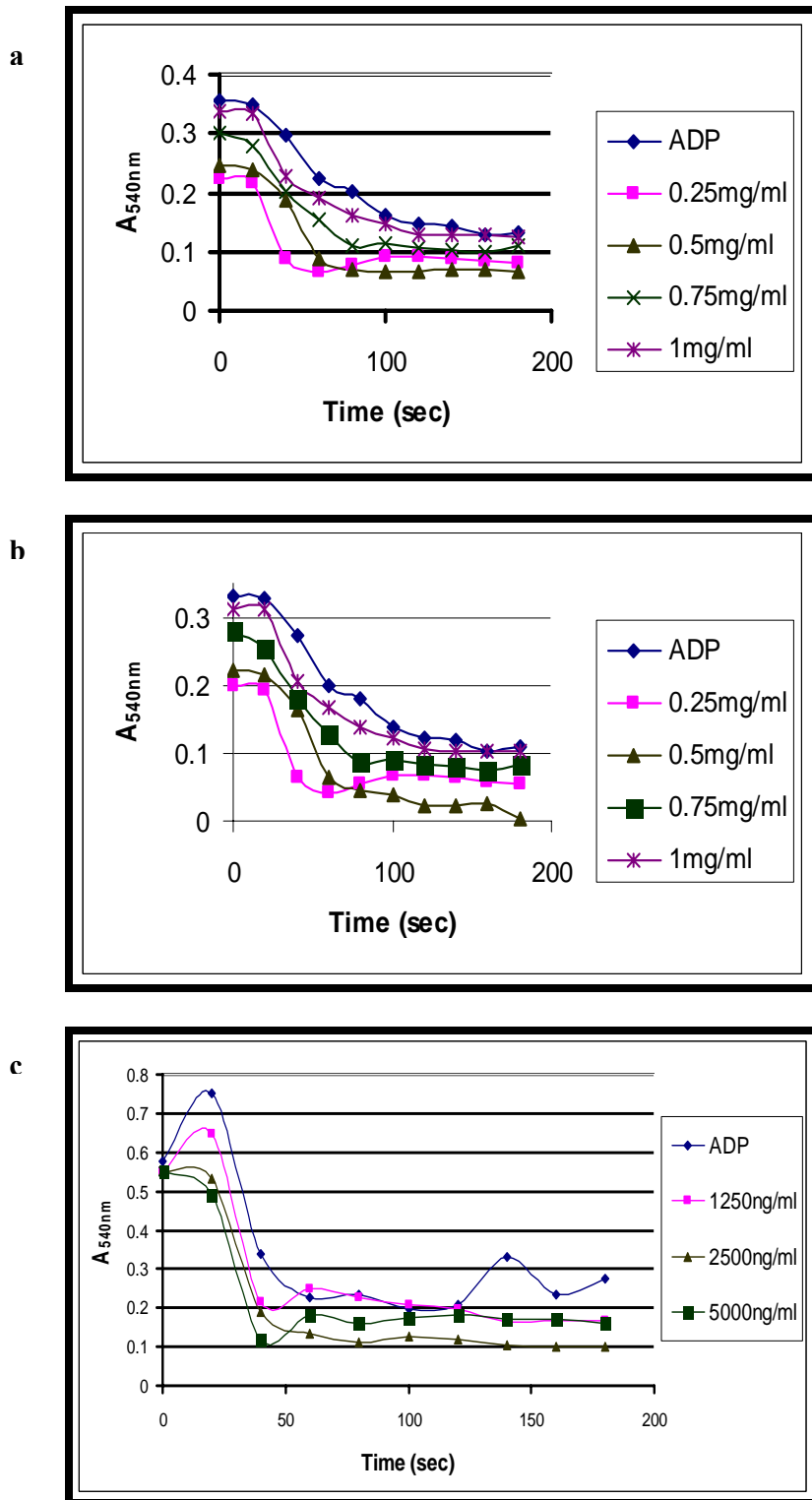
Figure 3.12: Typical platelet aggregation curves when exposed to varying concentrations (0.25-1mg/ml) of (a) the OL extract, (b) the AL extract and (c) marrubiin (1250-5000ng/ml). ADP = positive control, n= 3.

From the progress curves that are shown on figure 3.12, the percentage inhibition of each extract and marrubiin was calculated, and summarized on table 3.2.

Table 3.3: The effect of the OL extract and the AL extract and marrubiin on platelet aggregation, data presented as mean ± sem, n= 3

Test compound	Percent inhibition
OL extract (mg/ml)	
1	10.32 ± 4.23
0.75	10.41 ± 1.70
0.5	11.02 ± 4.95
0.25	11.88 ± 3.05
AL extract (mg/ml)	
1	15.54 ± 3.08
0.75	16.87 ± 4.91
0.5	23.47 ± 2.74
0.25	26.27 ± 2.31
Marrubiin (ng/ml)	
5000	18.00 ± 3.29
2500	10.32 ± 1.23
1250	2.04 ± 5.67

Table 3.2 shows that all three compounds did not have a significant effect on ADP-induced platelet aggregation. A small decrease in the extent of ADP response was observed as a result of pre-incubation with the extracts or marrubiin. An inverse concentration dependent relationship was found for the aqueous extract, i.e. the higher the concentration of the extract the lower the inhibitory effect possibly indicating an inhibitor in the aqueous extract. The OL extract did not have an effect on platelet aggregation, while marrubiin showed a concentration dependent relationship in response to ADP-induced platelet aggregation even though the inhibition was only 18% for the highest concentration (5000ng/ml).

3.4.2. Platelet Aggregation Studies using Flow Cytometry

Platelets play a fundamental role in hemostasis and thrombosis (Moran *et al.*, 2006). There is a conformational activation of GIIb/IIIa, fibrinogen binding, and finally platelet aggregation once the platelets are activated (De Meyer *et al.*, 2006). The effect of the extracts and marrubiin on thrombin activated platelets was determined using a monoclonal antibody (FITC-PAC-1). The effect of the extracts (1mg/ml) and marrubiin

(10µg/ml) to induce platelet aggregation was evaluated to determine if they had any inherent platelet aggregation potential (figure 3.13)
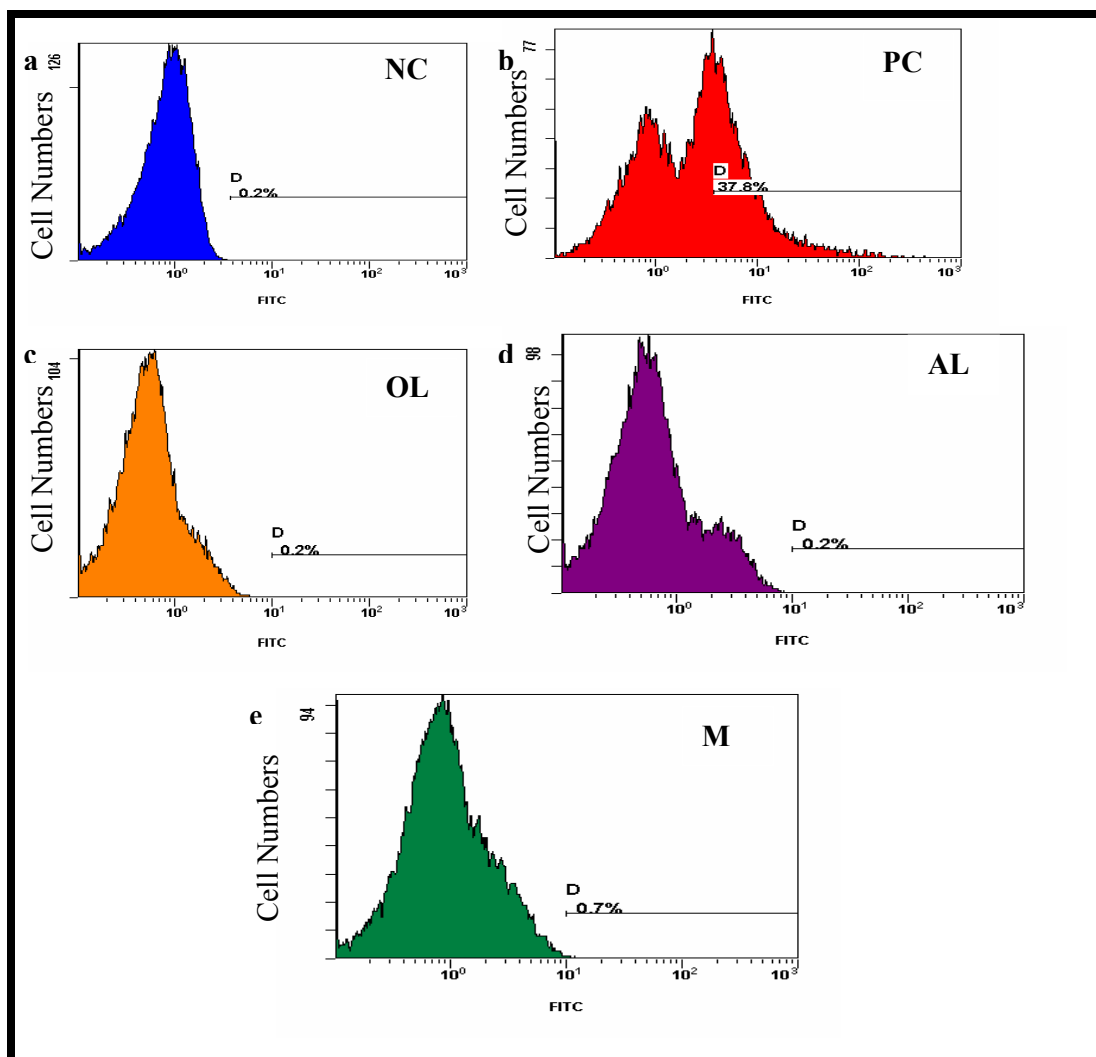
Figure 3.13: Flow cytometry histograms for platelet aggregation, where, NC = negative control, PC = positive control, OL = organic extract, AL = aqueous extract and M= marrubiin.

In figure 3.13 the positive control had two distinct populations, where one population was not aggregated and 37.8% of the population was aggregated. When comparing the test compounds with the negative control there is no distinction, indicating that the compounds did not have any inherent potential to cause platelet aggregation.

The effect of the extracts and marrubiin on thrombin-induced platelet aggregation was determined. As with the spectrophotometric method, whole blood was initially used for experimentation.

Therfore a double staining method was used, however, problems encountered during analysis with PE-Cy5 (platelet specific antibody) (BD Biosciences) resulted in it being difficult to quantify the activated from non-activated platelets. Thereafter only the PAC-1 monoclonal antibody was used as a marker of platelet aggregation to assess the inhibitory efficacy of the extracts and marrubiin using PRP and flow cytometry.

Figure 3.14 shows the results of the histograms obtained when thrombin-activated PRP was exposed to the OL and the AL extracts, and marrubiin. In the negative control (a), there is no platelet aggregation. When the platelets are activated by 10U/ml thrombin (b), there is a distinct tail formation as the platelets aggregated. The addition of the OL extract (c), the AL extract (d), or marrubiin (e) on thrombin-activated platelets decreased the expression of the surface marker of platelet aggregation.
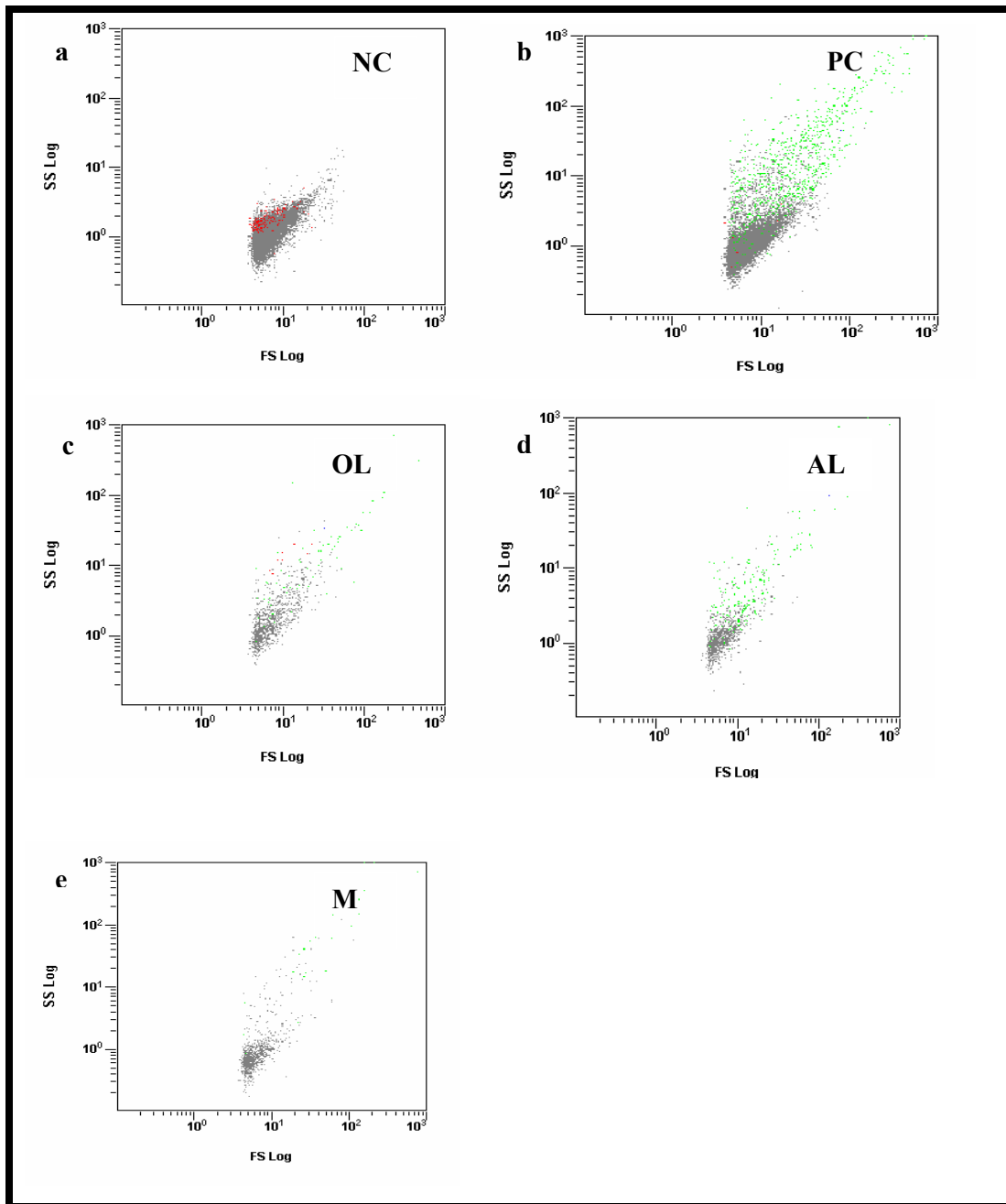
Figure 3.14: Dot plots of forward scatter (FS) vs side scatter (SS), light scattering properties of FITC-PAC-1 positive and FITC-PAC-1 negative platelets. Platelets were identified by their green fluorescence, where NC = negative control, PC = positive control OL = organic extract, AL = aqueous extract and M= marrubiin, n= 2.

From the results obtained from figure 3.14, an overlay was created to observe the extent of the inhibitory effects of the extract on GPIIb/IIIa activity (figure 3.15). From the overlay, the red peak is the positive control, the green peak the aqueous extract, the purple peak the organic extract, and the blue peak, marrubiin. The positive had the most number of aggregated platelets when compared to the other three samples. These results show that extracts and marrubiin decreased the expression of GIIb/IIIa activity when compared to the positive control, hence, decreasing platelet aggregation.
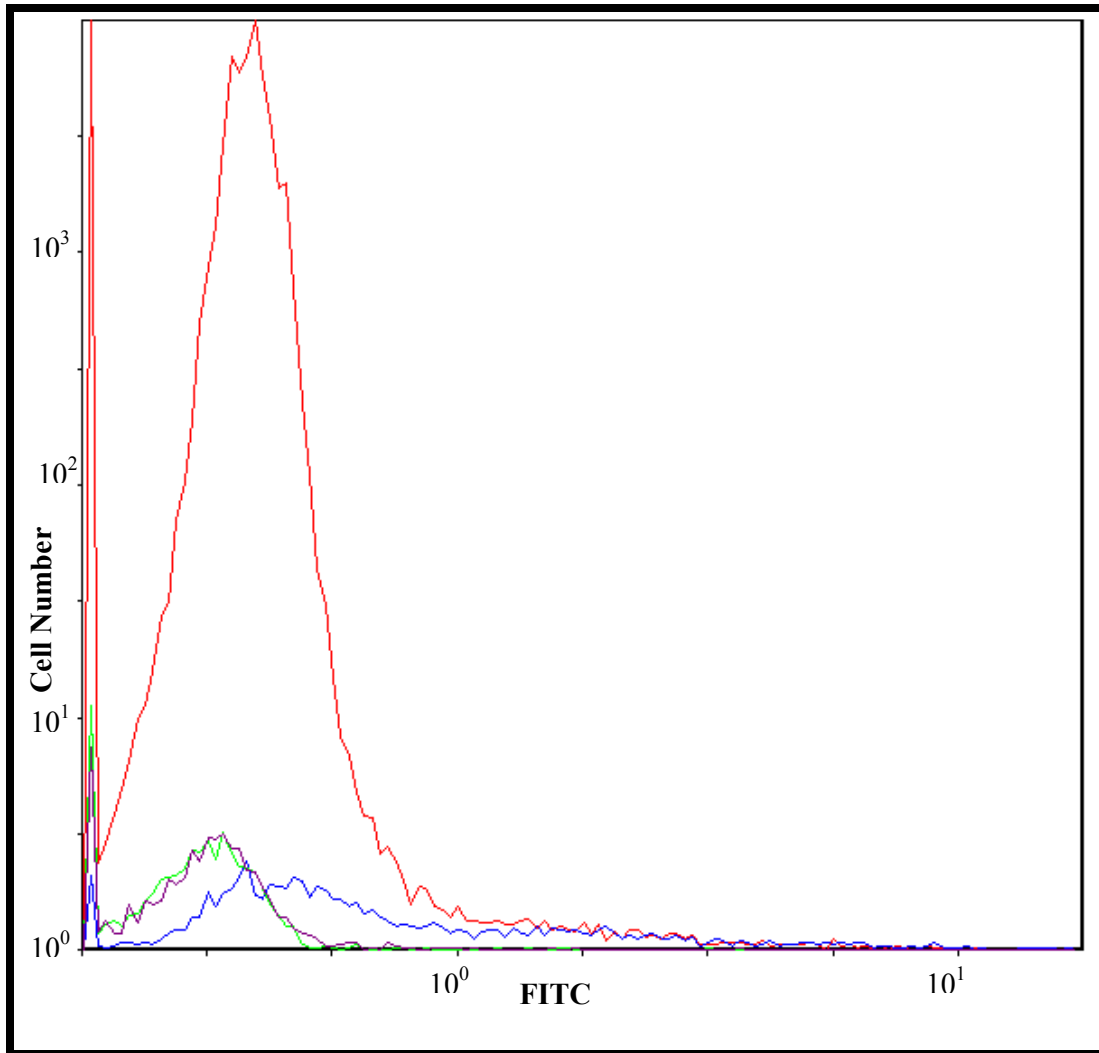
Figure 3.15: An overlay of the results after platelets were stimulated with thrombin, and the expression of GIIb/IIIa was measured using PAC-1, red =PC, green =AL, purple =OL, and blue = M.

Table 3.4 The inhibitory effect of the OL and AL extracts and marrubiin on thrombin induced PAC-1 expression on platelets (n=2)

Test compound	Percent inhibition
OL extract (mg/ml)	
2.00	92.50
1.00	64.74
0.50	45.32
AL extract (mg/ml)	
2.00	32.65
1.00	45.40
0.50	63.50
Marrubiin (µg/ml)	
100.00	68.00
10.00	8.14

Table 3.3 shows that both the extracts and marrubiin inhibited the expression of GIIb/IIIa which is found on the surface of platelets, therefore, the compounds inhibit platelet aggregation. The results obtained provide a trend (n= 2). The AL extract displayed concentration dependence, but again the lower the concentration the higher the inhibition. These results correspond with the results obtained from the previous assay on platelets conducted on a spectrophotometer for ADP-induced platelet aggregation.

3.4.3 Discussion

Platelet hyperactivity is one of the key factors involved in thrombosis and PAD (Mekhfi *et al.*, 2004). The thrombi formed during platelet hyperactivity can lead to thromboembolic complications, atherosclerosis, myocardiac infarct, stroke, and PAD if it is not dissolved. Collagen leads to the formation of TXA_2, a potent inducer of platelet aggregation. TXA_2 levels are increased in thrombotic disorders. Thus, the inhibition of platelet aggregation and TXA_2 is a promising approach in therapeutic strategies of thrombosis (Moon *et al.*, 2000).

In the current study, the effect of the OL, the AL extracts and marrubiin were determined by assessing a platelet membrane receptor as an index of platelet function. The extracts and marrubiin inhibited thrombin-induced platelet aggregation in whole blood (table 3.2). A more drastic inhibition of thrombin-induced platelet aggregation was observed in the flow cytometric method, where a monoclonal antibody (PAC-1) was used because this is a more sensitive method which is sensitive even in nanogram ranges. PAC-1 binds to the receptors of GPIIb/IIIa found on the platelets, a saturation levels of PAC-1 were used so that all or most binding sites of GPIIb/IIIa could be occupied by PAC-1. This makes it possible for platelet activity to be measured, PAC-1 positive platelets are counted and this co-relates to the number of platelets activated because PAC-1 only binds to activated platelets. The OL extract decreased the expression of GPIIb/IIIa in a dose-dependent manner, as did marrubiin. The OL extract displayed the strongest inhibition of 92% at 2mg/ml. The extracts and marrubiin displayed no inherent platelet aggregation activation (figure 3.13). Similar to the results obtained to the spectrophotometric method, the AL extract displayed an inverse concentration dependent relationship, where the lowest concentration (0.5mg/ml) yielded the highest inhibition of platelet aggregation (63.5%). The spectrophotometric method yielded low inhibitory action of the extracts and marrubiin, this method was less sensitive than the flow cytometric method because turbidity was used as a measure of platelet activity, and this method is sensitive in the microgram range.

Several studies have found that many plants have anti-platelet aggregation activity. These include the plants that have been mentioned to inhibit thrombin in section 3.2.1. In a study conducted by Mekhfi *et al*., (2004), the anti-platelet activity of five Morrocan plants (*Urtica dioıca, Arbutus unedo, Petroselinum crispum, Equisetum arvense,* and *Cistus ladaniferus)* was compared. Aggregometry was used to obtain the data. For the thrombin-induced platelet aggregation, *Equisetum arvense* (IC_{50} = 6.75mg/ml) displayed the strongest inhibition of thrombin-induced platelet aggregation by 86.7% while *Cistus ladaniferus* (IC_{50} = 6.75mg/ml) displayed the strongest inhibition of ADP-induced platelet aggregation by 81.5%. The AL extract of *Andrographis paniculata* and its active diterpenoid inhibited platelet aggregation by 90% with an IC_{50} value of 50-100μM (Thisoda *et al*., 2006). The mechanism of inhibition was through the extracellular signal-regulated kinase 1/2. When compared to these plants, the OL extract displayed the strongest inhibition of platelet aggregation because more than

90% inhibition of platelet aggregation was achieved at only 2mg/ml, thus, *Leonotis leonurus* could be a potential therapeutic agent in thrombosis.

In general, the results obtained from the coagulation studies show that both extracts of *Leonotis leonurus* exhibit antithrombotic and anticoagulant activity. Marrubiin also showed anticoagulant activity but no antithrombotic activity. The highest concentration of marrubiin used in these studies was 100µg as levels higher than this would have very little physiological relevance and therefore did not always correlate with the OL extracts concentration. Regardless, it is clear that, even if marrubiin has a contribution in the effects shown by the OL extract, it is not the only compound in this extract that has the anticoagulant effects. The AL extract also showed anticoagulant effects, although antiplatelet effects were not as pronounced as the OL extract. Therefore, both extracts need to be fractionated so that the compounds present in each extract can be tested for antithrombotic and anticoagulant activity to see what role they play in this regard.

CHAPTER 4: The *in vitro* Antidiabetic Effects of *Leonotis leonurus* and Marrubiin

4.1 Glucose Stimulated Insulin Secretion

From the standard curve generated in section 2.8, the insulin content of each sample was determined.

4.1.1 Chronic Insulin Release

Exposure of islets in 11.1mM glucose media to the extract and marrubiin for 48 hours decreased the insulin secretion of the β-cells. The OL 1, OL 2 extracts and marrubiin decreased insulin secretion by 260%, 230%, and 840% (P<0.05) (2.6, 2.3, and 8.4-fold) respectively. Islets cultured in 33.3mM glucose media and exposed to the same extracts showed an increase in the release of insulin with decreasing OL extract concentration. Marrubiin-treated islets showed a 340% (3.4- fold) (P<0.01) increase in the insulin secretion (figure 4.1).

Figure 4.1: Chronic insulin secretion after 48 hours exposure to the OL extract (OL 1= 2.5 and OL 2= 10μg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose. *P < 0.05 and #P < 0.01, indicating significance relative to the 11.1mM glucose control and 33.3mM glucose control, respectively. $P < 0.01 and %P < 0.05, indicating significance relative to the conditions.

The control islets cultured in 33.3mM glucose media showed a 220% (2.2-fold) decrease in insulin release (P<0.01). This was expected, because in a chronic state, hyperglycemia generally has an effect in insulin release. In islets exposed to OL 1, islets cultured in 33.3mM glucose media showed a 210% (2.1-fold) increase in insulin release (P<0.01). There was no significant difference in islets exposed to OL 2 under normoglycemic condition and hyperglycemic condition. The insulin release in islets exposed to marrubiin in 33.3mM glucose media increased by 1240% (12.4-fold) when compared to those cultured in 11.1mM (P<0.05).

4.1.2 Basal Insulin Secretion

Islets cultured in 11.1mM glucose media and exposed to the OL 2 extract increased the basal insulin secretion 330% (3.3-fold), while the OL 1 extract and marrubiin increased insulin secretion 210% (2.1- fold) and 220% (2.2-fold) (P<0.05), respectively, relative to the relevant control islets (figure 4.2). Islets cultured in media containing 33.3mM glucose media and exposed to the extracts decreased insulin secretion relative to the respective control islets. Marrubiin, however, showed a 300% (3-fold) increase in basal insulin secretion, relative to the relevant control islets (P<0.01).

Figure 4.2: Basal insulin secretion after 48 hours exposure to the OL extract (OL 1= 2.5 and OL 2= 10µg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose. *P < 0.05 and #P < 0.01, indicating significance relative to the 11.1mM glucose control and 33.3mM glucose control, respectively. $P < 0.01 and %P < 0.05, indicating significance relative to the conditions. $P < 0.01 and ^P < 0.05, indicating significance relative to the conditions.

The control islets cultured in 33.3mM glucose media showed a 220% (2.2-fold) increase in insulin release (P<0.01). In islets exposed to OL 1, islets cultured in 11.1mM glucose media showed a 210% (2.1-fold) increase in insulin release (P<0.01). In islets exposed to OL 1, islets cultured in 11.1mM glucose media showed a 230% (2.3-fold) increase in insulin release (P<0.01) The insulin release in islets exposed to marrubiin in 33.3mM glucose media increased by 305% (3.05-fold) when compared to those cultured in 11.1mM (P<0.05).

4.1.3 Stimulated Insulin Secretion

The effect of the extract and marrubiin on stimulating insulin release was determined (figure 4.3). In islets exposed to 11.1mM glucose media, the OL extract and marrubiin did not induce insulin release, and caused a reduction in insulin secretion. The OL 2 extract decreased insulin secretion by 125% (1.25-fold), while the OL 1 extract decreased insulin secretion by 240% (2.4-fold), and marrubiin decreased insulin secretion by 250% (2.5-fold) (P<0.05). In islets exposed to 33.3mM glucose media, both the OL extract and marrubiin stimulated insulin release. The OL 1 and OL 2 extract significantly increased insulin secretion 160% (1.6–fold) and 200% (2-fold) (P<0.05), respectively, with increasing extract concentration. The marrubiin standard, however, further increased insulin secretion 400% (4-fold), relative to the relevant controls (P<0.01).
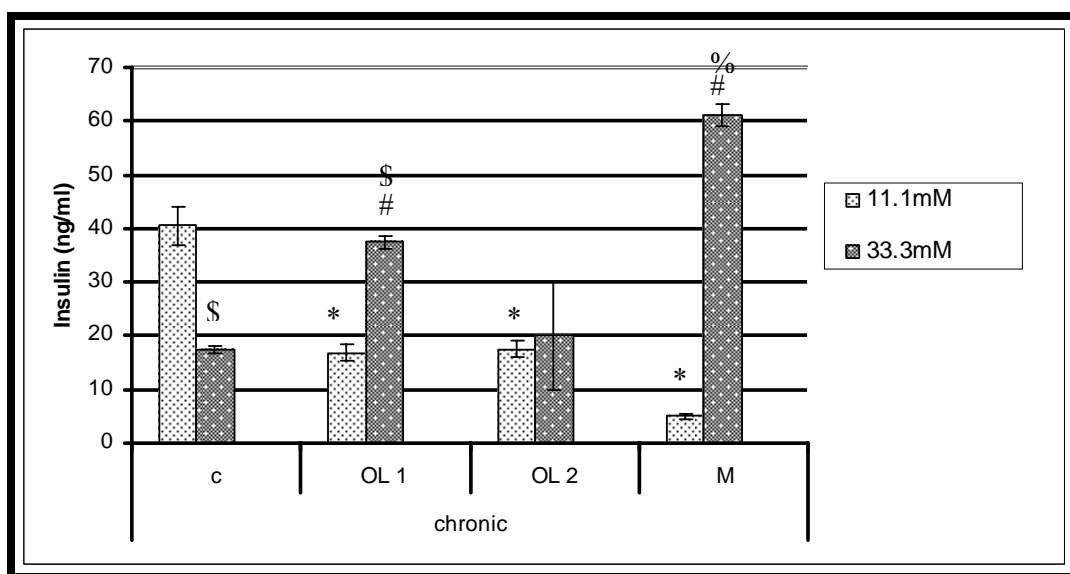
Figure 4.3: Stimulated insulin secretion after 48 hour exposure to the OL extract (OL 1= 2.5 and OL 2= 10μg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose. *P < 0.05 and #P < 0.01, indicating significance relative to the 11.1mM glucose control and 33.3mM glucose control respectively. $P < 0.01 and %P < 0.05, indicating significance relative to the conditions.

In islets exposed to OL 1, islets cultured in 33.3mM glucose media showed a 316% (3.16-fold) increase in insulin release (P<0.01). In islets exposed to OL 2, islets cultured in 33.3mM glucose media showed a 260% (2.6-fold) increase in insulin release (P<0.01) The insulin release in islets exposed to marrubiin in 33.3mM glucose media increased by 880% (8.8-fold) when compared to those cultured in 11.1mM (P<0.05). There was no significant difference in insulin release between the control islets cultured under normoglycemic condition those cultured under hyperglycemic condition.

4.1.4 Insulin Content

The insulin content of the islets was determined (figure 4.4). The 11.1mM islets exposed to extract showed similar insulin content to that of the relevant control islets. However, islets cultured in 11.1mM glucose media and exposed to marrubiin standard showed a significant reduction in insulin content, relative to the control islets (P<0.05). Control islets cultured in 33.3mM glucose media contained significantly less insulin relative to the 11.1mM control islets (P<0.05). There was no effect on insulin content

shown by both the OL 2 extract and marrubiin in 33.3mM glucose media. In hyperglycemic conditions the OL 1 extract significantly increased insulin (P<0.05).
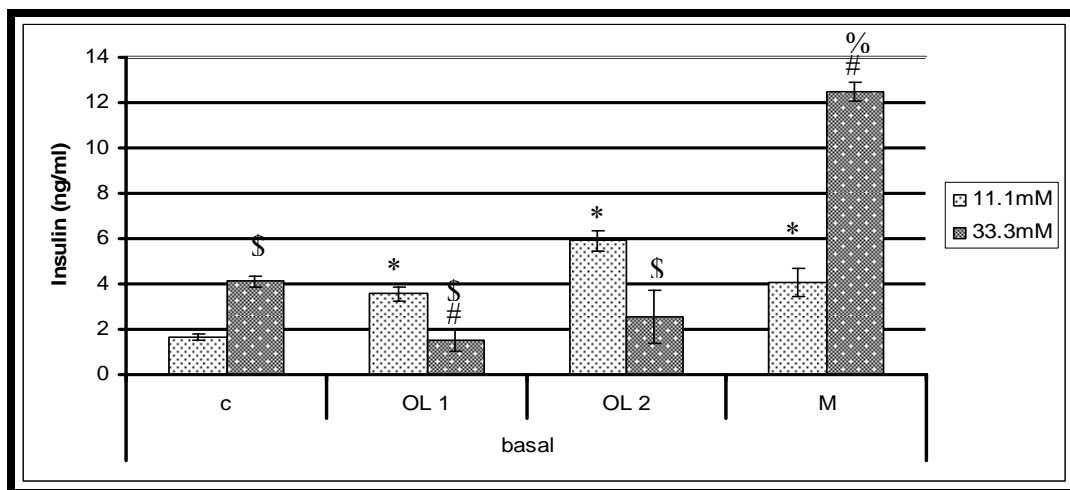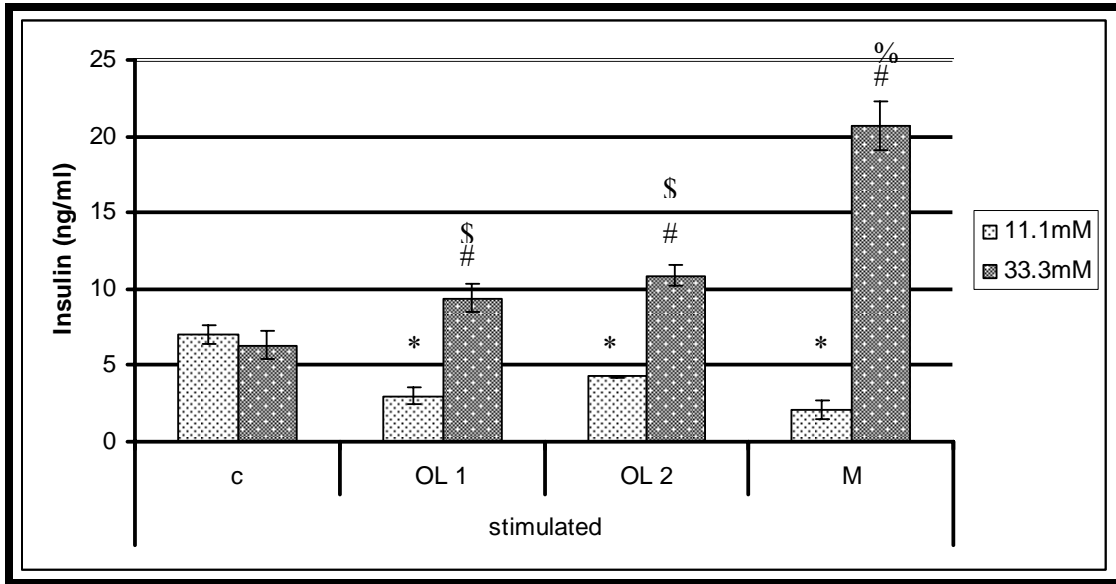

Figure 4.4: The insulin content of the islets after 48 hour exposure to the OL extract (OL 1= 2.5 and OL 2= 10μg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose. #P < 0.05, indicating significance relative to the control. $P < 0.01 and %P < 0.05, indicating significance relative to the conditions.

The control islets cultured in 11.1mM glucose media showed a 270% (2.7-fold) increase in insulin content (P<0.01). In islets exposed to OL 1, islets cultured in 11.1mM glucose media showed a 160% (1.6-fold) increase in insulin content (P<0.01). In islets exposed to OL 2, islets cultured in 11.1mM glucose media showed a 690% (6.9-fold) increase in insulin content (P<0.05) The insulin release in islets exposed to marrubiin in 33.3mM glucose media increased by 300% (3-fold) when compared to those cultured in 11.1mM (P<0.01).

4.1.5 Stimulatory Index
In islets cultured in 11.1mM glucose media, both the OL 2 extract and marrubiin showed a significant increase in stimulatory index, relative to control. The islets exposed to OL 2 extract in 33.3mM glucose media increased the stimulatory index by 450 (4.5–fold), while the marrubiin standard increased stimulatory index by 500% (11–fold), relative to the respective control islets (P<0.01).

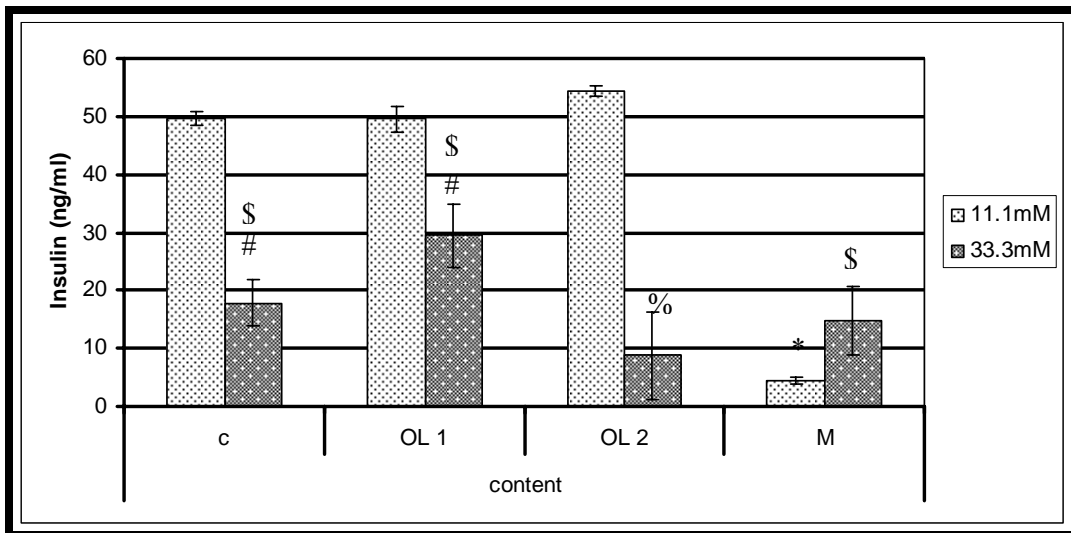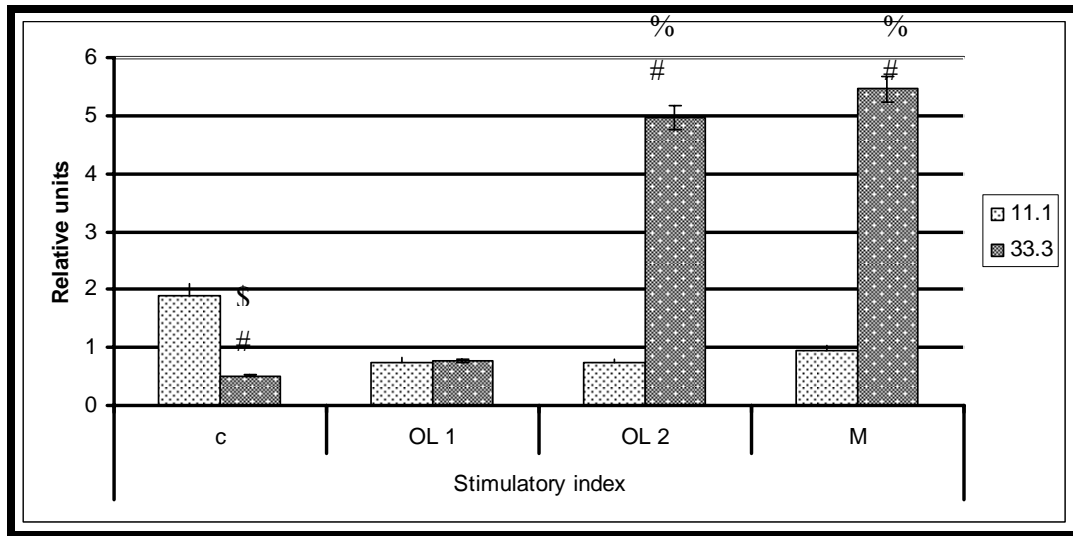**Figure 4.5: The insulin stimulatory index of islets after 48 hour exposure to the OL extract (OL 1= 2.5 and OL 2= 10μg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose. #P < 0.01, indicating significance relative to the 33.3mM glucose control. $P < 0.01 and %P < 0.05, indicating significance relative to the conditions.**

4.1.6 Discussion

Control of insulin secretion by pancreatic β-cells is critical for glucose homeostasis. Hypoglycemia is caused when excessive insulin is secreted but diabetes onset occurs when insufficient insulin is secreted. Intracellular glucose changes are detected by glucose machinery, and this information is transmitted to the secretory and insulin biosynthetic pathways. This sensing system uses key metabolic pathways that are present in pancreatic β-cells. Glucose triggers the secretion of insulin via a K_{ATP} channel-dependent Ca^{2+} influx and rise of cytosolic (Ca^{2+}_{c}), and a K_{ATP} channel-independent amplification of secretion without further increase of Ca^{2+}_{c}. The mechanism by which glucose induces insulin release involves the closing of the ATP-sensitive potassium channels because of an increase in glucose metabolites. This is then followed by a depolarization of the plasma membrane and increase influx of Ca^{2+} via voltage-dependent gating of Ca^{2+} channels. Insulin transportation into plasma membrane and exocytosis is triggered because of the increase in intracellular levels of Ca^{2+} (Ca^{2+}_{i}). (Szollosi *et al.*, 2007; Santana *et al.*, 2006; Uchida *et al.*, 2007).

The β-cells of diabetic animal models have been found to lose their unique differentiation that optimizes glucose-induced insulin secretion and synthesis. As a result genes such as insulin that are highly expressed are decreased with diabetes (Laybutt *et al*., 2002). The capacity to produce insulin is determined by the total β-cell number and β-cell functional activity (Maedler *et al*., 2001).

The drugs that are currently available for diabetes management have certain drawbacks, therefore, there is a need for a more effective and safer agent that can be used to manage this disorder (Kaleem *et al*., 2006). This study was undertaken to investigate the *in vitro* effects of *Leonotis leonurus* extracts and marrubiin on insulin secretion and gene expression. Under hyperglycemic conditions, the OL 1 extract and marrubiin significantly increased the insulin secretion. Insulin secretion was increased by 194% (1.94-fold) and 340% (3.4-fold) by the OL 1 extract and marrubiin, respectively (figure 4.1). This correlated with the results obtained for insulin content, since insulin secretion was very high under stimulatory conditions (33.3mM glucose media), resulting in low insulin content. The islets could be upregulating the expression of insulin as a mechanism to maintain minimal levels of glucose, hence, protecting themselves against glucotoxicity, thus in a low insulin content. The marrubiin content in the OL 1 is 0.13μg/ml (125ng/ml). It is therefore possible that marrubiin could be responsible for the increase in insulin secretion under chronic hyperglycemic conditions. Under chronic hyperglycemic conditions (control islets), the insulin secretion is decreased, which could be due to high glucose concentrations that lead to glucotoxicity. The OL 1 extract and marrubiin improved insulin secretion as the glucose concentration increased (figure 4.1). In a basal state, where the islets are stimulated with a low glucose concentration, there is a significant increase in insulin secretion in control and marrubiin islets, but the islets exposed to the OL extract did not show the similar effects as marrubiin. When the glucose concentration is increased to stimulate insulin release, there is a significant increase in insulin secretion in all experimental islets. This was not observed in islets cultured under normoglycemic conditions.

The stimulatory index of islets cultured under hyperglycemic conditions was increased by 450% (4.5-fold) and 500% (5-fold) in islets exposed to the OL 2 extract and

marrubiin, respectively. This increased stimulatory effect is expected to induce hypoglycaemia, a feature which had been reported by Ojewole, 2003. Ojewole (2003) reported that *Leonotis leonurus* possesses hypoglycemic/antidiabetic, antinociceptive, and anti-inflammatory effects when tested on Wistar rats. He concluded that the chemical constituents of this plant such as diterpenoids, flavanoids, and polyphenols may be responsible for these effects. From this study, the antidiabetic effect of OL could possibly be attributed to marrubiin (diterpenoid) since this compound greatly influenced the secretion of insulin in ß-cells. Various plants from the Lamiaceae family have been found to have hypoglycemic effects, these include *Marrubium vulgare* (Ramos *et al*., 1992, Eddouks *et al*., 2002), *Lavadula dentana, Origanum vulgare*, and *Salvia officinalis* (Eddouks *et al*., 2002, Eidi *et al*., 2005, Koukoulitsa *et al*., 2006) to mention a few.

Four milligram per kilogram of the AL extract of *Marrubium vulgare* had an antidiabetic effect due to its ability to increase insulin sensitivity. The antidiabetic effect of *Origanum vulgare* has been attributed to the fact that five constituents of this plant had the ability to inhibit aldose reductase. This enzyme is a key enzyme in the polyol pathway since it catalyzes the conversion of glucose to sorbitol. The compound 12-hydroxyjasmonic acid 12-O-*b*-glucopyranoside displayed 96% inhibition of the aldose reductase enzyme. Intracellular increase in sorbitol concentration has been implicated in chronic complications of diabetes. Therefore, the inhibition of the polyol pathway is a promising approach in finding an antidiabetic agent (Koukoulitsa *et al*., 2006). In another study 20mg/kg of the AL extract of *Origanum vulgare* was found to normalise blood glucose levels in severely STZ-induced diabetic rats. This extract was administered orally in a single dosage for a period of two weeks. The plant extract had no effect on basal insulin concentration. It was concluded that the plant extract had blood lowering glucose activity that was independent of insulin secretion by β-cells, therefore, the hypoglycemic activity was due to inhibition of hepatic glucose production and/or stimulation of glucose utilization by peripheral tissues (Lemhadri *et al*., 2004).

Salvia officinalis (100-500mg/kg) was found to decrease the concentration of glucose in STZ-induced diabetic rats, but molecular studies were not conducted to explain the mechanism of antidiabetic action (Eidi *et al*., 2005). Lima *et al*., (2006) exposed rats to

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this plant extract as a tea, and found that it decreased fasting plasma glucose levels but had no effect on glucose clearance. This indicated possible effects on gluconeogenesis. *Salvia officinalis* displayed effects that are similar to metformin, but these effects were not observed in primary cultures obtained from STZ-treated rats. Therefore, this plant had no direct effect on ß-cells, but it could be used as a food supplement to prevent DM in individuals at risk of developing this disease.

4.2 Apoptotic Studies Detected with TUNEL

Figure 4.6 shows results obtained from TUNEL staining, after the β-cells were exposed to the OL extract and marrubiin for 48 hours. From figure 4.6, the OL extract induced an increase in apoptosis of β-cells in an inverse dose-dependent manner under both normo- and hyperglycemic conditions. In 11.1mM glucose media the OL 1 extract increased apoptosis 139% (1.39-fold) (P<0.05) and OL 2 extract had no effect relative to the relevant controls. In 33.3mM glucose media OL 2 extract increased apoptosis by 150% (1.5-fold) (P<0.05) and OL 1 extract increased the occurrence by 1.01-fold. Marrubiin standard showed a had no effect in the occurrence of apoptotic cells in 11.1mM and 33.3mM glucose media, respectively (P<0.05). There was no significant difference in the number apoptotic islets between the normoglycemic and hyperglycemic conditions.

4.2.1 Discussion

During adulthood and organogenesis, tissues are remodelled by a physiological process called apoptosis. Apoptosis is a key mechanism in the remodelling of the pancreas after birth. Energy is required in this process and proteins are synthesized. There are morphological changes that occur during apoptosis, these include DNA fragmentation, condensation of nuclear chromatin, cellular shrinkage, and the development of apoptotic bodies (Farilla *et al*., 2002).

In 11.1mM glucose RPMI media, the OL 2 extract decreased the occurrence of apoptosis by 2% while marrubiin decreased it by 50%. The OL 1 extract increased the occurrence of apoptosis by 39%. In 33.3mM RPMI media, marrubiin decreased the occurrence of apoptosis by 30%. The OL extract increased the occurrence of apoptosis by 50% and 1% for OL 1 and OL 2, respectively. One would have expected the OL 1 extract to show the same effect as marrubiin on apoptosis, since the marrubiin content

of these two is similar, but this was not the case. There could be compounds in the OL extract that have the opposite effect to that of marrubiin on apoptosis.

Cytotoxic analyses that have been conducted on the Lamiaceae family has been targeting cancer cells. Diterpene lactones were isolated from *Leonurus sibiricus*, these lactones and the plant itself exhibited moderate cytotoxic effects (IC$_{50}$ = 50-60 μg/ml) against leukaemia cells (L 1210) (Satoh *et al*., 2003). *Mentha x piperita (*100μg/ml), popularly known as peppermint displayed that this plant had no toxic effects on the intestinal cell line IEC-6 (Vidal *et al*., 2007). Similar to these plants, in this study *Leonotis leonorus* displayed a moderate apoptotic effect in low concentrations, but as the concentration increased, the occurrence of apoptosis decreased.

a
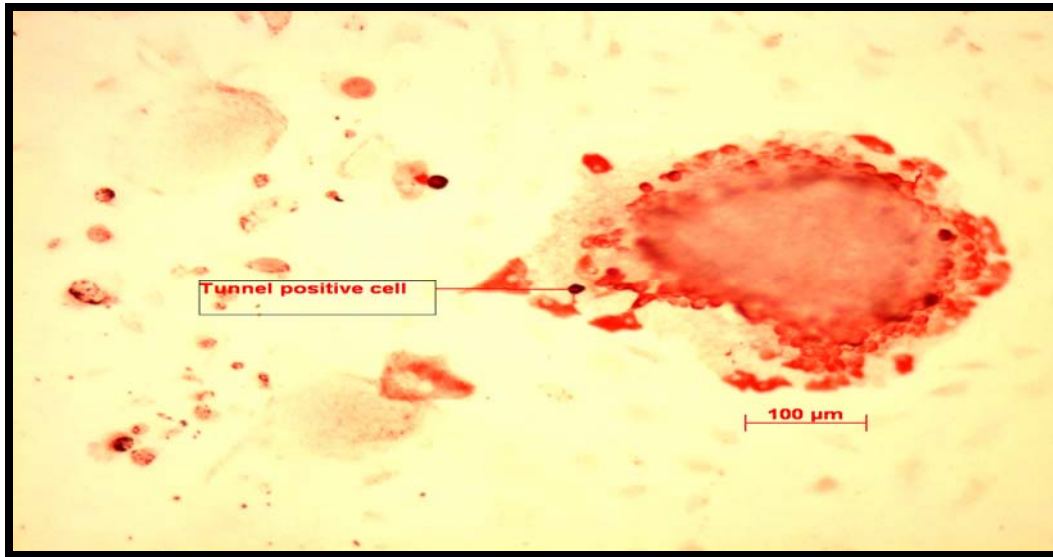
b

Figure 4.6: (a) An illustration of a TUNEL positive islet nucleus, (b) the effects of the OL extract (OL 1= 2.5 and OL 2= 10μg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose on DNA fragmentation using TUNEL. *P < 0.05 and #P < 0.01, indicating significance relative to the 11.1mM glucose control, and 33.3mM glucose control respectively.

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4.3 Proliferation Studies Detected with Ki-67

Ki-67 is a marker of cell proliferation that stains in the early to mid-G_1 phase of the cell cycle through mitosis (Laybutt *et al*., 2002). The effect of the extract and marrubiin on β-cell proliferation was determined.

Figure 4.7 shows that marrubiin had the most effect on proliferation of β-cells. Marrubiin increased proliferation by 180% (1.8-fold) and 200% (2-fold) for 11.1mm and 33.3mM glucose media, respectively (P<0.05). The islets exposed to the OL 2 extract showed an increase in proliferative capacity, in both 11.1 and 33.3mM glucose in RPMI. Under normoglycemic conditions, the OL 2 extract increased the occurrence of proliferation by 148% (1.48-fold) (P<0.05) and under hyperglycemic conditions there was a 155% (1.55-fold) increase (P<0.05). The OL 1 had no effect on proliferation in 11.1mM and 33.3mM glucose media, relative to the relevant control. There was no significant difference in the number proliferatory islets between the normoglycemic and hyperglycemic conditions.

4.3.1 Discussion

A dynamic response to a changing demand in insulin is ensured by a constant remodelling of islet mass mediated by proliferative and apoptotic stimuli (Farilla *et al*., 2002). The effect of the OL extract and marrubiin on β-cell proliferation was investigated by studying the expression of Ki-67 antigen, a nuclear protein expressed by dividing cells. Cell cycle analysis has shown that Ki-67 protein is exclusively expressed by dividing cells in the late G_1, S, and G_2 phases, but absent in the G_0 and early G_1 phase (Socha *et al*., 2003; Farilla *et al*., 2002). The phase of the cell cycle can be obtained by the pattern of localization of the Ki-67 protein. In the type I pattern, the Ki-67 protein is located close to the telomere and centromere, and this corresponds with the early G_1 phase. In the type II pattern, the Ki-67 protein is translocated to the nucleolus, defining the transition from the G_1 to the S phase. During mitosis the Ki-67 protein is situated near the chromosomes. The presence of these patterns makes it possible to use Ki-67 protein as a marker of proliferation (Socha *et al*., 2003).

By immunofluorescence, it was observed that the Ki-67 antigen was expressed in the controls, the OL extract, and marrubiin-treated islets. However, after the cell number counted was normalized with respect to the controls, the percentage of Ki-67 positive

cells was higher in the OL 2 extract and marrubiin-treated cells compared to the controls. This number was higher in islets exposed to hyperglycemic conditions. The OL extract displayed a concentration dependent increase in proliferation. In 11.1mM and 33.3mM glucose media, the OL 1 did not affect proliferation. However, proliferation was significantly increased by 45% and 80% by the OL 2 extract and marrubiin, respectively. In 33.3mM glucose media, proliferation was increased by 50%, and 100% by the OL 2 extract and marrubiin, respectively. Again, the OL 1 extract failed to exhibit the effects shown by marrubiin, however, the OL 2 showed half the effect of marrubiin. This could possibly be due to the presence of a compound in the OL extract that has an inhibitory effect on the compound(s) inducing proliferation in this extract.

Conentrations of *Salvia miltiorrhiza* (belongs to the Lamiaceae family) between 0.2-8μg/ml, were found to strongly stimulate proliferation of a human hepatoma (HepG$_2$ cells) cell line instead of enhancing apoptosis (Liu *et al*., 2005).
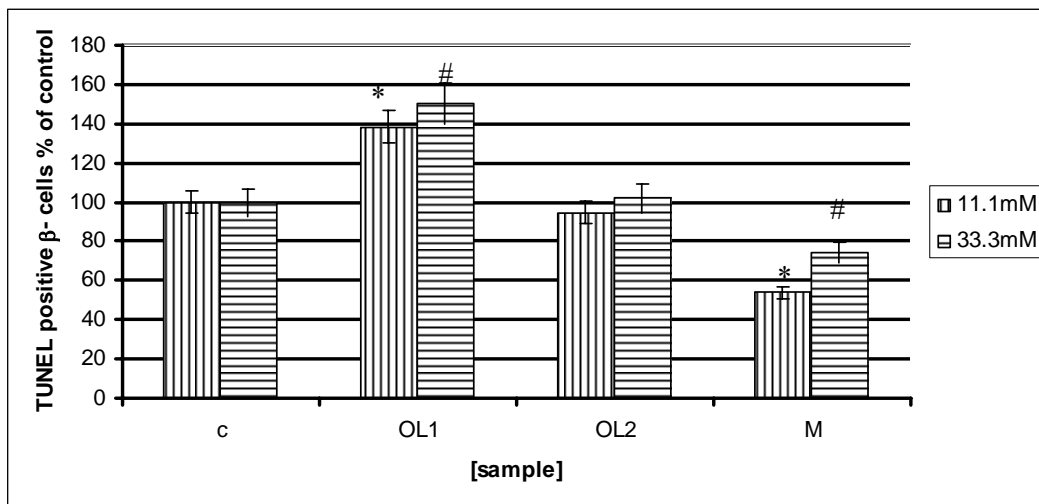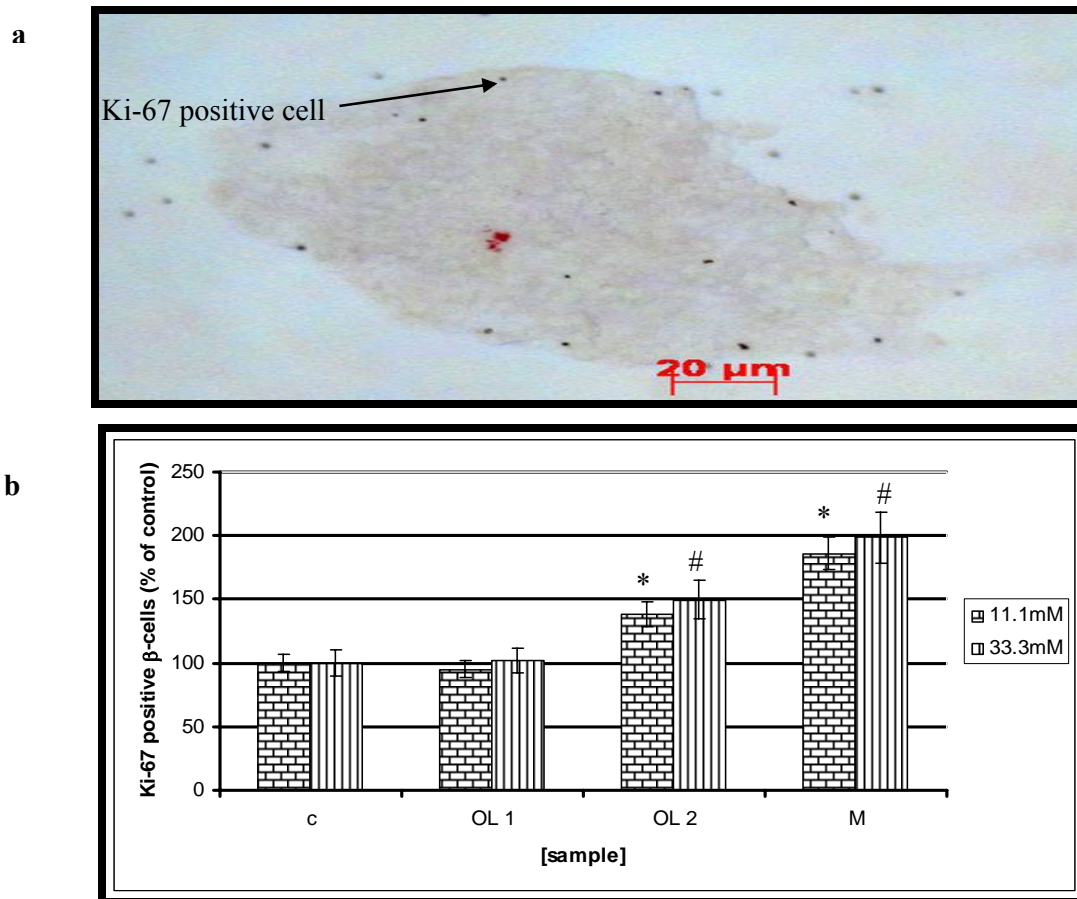
Figure 4.7: (a) An illustration of a Ki-67 positive islet, (b) the effects of the OL extract (OL 1= 2.5 and OL 2= 10µg/ml) and marrubiin (M) (150ng/ml) in RPMI media containing 11.1mM and 33.3mM glucose on proliferation using Ki-67 as a proliferative marker. *P < 0.05 and #P< 0.05, indicating significance relative to the relevant control.

4.4 Real-Time Polymerase Chain Reaction

The gene of interest in this study was insulin. RT-PCR was used to quantify expression of insulin in islets exposed to OL extract and marrubiin.

The melting curve obtained for the insulin gene expression under the six conditions is shown on figure 4.8. From the melting curve, one can observe that a single product was formed at 90˚C for all conditions and there were no primer dimers formed.
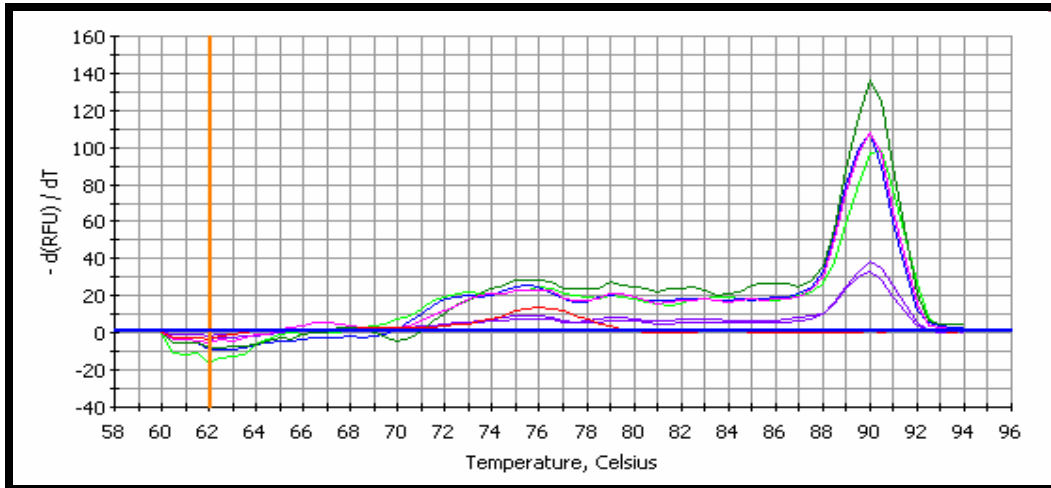
Figure 4.8: A melt curve for insulin expression by islet cells exposed to the OL extract (10μg/ml) and marrubiin (150ng/ml) in 11.1mM and 33.3mM glucose RPMI.

Figure 4.9 displays the expression levels of insulin by untreated islets and islets treated with marrubiin under normoglycemic condition. Since only M was stable relative to the housekeeping gene expression levels, it was the only condition that could be adequately evaluated and normalized, against cyclo A and tubulin. There was a 50% decrease in insulin gene expression relative to the control.
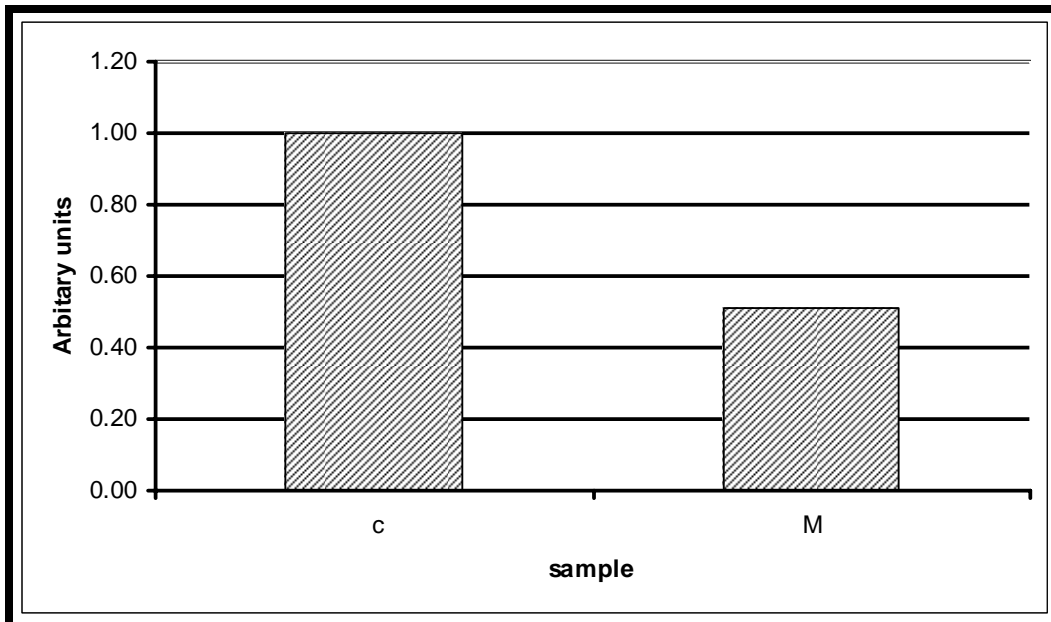
Figure 4.9: The expression of insulin by untreated islets (control) and islets treated with 150ng/ml marrubiin (M) under normoglycemic conditions, using tubulin and cyclo A as housekeeping genes (n= 2).

Figure 4.10 displays the trend of insulin expression levels that have not been normalized, across all conditions.
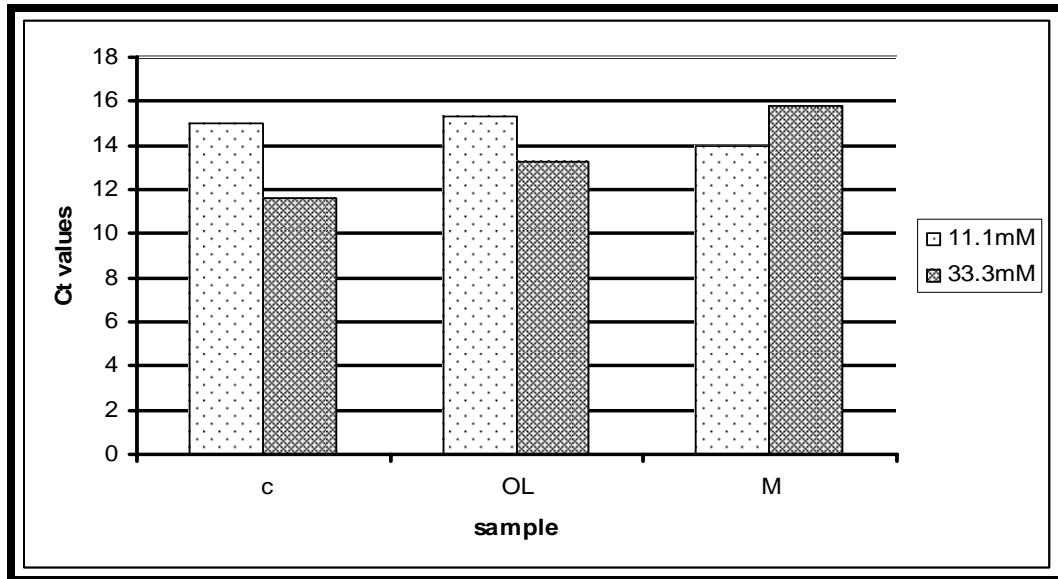
Figure 4.10: A graph showing the gene expression across all conditions for untreated islets (control), islets exposed to the OL extract (10μg/ml), and marrubiin (150ng/ml), under normoglycemic and hyperglycemic conditions (n = 2)

4.4.1 Discussion

To maintain a β-cell secretory capacity, a therapeutic insulin agent should increase insulin synthesis and insulin secretion (Alarcon *et al*., 2006). Chronic exposure to the OL 1 extract and marrubiin under hyperglycemic conditions stimulate insulin release, while OL extracts and marrubiin decreased insulin release under normoglycemic conditions. Marrubiin therefore appears to have a protective effect against glucotoxicity.

The -340 to -90 (relative to the transcription start site) base pair region of the promoter of the insulin gene contains a number of elements that are bound by various elements that regulate insulin gene transcription (Jang *et al*., 2007). Transfection of β-cell lines with fragments of the insulin gene upstream region linked to a reporter gene have demonstrated that specific sequences exert both positive and negative effects on gene expression (Goodison *et al*., 1992).

In figure 4.9, marrubiin decreased the expression of insulin by 50%, which correlated with the results obtained from the chronic sample GSIS assay, in which the marrubiin standard decreased insulin secretion by more than 40% under normoglycemic conditions.

In this study, marrubiin has also been found to have antidiabetic activity due to stimulatory effect on insulin secretion under hyperglycemic conditions and it induced β-cell proliferation. Based on the results that have been obtained for plants from the same family as *Leonotis leonurus* that show antidiabetic activity (section 4.1.6), the antidiabetic activity observed in this study could possibly be due to the lactone (marrubiin) in the plant extract.

CHAPTER 5: Conclusion and Future Studies

The aim of this study was to determine the effect of *Leonotis leonurus* extracts and its active compound, marrubiin, on blood coagulation, insulin secretion, and insulin expression.

Few studies have been conducted in determining marrubiin's biological activity. Similar to *Leonotis leonurus*, marrubiin has been found to have anti-inflammatory activity, antispasmodic, antinociceptive, vasorelaxant effects (Stulzer *et al.*, 2006; Meyre-Silva *et al.*, 2005; Bardai *et al.*, 2003; and De Jesus *et al.*, 2000).

The thrombin assay, fibrinolysis assay, and platelet aggregation study were used to determine the effect of the *Leonotis leonurus* extracts and marrubiin. The overall studies on blood coagulation showed that the *Leonotis leonurus* extracts and marrubiin had an antithrombotic and anticoagulant activity. The extracts inhibited thrombin by more than 90%, with IC_{50} values of 6.2 and 3.0mg/ml for the OL and the AL extract respectively. Anticoagulant activity was displayed by the extracts and marrubiin by increasing the rate of fibrinolysis and having no effect on clot formation. Platelet aggregation studies were also investigated and the extracts and marrubiin displayed no inherent platelet aggregation. Platelet aggregation was also inhibited by both the *Leonotis leonurus* extracts and marrubiin. The OL extract displayed the strongest inhibition (92%) at 2mg/ml, and marrubiin displayed 68% inhibition at 100μg/ml.

In the antidiabetic studies, the OL extract and marrubiin increased the secretion of insulin. Even though the OL extract and marrubiin displayed apoptotic activity, this was counteracted by an even higher fold increase in proliferation of ß-cells. Therefore, it can be concluded that the *Leonotis leonurus* extract and marrubiin have promoted the proliferation of ß-cells. Overall, the extracts and marrubiin did not have a negative effect on apoptosis and increased the proliferation of the ß-cells. RT-PCR results confirmed the finding of GSIS from the chronic sample, marrubiin decreased insulin expression in normoglycemic condition, hence there was a decrease in insulin secretion under the same conditions in the chronic sample.

Based on these results, a possible therapeutic agent can be developed from this plant, since it displays moderate apoptic effects at lower concentrations, but greatly enhances proliferation.

The key role played by thrombin in the pathogenesis of thrombosis and atherosclerosis makes this enzyme a target for antithrombotics. There are two positively charged regions on the thrombin surface that play a role in the specificity of thrombin towards macromolecular substrates such as fibrinogen, co-factors, and some inhibitors such as heparin (Mendes-Silva *et al*., 2003). For future blood coagulation studies the mechanism by which the *Leonotis leonurus* extracts inhibit thrombin can be elucidated to determine if this plant blocks the catalytic activity of thrombin or whether it interacts with the exosite region responsible for the specificity of thrombin. An *in vivo* model can also be used to evaluate the effect of these extracts and marrubiin on thrombosis by inducing thrombosis in rats, to verify the *in vitro* findings. As mentioned before, the inhibition of TXA$_2$ is a promising direction towards the development of a therapeutic agent against thrombosis. The effect of the OL and the AL extracts and marrubiin on cyclooxygenase and the TXA$_2$ pathway can be evaluated to observe if the antiplatelet activity displayed by the plant and marrubiin is through the inhibition of this pathway. The double staining method for detecting platelet activity has to be optimized in order to obtain data that would further explain the mechanisms involved in the antiplatelet activity of this plant.

Lipid peroxidation of cell membranes is associated with thrombosis in T2DM (Okamoto *et al*., 2006). The effect of the plant and marrubiin on the production of ROS could also be evaluated.

The mechanism of antidiabetic effect displayed by *Leonotis leonurus* and marrubiin can be determined by finding their effect on the insulin signalling pathway. Specific proteins in the insulin signalling pathway can be blocked in the presence of the extracts or marrubiin to determine the mechanism by which the effects observed in these studies are brought about. For the insulin expression studies, more stable housekeeping genes have to be identified in order to effectively

evaluate the expression levels of insulin across both normoglycemic and hyperglycemic conditions.

The development of T2DM in hypertensive patients multiplies the risk of cardiovascular complications (Cea-Calvo *et al.*, 2006). *Leonotis leonurus* can serve as multifactorial therapeutic agent in hypertensive patients as it has been found to have hypotensive activity (Kenechukwu, 2004), and antidiabetic activity (in this study) to protect the patients against the development of T2DM.

Chapter 6: References

Adams T.A. (1999). South African Plants. Hallmark Publishers. Cape Town. 166-167

Alarcon C., Wicsteed B., and Rhodes C.J. (2006). Exendin 4 controls insulin production in rat islet ß-cells predominantly by potentiation of glucose-stimulated proinsulin biosynthesis at the translational level. *Diabetologia*. **49**, 2920-2929

Almind K., Frederiksen S.K., and Bernal D. (1999). Search for variants of the gene promoter and the potential phosphotyrosine encoding sequence of the insulin receptor substrate-2 gene. *Diabetologia*. **42**, 1244-1249

Agirbasli M., and Vaughan D.E. (2000). The role of renin-angiotensin system in regulating fibrinolytic balance: potential mechanism in prevention of myocardial infarction. *Turkish Journal of Medical Science*. **30**, 95-100

Araki E., M. A. Lipes, Patti M. E., Bruning J.C., Haag B. L., Johnson R.S., and Kahn C.R. (1994). Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature*. **372**, 186–190

Banerjee A., Chisti Y., and Banerjee U.C. (2004). Streptokinase‑a clinically useful thrombolytic agent. *Biotechnology Advances*. **22**, 287-307

Bardai S.E, Morel N., Wibo M., Fabre N., Llabres G., Lyoussi B., and Quetin-Leclercq J. (2003). The vasorelaxant activity of marrubenol and marrubiin from Marrubium vulgare. *Planta Medica*. **69**, 75-77

Beisswenger P.J, Howell S.K, Touchette A.D., Lal S., and Szwergold B.S. (1999). Metformin reduces systemic methylglyoxal levels in type 2 diabetes. *Diabetes*. **48**, 198-202

Bellavite P., Andrioli G., Guzzo P., Arigliano P., Chirumbolo S., Manzato F., and Santonastaso C. (1993). A colorimetric method for the measurement of platelet adhesion in microtiter plates. *Analytical Biochemistry*. **216**, 444-450

Bergman R.N. (1989). Toward physiological understanding of glucose tolerance. Minimal model approach. *Diabetes*. **38**, 1512-1527

Bernard-Kargar C. and Ktorza A. (2001). Endocrine pancreas plasticity under physiological and pathological conditions. *Diabetes*. **50**, S30-S35

Bienvenu E., Amabeoku G.J., Eagles P.K., Scott G., and Springfield E.P. (2002). Anticonvulsant activity of aqueous extract of *Leonotis leonurus*. *Phytomedicine*. **9**, 217-223

Bjornholm H. and Zierath J.R. (2005). Insulin signal transduction in human skeletal muscle: identifying the defects in type II diabetes. *Biochemical Society Transaction*. **33**, 354–361

Boyer, R.F. (1993). Modern experimental biochemistry, 2nd edition. The Benjamin/ Cummings Publishing Company, Carlifornia. 61-64, and 90-102

Briaud I., Dickson L.M., Lingohr M.K., McCuaig J.F., Lawrence J.C., and Rhodes C.J. (2005). Insulin receptor substrate-2 proteasomal degradation mediated by a mammalian target of rapamycin (mTOR)-induced negative feedback down-regulates protein kinase B-mediated signaling pathway in β-cells. *Journal of Biological Chemistry*. **280**, 2282-2293

Brozinick J.T., Roberts B., and Dohm G.L. (2003). Defective insulin signalling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle potential role in insulin resistance. *Diabetes*. **52**, 935-941

Bruning J.C., Winnay J., Cheatham B., and Kahn R.C. (1997). Differential signaling by insulin receptor substrate 1 (IRS-1) and IRS-2 in IRS-1-deficient cells. *Molecular and Cellular Biology*. **17**, 1513-1521

Butler A.E., Janson J., and Soeller W.C. (2003). Increased β-cell apoptosis prevents adaptive increase in β-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes*. **52**, 2304-2314

Butler A.E., Janson J., Bonner-Weir S., Ritzel R., Rizza R.A., and Butler P.C. (2003). β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes*. **52**, 102-110

Casellas A., Salavert A., Agudo J., Ayuso E., Jimenez V., Moya M., Muñoz S., Franckhauser S., and Bosch F. (2006). Expression of IGF-I in pancreatic islets prevents lymphocytic infiltration and protects mice from type 1 diabetes. *Diabetes*. **55**, 3246-3255

Carmassi F., Morale M., and Pucetti R. (1992). Coagulation and fibrinolytic system impairement in insulin dependent diabetes mellitus. *Thrombosis Researh*. **67**, 643-654

Cassar K., Bachoo P., and Brittenden J. (2003). The role of platelets in peripheral vascular disease. *European Journal of Vascular and Endovascular Surgery*. **25**, 6-15

Cea-Calvo L., Conthe P., Gómez-Fernández P., de Alvaro F., Fernández-Pérez C., and RICARHD investigators. (2006). Target organ damage and cardiovascular complications in patients with hypertension and Type 2 diabetes in Spain: a cross-sectional study. *Cardiovascular Diabetologie*. **3**, 5-23

Cerf M.E. (2006). Transcription factors regulating β-cell function. *European Journal of Endocrinology*. **155**, 671-679

Ceriello A. (1997). Fibrinogen and diabetes mellitus: Is it time for intervention trials? *Diabetologia*. **40**, 731-734

Ceriello A. (1993). Fibrinogen and diabetes mellitus. The role of hyperglycemia and therapeutic aspects. *Diabetologia*. **36**, 1119-1125

Ceriello A., Giugliano D., and Quatraro A. (1990). Evidence for a hyperglycemia-dependent decrease of antithombin III-thrombin complex formation in humans. *Diabetologia*. **33**, 163-167

Charles M.A., Morange P., and Eschwege E. (1998). Effect of weight change and metformin on fibrinolysis and the von Willebrand factor in obese non-diabetic subjects. *Diabetes Care*. **21**, 1967-1972

Cheng D. (2005). Prevalence, predisposition and prevention of type II diabetes. *Natural Metabolism*. **2**, 29-40

Cho H., Thorvaldsen J.L., Chu Q., Feng F., and Birnabaum M.J. (2001). Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *Journal of Biological Chemistry*. **276**, 38349-38352

Cho H., Thorvaldsen J.L., Chu Q., Mu J., Kim J.K., and Crenshaw E.B. (2004). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKBβ). *Science*. **292**, 1728-1731

Chou C.K., Dull, T.J., Russel, D.S., Gherzi, R., Lebwohl, D., Ullrich A. and Rosen, O.M. (1987). Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *Journal of Biological Chemistry*. **262**, 1842–1847

Clark A., Wells C.A., Buley I.D., Cruickshank J.K., Vanhegan R.I., Matthews D.R., Cooper G.J.S., Holman R.R., and Turner R.C. (1988). Islet amyloid, increased α-cells, reduced β-cells and exocrine fibrosis - quantitative changes in the pancreas in type-2 diabetes. *Diabetes Research and Clinical Experimentaion*. **9**, 151-159

Ciprandi A., Kobe de Oliveira S., Masuda S., Horn F., and Termignoni C. (2006). *Boophilus microplus*: Its saliva contains microphilin, a small thrombin inhibitor. *Experimental Parasitology*. **114**, 40-46

Clemetson K.J. (1999). Primary haemostasis: Sticky fingers cement the relationship. *Current Biology*. **9**, 110-112

Clutton P., Folts J.D., and Freedman J.E. (2001). Pharmacological control of platelet function. *Pharmacological Research*. **44**, 255-264

Coller B.S. (1995). Blockade of platelet GPIIb/IIIa receptors as an antithrombotic strategy. *Circulation*. **92**, 2373-2380

Colwell J.A. (2001). Treatment for the procoagulant state in Type 2 diabetes. *Endocrinology and Metabolism Clinics of North America*. **30**, 1011-1029

Colwell J.A. (1986). Effects of exercise on platelet function, coagulation, and fibrinolysis. *Diabetes Metabolism Research and Reviews*. **1**, 501-512

Colwell J.A. and Jokl R. (1996). Vascular thrombosis in diabetes. Diabetes Mellitus: Theory and Practice, edition 5. Norwalk, Appleton and Lange. London. 207-216

Davi G., Belvedere M., and Vigneri S. (1996). Influence of metabolic control on thromboxane biosynthesis and plasma plasminogen activator inhibitor 1 in non-insulin-dependent diabetes mellitus. *Thrombosis Haemostasis*. **76**, 34-37

Degawa-Yamauchi M., Bovenkerk J.E., and Juliar B.E. (2003). Serum resistin (FIZZ3) protein is increased in obese humans. *Journal of Clinical Endocrinology Metabolism*. **88**, 5452-5455

De Jesus R.A., Cechinel-Filho V., Oliveira A.E., and Schlemper V. (2000). Analysis of the antinociceptive properties of marrubiin isolated from *Marrubium vulgare*. *Phytomedicine*. **7**, 111-115

De Meyer S.F., Vanhoorelbeke K., Ulrichts K., Staelens S., Feys H.B., Salles I., Fontayne A., and Deckmeyn H. (2006). Development of monoclonal antibodies that inhibit platelet adhesion or aggregation as potential anti-thrombotic drugs. *Cardiovascular Hematology Disorder Drug Targets*. **6**, 191-207

Derberg M., Houssa P., Frank B.H., Sodoyez-Goffaux, and Sodoyez J-C. (1998). Highly specific radioimmunoassay for human insulin based on immune exclusion of all insulin precursors. *Clinical Chemistry*. **44**, 1504-1513

De Taeye B., Harris Smith L., and Vaughn D.E. (2005). Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease. *Current Opinion in Pharmacology*. **5**, 149-154

Drew A.K. and Meyers S.P. (1997). Safety issues in herbal medicine: implications for the health professions. *Medical Journal of Australia*. **166**, 538-541

Dvorák Z., Pascussi J-M., and Modrianský M. (2003). Approaches to messenger RNA detection- comparison of methods. *Biomedical Papers*. **147**, 131-135

Eddouks M., Maghrani M., Lemhadri A., Ouahidi M.L., and Jouad H. (2002). Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in south-east region of Morocco (Tafilalet). *Journal of Ethnopharmacology*. **82**, 97-103

Ehrmann D.A., Schneider D.J., and Sobel B.E. (1997). Troglitazone improves defects in insulin action, insulin secretion, ovarian steroidogenesis, and fibrinolysis in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology Metabolism*. **82**, 2108-2116

Eidi M., Eidi A., and Zamanizadeh H. (2005). Effect of *Salvia officinalis* L. leaves on serum glucose and insulin in healthy and streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology*. **100**, 310-313

Fantuzzi G. (2005). Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical. Immunology*. **115**, 911–919

Farilla L., Hui H., Bertolotto C., Kang E., Bulotta A., Di Mario U., and Perfetti R. (2002). Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in zucker diabetic rats. *Endocrinology*. **143**, 4397-4408

Ferré F., A., Marchese P., Pezzoli S., Griffin E., Buxton E., and Boyer V. (1994). Quantitative PCR: an overview. Birkhäuser. Boston. 67-88

Freed M., Fuell D., and Menci I. (2000). Effect of combination therapy with rosiglitazone and glibenclamide on PAI-1 antigen, PAI-1 activity, and tPA in patients with type 2 diabetes. *Diabetologia*. **43**, 1024-1030

Fujikawa H., Shimojima Y., and Yano K. (2006). Novel method for estimating viable Salmonella cell counts using real-time PCR. *Shokuhin Eiseigaku Zasshi*. **47**, 151-6

Fujimoto W.Y. (2000). The importance of Insulin Resistance in the Pathogenesis of Type 2 Diabetes Mellitus. *Excerpta Medica*. **108**, 9S-14S

Fulop T., Tessier D., and Carpentier A. (2006). The metabolic syndrome. *Pathologie Biologie*. **54**, 375-386

Gilliland G., S. Perrin, K. Blanchard and H.F. Bunn. (1990). Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proceedings of the National Academy of Science*. **87**, 2725-2729

Goldenberg N.A., Hathaway W.E., Jacobson L., and Manco-Johnson M.J. (2005). A new global assay of coagulation and fibrinolysis. *Thrombosis Research*. **116**, 345-356

Goodison S., Kenna S., and Ashcroft S. (1992). Control of gene expression by glucose. *Biochemistry Journal*. **285**, 563-568

Gou Y.L., Sze A., Rowlands D.K., Chung Y.W., and Chan H.C. (2003). Effects of bak foong pill on blood coagulation and platelet aggregation. *Biological Pharmacology Bulletins*. **26**, 241-246

Goun A.E., Petrichenko V.M., Solodnikov S.U., Suhinina T.V., Martin A.K., Cunningham G., Nguyen C., and Miles H. (2002). Anticancer and antithrombin activity of Russian plants. *Journal of Ethnopharmacology*. **81**, 337- 342

Hall R.K., Yamaski T., Kucera T., and Waltner-Law M. (2000). Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. *Journal of Biological Chemistry*. **275**, 30169-30175

Halushka P.V., Rogers R.C., and Loadholt C.B. (1981). Increased platelet thromboxane synthesis in diabetes mellitus. *Journal of Laboratory Clinical Medicine*. **97**, 87-96

Harrison P. (2005). Platelet function analysis. *Blood Reviews*. **19**, 111-123

Hart L.M., Lindhout D., Van der Zon G.C., Kayserilli H., Apak M.Y., Kleijer W.J., Van der Vorm E.R., Maassen J.A. (1996). An insulin receptor mutant (Asp707 --> Ala), involved in leprechaunism, is processed and transported to the cell surface but unable to bind insulin. *Journal of Biological Chemistry*. **271**, 18719-18724

Heijnen H.F.G., Schiel A.E., Fijnheer R, Geuze H.J., and Sixma J.J. (1999). Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and α-Granules. *Blood*. **94**, 3791-3799

Hemker H.C., Giesen P., Dieri R.A., Regnault V., de Smedt E., Wagenwoord R., Lecompte E., and Beguin S. (2003). Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiology of Haemostasis and Physiology*. **33**, 4-15

Hilberg, T., Eichler, E., Glaser, D., Prasa, D., Sturzebercher, J. and Gabriel, H. H. W. (2003). Blood coagulation and fibrinolysis before and after exhaustive exercise in patients with IDDM. *Thrombosis and Haemostasis. 90*, 1065-1073

Hirota K., Daitoku H., and Araya N. (2003). Hepatocyte nuclear factor-4 is a novel downstream target of insulin via FKHR as a signal-regulated transcriptional inhibitor. *Journal of Biological Chemistry*. **278**, 13056-13060

Hoareau L. and Da Silva E.J. (1999). Medicinal plants: a re-emerging aid. *Electronic Journal of Biotechnology*. **2**, 296-300

Homsby W.G., Boggess K.A., and Lyons T.J. (1990). Hemostatic alterations with exercise conditioning in NIDDM. *Diabetes Care*. **13**, 87-92

Honegger J., Prettin C., Feuerhake F., Manfred P., Schutle-Monting J., Reincke M. (2003). Expression of Ki-67 antigen in nonfunctioning pituitary adenomas: correlation with growth velocity and invasiveness. *Journal of Neurosurgery*. **99**, 674-679

Hoppener J.W.M., Oosterwijk C., Nieuwenhuis M.G., Posthuma G., Thijssen J.H.H., Vroom T.M., Ahrén B., and Lips C.J.M. (1999). Extensive islet amyloid formation is induced by development of type II diabetes mellitus and contributes to its progression: pathogenesis of diabetes in a mouse model. *Diabetologia*. **42**, 427-434

Hoppener J.W.M, Verbeek J.S., de Koning E.J.P., Oosterwijk C., van Hulst K.L., Visser-Vernooy H.J., Hofhuis F.MA, van Gaalen S., Berends M.J.H., Hackeng

W.H.L., Jansz H.S., Morris J.F., Clark A., Capel P.J.A., and Lips C.J.M. (1993). Chronic overproduction of islet amyloid polypeptide 21(amylin) in transgenic mice - Iysosomal localization of human islet amyloid polypeptide and lack of marked hyperglycemia or hyperinsulinemia. *Diabetologia*. **36**, 1258-1265

Houghton P.J. and Raman A. (1998). Laboratory handbook for the fractionation of natural extracts. Chapman and Hall, 171-173

Huber K. (2001). Defective fibrinolytic states as triggers of myocardial infarction: the cardiologist's view. *Italian Heart Journal*. **2**, 646-651

Hundley A.F., Yuan L., and Visco A.G. (2006). Skeletal muscle heavy-chain polypeptide 3 and myosin binding protein H in the pubococcygeus muscle in patients with and without pelvic organ prolapse. *American Journal of Obstetrics and Gynecology*. **194**, 1404-1410.

Jager J., Gremeaux T., Cormont M., Le Merchand-Brustel Y., and Tanti J-F. (2007). Interleukin-1 β− induced insulin resistance in adipocytes through down-regulation of IRS-1. *Endocrinology*. 148, 241-251

Jang W.G., Kim E.J., Park K-G., Park Y.B., Choi H-S., Kim H-J., Kim Y.D., Kim K-S., Lee K-U., and Lee I-K. (2007). Glucocorticoid receptor mediated repression of human insulin gene expression is regulated by PGC-1α. *Biochemical and Biophysical Research Communications*. **352**, 716–721

Jarvi A.E., Karlstrom B.E., and Granfeldt Y.E. (1999). Improved glycemic control and lipid profile and normalized fibrinolytic activity on a low-glycemic index diet in type 2 diabetic patients. *Dieabetes Care*. **22**, 10-18

Johnstone M.T. and Veves A. (2001). Diabetes and cardiovascular disease. Human Press Publishers, New Jersey. 149-163

Jorkl R. and Colwell J.A. (1997). Clotting disorders in diabetes. *Diabetes Care*. **65**, 1543-1557

Jorkl R. and Colwell J.A. (1997). Arterial thrombosis and atherosclerosis in diabetes. *Diabetes Metabolism Research and Reviews*. **5**, 1-15

Jorkl R. Laimins M., and Klein R.L. (1994). Platelet plasminogen activator inhibitor 1 in patients with type 2 diabetes. *Diabetes Care*. **17**, 818-823

Juhan-Vague I., Roul C., and Alessi M.C. (1989). Increased plasma plasminogen activator inhibitor-1 activity in non-insulin-dependent diabetic patients. *Thrombosis Haemostasis*. **3**, 370-373

Jung S.H., Park S.Y., Kim-Pak Y., Lee H.K., Park K.S., Shin K.H., Ohuchi K., Shin H-K, Keum S.R., and Lim S.S. (2006). Synthesis and PPAR-γ ligand-binding activity of the new series of 2'-Hydroxychalcone and Thiazolidinedione derivatives. *Chemical Pharmacology Bulletins.* **54**, 368-371

Kahn S.E. (2000). The importance of the ß-Cell in the Pathogenesis of Type 2 Diabetes Mellitus. *American Journal of Medicine*. **108**, 2-8

Kahn S.E. (2003). The relative contributions of insulin resistance and ß-cell dysfunction to pathophysiology of Type 2 Diabetes. *Diabetologia*. 46, 3-19

Kaleem M., Asif F., Ahmed Q.U., and Bano B. (2006). Antidiaebtic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats. *Singapore Medical Journal*. **47**, 670-675

Karlheinz P., Meike, S., Jari, S., Benedikt, K., Martin, M, Thomas, N., Peter, S., Wolfgang, K., and Christoph, B. (1998). Induction of fibrinogen binding and platelet aggregation as a potential intrinsic property of various glycoprotein IIb/IIIa ($\alpha IIb\beta_3$) inhibitors. *Blood*. **92,** 3240-3249

Kasuya N., Kishi Y., Isobe M., Yoshida M., and Numano F. (2006). P-Selectin Expression, but not GPIIb/IIIa activation, is enhanced in the inflammatory stage of Takayasu's arteritis. *Circulation Journal*. **70**, 600-604

Kauffman M.J. (2000). Should you take aspirin to prevent heart attack? *Journal of Scientifc Exploration*. **14**, 623-641

Kaushal V., Kaushal G.P., Melkaveri S.N., and Mehta P. (2004). Thalidomide protects endothelial cells from doxorubicin-induced apoptosis but alters cell morphology. *Thrombosis Haemostasis.* **2**, 327–334

Kenechukwu O. (2004). Cardiovascular effects of *Leonotis leonurus* extracts in normotensive rats and in isolated perfused rat heart. Masters Thesis. University of Western Cape. 89-90

Kile B.T., Schulman B.A., Alexander W.S., Nicola N.A., Martin H.M. and Hilton D.J. (2002). The SOCS box: a tale of destruction and degradation. *Trends in Biochemical Science*. **27**, 235–241

Kim Y.B., Nikoulina S.E., Ciaraldi T.P., Henry R.R., and Kahn B.B. (1999). Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type diabetes. *Journal of Clinical Investigation*. **104**, 733-741

Kirpichnikov D. and Sowers JR. (2001). Diabetes Mellitus and diabetes-associated vascular disease. *Trends in Endocrinology and Metabolism*. **12**, 225-230

Knoss W., Reuter B., and Zapp J. (1997). Biosynthesis of the labdane diterpene marrubiin in *Marrubium vulgare* via a non-mevalnate pathway. *Biochemistry Journal*. **326**, 449-454

Koukoulitsa C., Zika C., Geromichalos G., Demopoulos V., and Skaltsa H. (2006). Evaluation of aldose reductase inhibition and docking studies of some secondary metabolites, isolated from *Origanum vulgare* L. ssp. hirtum. ***Bioorganic and Medicinal Biochemistry***. **14**, 1653-1659

Kovacs P. and Stumvoll M. (2005). Fatty acids and insulin resistance in muscle and liver. ***Best Practice and Research in Clinical Endocrinology and Metabolism***. **19**, 625–635

Krook A., Roth R.A., Jiang X.J., Zierath J.R., and Wallberg-Henriksson H. (1998). Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. ***Diabetes***. **47**, 1281-1286

Kubota N., Terauchi Y., and Yamauchi T. (2002). Disruption of adiponectin causes insulin resistance and neointimal formation. ***Journal of Biological Chemistry***. **277**, 25863-25866

Kwaan H.C. (1992). Changes in blood coagulation, platelet function, and plasminogen-plasmin system in diabete. ***Diabetes***. **41**, 32-35

Lange U., Nowak G., and Bucha E. (2004). Ecarin chromogenic assay – A new method for quantitative determination of direct thrombin inhibitors like hirudin. ***Pathophysiology of Haemostasis and Thrombosis***. **33**, 184-191

Laybutt R., Weir G.C., Kaneto H., Lebet J., Palmiter R.D., Sharma A., and Bonner-Weir S. (2002). Overexpression of c-Myc in β-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. ***Diabetes***. **51**, 1793-1804

Leahy J.L. (2005). Pathogenesis of Type 2 diabetes mellitus. ***Archives of Medical Research***. **36**, 197-209.

Leiter L.A. and Lewanczuk R.Z. (2005). The renin-angiotensin system and reactive oxygen in Type 2 diabtes and angiotensin II inhibition. *American Journal of Hypertension*. **18**, 121-128

Lemhadri A., Zeggwagh N.A., Maghrani M., Jouad H., and Eddouks H. (2004). Anti-hyperglycaemic activity of the aqueous extract of *Origanum vulgare* growing wild in Tafilalet region. *Journal of Ethnopharmacology*. **92**, 251-256

LeRoith D., and Gavrilova O. (2005). Mouse models created to study the pathophysiology of Type 2 diabetes. *The International Journal of Biochemistry & Cell Biology*. **38**, 904-912

Li J., Yu X., Pan W, and Unger R.H. (2002). Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *American Journal of Physiological Endocrinological Metabolism*. **282**, 1334-1341

Light M.E., McGraw L.J., Sparg S.G., Taylor M.N., Erasmus D.G., Jager A.K., and Van Staden J. (2002). Investigation of the biological activities of *Siphonochilus aethiopicus* and the effect of seasonal senescence. *South African Journal of Botany*. **68**, 55-61

Lima C.F., Azevedo M.F., Araujo R., Fernandes-Ferreira M., and Pereira-Wilson C. (2006). Metformin-like effect of *Salvia officinalis* (common sage): is it useful in diabetes prevention? *British Journal of Nature*. **96**, 326-333

Liu G., Guan G.J., Qi T.G., Fu Y.Q., Li X.G., Sun Y., Wu T., and Wen R.Z. (2005). Protective effects of *Salvia miltiorrhiza* on rats with streptozotocin diabetes and its mechanism. *Journal of Chemical Information and Remodelling*. **3**, 459-462

Low-Ah-Kee N. (2005). Purification of a cancer procoagulant for screening antithrombotic activity of several indigenous plant extracts. Master Thesis. Nelson Mandela Metropolitan University. 52-61

Luna M.T.T. (2004). Genes and Type 2 Diabetes Mellitus. *Archives of Medical Research*. **36**, 210-222

Lũthje J., and Ogilvie A. (1999). Platelet aggregation in whole blood: A laboratory experiment for a medical biochemistry course. *Methods*. **26**, 215-216

Lyon C.J., and Hsueh W.A. (2003). Effect of plasminogen activator inhibitor-1 in diabetes mellitus and cardiovascular disease. *Journal of Medicine*. **115**, 62-68

Maassen J.A., Tobias E.S., Kayserilli H., Tukel T., Yuksel-Apak M., d'Haens E., Kleijer W.J., Féry F. and van der Zon G. (2003). Identification and functional assessment of novel and known insulin receptor mutations in five patients with syndromes of severe insulin resistance. *Journal of Clinical Endocrinology and Metabolism*. **88**, 4251-4257

Macfarlane R.G., and Biggs R. (1953). A thrombin generation test. *Journal of Clinical Pathology*. **6**, 3–7

McElduff A., Hedo J.A., Taylor S.I., Roth J., and Gorden P. (1984). Insulin receptor degradation is accelerated in cultured lymphocytes from patients with genetic syndromes of extreme insulin resistance. *Journal of Clinical Investigation*. **74**, 1366–1374

Maeda N., Shimomura I., and Kishida K. (2002). Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *National Medicine*. **8**, 731-737

Maedler K., Spinas G.A., Dyntar D., Moritz W., Kaiser N., and Donath M.Y. (2001). Dinstinct effects of saturated and monosaturated fatty acids on β-cell turnover and function. *Diabetes*. **50**, 69-76

Mao S.J.T. and Tucci M.A. (1991). A colorimetric assay for measuring the lysis of a plasma clot. *Analytical Biochemistry.* **192**, 6-10

Marchand B., Behl M., and Rodriguez E. (1983). Application of high-performance liquid chromatography and isolation of sesquiterpene lactones. *Journal of Chromatography*. **265**, 97-104

Masika P.J. and Afolayan A.J. (2003). An ethnobotanical study of plants used for the treatment of livestock diseases in the Eastern Cape Province, South Africa. *Pharmaceutical Biology*. **41**, 16-21

Matsuda T., Morishita E., Jokaji H., Asakura H., Saito M., Yoshida T., and Takemoto K. (1996). Mechanism on disorders of coagulation and fibrinolysis in diabetes. *Diabetes*. **45**, 109-110

Mekhfi H., Haouari M.E, Legssyer A., Bnouham M., Aziz M., Atmani F., Remmal A., and Ziyyat A. (2004). Platelet anti-aggregant property of some Moroccan medicinal plants. *Journal of Ethnopharmacology*. **94**, 317-322

Mendes-Silva W.,Assafima M.,Rutaa B., Monteiroa R.Q., Guimara J.A., and Zingali R.B. (2003). Antithrombotic effect of Glycyrrhizin, a plant-derived thrombin inhibitor. *Thrombosis Research*. **112**, 93-98

Meyre-Silva C., Yunes R.A., Schlemper V., Campos-Buzzi F., and Cechinel-Filho V. (2005). Analgesic potential of marrubiin derivatives, a bioactive diterpene present in *Marrubium vulgare* (Lamiaceae). *IL Farmaco*. **60**, 321-326

Michelson A.D., Barnard M.R., Kreuger L.A., Frelinger A.L., and Furman M.I. (2000). Evaluation of platelet function by flow cytometry. *Methods*. **21**, 259-270

Michelson A.D, Ellis P.A., Barnard M.R., Matic G.B., Viles A.F., and Kestin A.S. (1991). Downregulation of the platelet surface glycoprotein Ib-IX complex in whole blood stimulated by thrombin, adenosine diphosphate, or an i*n vivo* wound. *Blood*. **77**, 770-779

Mlinar B., Janja M., Janez A., and Pfeifer M. (2007). Molecular mechanisms of insulin resistance and associated diseases. *Clinica Chimica Acta*. **375**, 20-35

Moon C.H., Jung Y.S., Kim H.M., Lee S.H., Baik E.J., and Park S.W. (2000). Mechanism for antiplatelet effect of onion: AA release inhibition, thromboxane A2 synthase inhibition and TXA$_2$/PGH2 receptor blockade. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **62**, 277-283

Moran N., Kiernan A., Dunne E., Edwards R.J., Shields D.C., and Kenny D. (2006). Monitoring modulators of platelet aggregation in a microtiter plate assay. *Analytical Biochemistry*. **357**, 77-84

Mullen K.L., Smith A.C., Junkin K.A., and Dyck D.J. (2007). Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat fed rats. *American Journal of Physiological Endocrinology Metabolism*. **293**, 83-90

Nagaoka M.R., Kouyoumdjian M., and Borges D.R. (2000). The hepatic clearance of recombinant tissue-type plasminogen activator decreases after an inflammatory stimulus. *Brazilian Journal of Medical and Biological Research*. **33**, 119-125

Nagashima H. (2000). Studies on the different modes of action of the anticoagulant protease inhibitors DX-9065a and argatroban. *Journal of Biological Chemistry.* **277**, 50439-50444

Nakae J., Biggs W.H., and Kitamura T. (2002). Regulation of insulin action and pancreatic β-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *National Genetics*. **32**, 245-253

Nedelman J., Heagerty P., and Lawrence H. (1992). Quantitative PCR: procedures and precisions. *Bulletin of Mathematical Biology*. **54**, 477-502

Ojewole A.O. (2003). Hypotensive effect of *Leonotis leonurus*. *The American Journal of Hypertension*. **16**, 213-225

Okamoto A., Iwamoto Y., and Maru Y. (2006). Oxidative stress-responsive transcription factor ATF3 potentially mediates diabetic angiopathy. *Molecular and Cellular Biology*. **26**, 1087-1097

Omran, A. (2003). Cytotoxic and apoptotic effects of scorpion Leiurus quinquestriatus venom on 293T and C2C12 eukaryotic cell lines. *Journal of Venomous Animal Toxins including Tropical Disorders*. **9**, 68-73

Ozcan Q. Cao, Tilmaz E., Lee A.H., Iwakoshi N. and Ozdelen E. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. **306**, 457–461

Peccoud J., and Jacob C. (1998). Statistical estimations of PCR amplification rates. Birkhäuser. Boston. 111-128

Peccoud J., and Jacob C. (1996). Theoretical uncertainty of measurements using quantitative polymerase chain reaction. *Biophysics Journal*. **71**, 101-108

Petrik J, Pell J.M, and Arany E. (1999). Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology*. **140**, 2353–2363

Persson E., Bergesten P., and Eriksson U.J. (1995). Appearance of glucose-induced insulin release in fetal rat beta-cells. *Journal of Endocrinology*. **58**, 115-120

Pitney W.R. and Dacie J. (1953). A simple method of studying the generation of thrombin in recalcified plasma. *Journal of Clinical Pathology*. **6**, 9–13

Pravenec M., Kazdova L., and Landa V. (2003). Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. *Journal of Biological Chemistry*. **278**, 45209-45215

Prentki M., Joly E., and El-Assaad W. (2002). Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in β-cell adaptation and failure in the etiology of diabetes. *Diabetes*. **5**, 405-413

Previs S.F., Withers D.J., Ren J.M, White M.F., and Shulman G.I. (2000). Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism *in vivo*. ***Journal of Biological Chemistry***. **275**, 38990-38994

Prochazkova J., Kylarova D., Vranka P., and Lichnovsky V. (2003). Comparative study of apoptosis-detecting techniques: TUNEL, apostain, and lamin B. ***Journal of Biotechniques***. **35**, 528-534

Puigeserver P., Rhee J., and Donovan J. (2003). Insulin regulated hepatic gluconeogenesis through Foxo1-PGC-1α interaction. ***Nature***. **32**, 550-555

Rajala M.W., Obici S., and Scherer P.E. (2003). Adipose-derived resistin and gut-derived resistin-like molecule-β selectively impair insulin action on glucose production. ***Journal of Clinical Investigation***. **111**, 225-230

Ramos K.R., Alarcon-Aguilar F., Lara-Lemus A, and Flores-Saenz J.L. (1992). Hypoglycemic effect of plants used in Mexico as antidiabetics. ***Archives of Medical Research***. **23**, 59-64

Rand M.L., Roland L., and Packham M.A. (2003). Platelet function assays. ***Transfusion and Apheresis Science***. **28**, 307-317

Reininger A.J. (2006). Primary haemostasis and its assessment by laboratory tests. ***Haemostaseologie***. **1**, 42-44, 46-47

Rhodes C.J. (2005). Type 2 diabetes – a matter of β-cell life and death? ***Science***. **307**, 380-384.

Ribnicky D.M., Poulev A., Watford M., Cefalu W.T., and Raskin I. (2006). Antihyperglycemic activity of Tarralin, an ethanolic extract of *Artemisia dracunculus* L. ***Phytomedicine***. **13**, 550-557

Ritzel R.A., Butler A.E., Rizza R.A., Veldhuis J.D. (2006). Butler P.C. Relationship between beta-cell mass and fasting blood glucose concentration in humans. ***Diabetes Care***. **29**, 717–718.

Rob J.A., Tollefsen S., and Helgeland L. (1997). A rapid and highly sensitive chromogenic microplate assay for quantification of rat and human prothrombin. *Analytic Biochemistry*. **245**, 222-225

Rondinone C.M., Carvalho E., Wesslau C., and Smith U.P. (1999). Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus. *Diabetologia*. **42**, 819-825

Rosenstein E.D., Advani S., Reitz R.E., and Kramer N. (2001). The prevalence of insulin receptor antibodies in patients with systemic lupus erythematosus and related conditions. *Journal of Clinical Rheumatology*. **6**, 371-373

Rossetti L, Barzilai N. and Chen W. (1996). Hepatic overexpression of insulin-like growth factor-II in adulthood increases basal and insulin-stimulated glucose disposal in conscious mice. *Journal of Biological Chemistry*. **271,** 203–208

Ruan H. and Lodish H.F. (2004) Regulation of insulin sensitivity by adipose tissue-derived hormones and inflammatory cytokines. *Current Opinion in Lipidology*. **15**, 297–302

Ruan H. and H.F. Lodish, Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. (2003). *Cytokine Growth Factor Reviews*. **14**, 447–455

Saltiel A.R. (2000). Series Introduction: The molecular and physiological basis of insulin resistance: emerging implications for metabolic and cardiovascular diseases. *The Journal of Clinical Investigation*. **106**, 163-164

Saltiel A.R. and Kahn C.R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. **414**, 799-806

Santana A., Enseñat-Waser R., Arribas M.I., Reig J.A., and Roche E. (2006). Insulin-producing cells derived from stem cells: recent progress and future directions. *Journal Cellular Molecular Medicine.* **10**, 866-883

Satoh M., Satoh Y., Isobe K., and Fujimoto Y. (2003). Studies on constituents of *Leonurus sibiricus* L. *Chemical Pharmacology Bulletins*. **51**, 341-342

Schatteman K.A., Goossens F.J., Scharpé S.S., Neels H.M. and Hendriks D.F. (1999). Assay of procarboxypeptidase U, a novel determinant of the fibrinolytic cascade, in human plasma. *Clinical Chemistry*. **45**, 807-813

Shimomura I., Hammer R.E., and Ikemoto S. (1999). Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature*. **401**, 73-76

Schinner S., Scherbaum W.A., Bornstein S.R., and Barthel A. (2005). Molecular mechanisms of insulin resistance. *Diabetic Medicine*. **22**, 674-682

Scott G., Springfield E.P., and Coldrey N. (2004). A pharmacognostical study of 26 South African plant species used as traditional medicines. *Pharmaeutical Biology*. **42**, 182-213

Senis Y.A., Paul Y., Kim P.Y., Gemma L.J., Fuller G.L.J., Prabhakar A.G., Wilkinson M.C., Brittan H., Zitzmann N., Wait R., Warrell D.A., Watson S.P., Kamiguti A.S., Theakston D.G., Nesheimb M.E., and Laing D. (2006). Isolation and characterization of cotiaractivase, a novel low molecular weight prothrombin activator from the venom of *Bothrops cotiara*. *Biochimica et Biophysica Acta*. **1764**, 863–871

Shattil S.J., Cunningham M., and Hoxie J.A. (1987). Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. *Blood*. **70**, 307-315

Sheehan D. (2000). Physical biochemistry: principles and applications, John Wiley and Sons, Ltd. Chichester. 12-34

Shulman, G.I. (2000). Cellular mechanisms of insulin resistance. *The Journal of Clinical Investigation*. **106**, 171-176

Simpson R.W., Shaw J.E., and Zimmet P.Z. (2003). The prevention of type 2 diabetes – lifestyle change or pharmacotherapy? A challenge for the 21st century. *Diabetes Research and Clinical Practice*. **59**, 165-180

Socha K., Socha K., and Fiedor P. (2003). Proliferation of transplanted allogenic pancreatic islets. *Transplantation Proceedings*. **35**, 2341-2342

Soeller W.C., Janson J., Hart S.E., Parker J.C., Carty M.D., Stevenson R.W., Kreutter D.K., and Butler P.C. Islet amyloid-associated diabetes in obese $A^{(vy)}$/a mice expressing human islet amyloid polypeptide. *Diabetes*. **47,** 743-750

Song S.H., Rhodes C.J., and Veldhuis J.D. (2003). Diazoxide attenuates glucose-induced defects in first-phase insulin release and pulsatile insulin secretion in human islets. *Endocrinology*. **144**, 3399-3405

Stafford G.I., Jager A.K., and van Staden J. (2005). Effect of storage on the chemical composition and biological activity of several popular South African plants. *Journal of Ethnopharmacology*. **97**, 107-115

Stamler J., Mendelsohn M.E., Amarante P., Smick D., Andon N., Davies P.F., Cooke J.P., and Loscalzo J. (1989). N-acetylcysteine potentiates platelet inhibition by endothelium-derived relaxing factor. *Circulation Research*. **65**, 789–795

Steinke F.A. and Driscoll S.G. (1967). *In vitro* studies of insulin inactivation with reference to erythroblastosis fetalis. *Blood*. **3**, 359-363

Steppan C.M., Bailey S.T., and Bhat S. (2001). The hormone resistin links obesity to diabetes. *Nature*. **409**, 307-312

Streeper R.S., Eaton E.M., and Ebert D.H. (1998). Hepatocyte nuclear factor-1 acts as an accessory factor to enhance the inhibitory action of insulin on mouse glucose-6-phosphatase gene transcription. *Proceedings of the National Academy of Sciences*. **95**, 9208-9213

Stulzer H.K., Tagliari M.P, Zampirolo J.A., Cechinel-Filho V., and Schlemper V. (2006). Antioedematogenic effect of marrubiin obtained from *Marrubium vulgare*. *Journal of Ethnopharmacology*. **108**, 379-384

Stumvoll M. and Haring H. (2002). Resistin and adiponectin of mice and men. *Obesisty Research*. **10**, 1197-1199

Summers S.A., Kao A.W., and Kohn A.D. (1999). The role of glycogen synthase kinase 3β in insulin-stimulated glucose metabolism. *Journal of Biological Chemistry*. **274**, 17934-17940

Szollosi A., Nenquin M., Aguilar-Bryan L., Bryan J., and Henquin J-C. (2007). Glucose stimulates Ca^{2+} influx and insulin secretion in 2 week old β-cells lacking ATP-sensitive K^+ channels. *Journal of Biological Chemistry*. **282**, 1747-1756

Talalay P. and Talalay P. (2001). The importance of using scientific principles in the development of medicinal agents from plants. *Academic Medicine*. **76**, 238-247

Tilburg J., van Haeften T., Pearson P., and Wijmenga C. (2001). Defining the genetic contribution of Type 2 diabetes mellitus. *Journal of Medical Genetics*. **38**, 569-578

Tkac, I., Salagovic, J., Kozarova, M., Javorsky, M., Tkacova, R. and Kalina, I. (2003). Angiotenin-converting genotype, albuminuria and plasma fibrinogen in Type 2 diabetes mellitus. *Wiener Klinische Wochenschrift*. **150**, 835-839

Thisoda P., Rangkadilok N., Pholphana N.,Worasuttayangkurn L., Ruchirawat S., and Satayavivad J. (2006). Inhibitory effect of Andrographis paniculata extract and its

active diterpenoids on platelet aggregation. *European Journal of Pharmacology*. **553**, 39-45

Tolleshaug H., Goldstein J. L., Schneider W. J., and Brown M. S. (1982). Post-translational processing of the LDL receptor and its genetic disruption in familial hypercholesterolemia. *Cell*. **30**, 715-724

Tschoepe D., Driesch E., and Schwippert B. (1995). Exposure of adhesion molecules on activated platelets in patients with newly diagnosed IDDM is not normalized by near-normoglycemia. *Diabetes*. **44**, 890-894

Tschoepe D., Rosen P., and Schwippert B. (1993). Platelets in diabetes: The role in haemostatic regulation in atherosclerosis. *Thrombosis Haemostasis*. **19**, 122-128

Tzeng T., Liu I. and Cheng J. (2005). Activation of opioid u-receptors by loperamide to improve interleukin-6-induced inhibition of insulin signals in myoblast C_2C_{12} cells. *Diabetologia*. **48**, 1386–1392

Uchida T., Iwashita N., Ohara-Imaizumi M., Ogihara T., Nagai S., Choi J.B., Tamura Y., Tada N., Kawamori R., Nakayama K., Nagamatsu S., and Watada H. (2007). PKC-DELTA plays non-redundant role in insulin secretion in pancreatic ß-cell. *Journal of Biological Chemistry.* **282**, 2702-2716

Unger R.H. (1995). Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. *Diabetes*. **44**, 863-870

Vanhaesebroeck B. and Alessi D.R. (2000). The P13K-PDK1 connection: more than just a road to PKB. *Biochemistry Journal*. **346**, 561-576

Vanhaelen M., Vanhaelen-Fastre R., But P., and Vanherweghem J. (1994). Identification of aristolochic acid in Chinese herbs. *Lancet*. **343**, 174-179

Vanherweghem J.L., Depierreux M., and Tielemans C. (1993). Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. *Lancet*. **341**, 387-391

Van Ypersele C. and Vanherwghem J.L. (1995). The tragic paradigm of Chinese herbs nephropathy. *Nephrology Dialysis Transplantation*. **10**, 157-160

Varughese G.I., Tomson J., and Lip G.Y.H. (2005). Type 2 diabetes mellitus: a cardiovascular perspective. *International Journal of Clinical Practice*. **59**, 798-816

Vague P.M., Juhan-Vague C., and Alessi M.C. (1987). Mtfromin decreases the high plasminogen activator inhibition capacity, plasma insulin and triglycerides levels in non-diabetic obese subjects. *Thrombosis Haemostasis*. **57**, 326-328

Vidal F., Vidal J.C., Gadelha A.P.R., Lopes C.S., Coelho M.G.P., and Monteiro-Leal L.H. (2007). *Giardia lamblia*: The effects of extracts and fractions from *Mentha x piperita* Lin. (Lamiaceae) on trophozoites. *Experimental Parasitology*. **115**, 25-31

Vinik A.I., Erbas T., Park T.S., Nolan R., and Pittenger G.L. (2001). Platelet dysfunction in Type 2 diabetes. *Diabetes Care*. **24,** 1476-1485

Walkowiak B., Kesy A., and Michalec L. (1997). Microplate reader: A convenient tool in studies of blood coagulation. *Thrombosis Research*. **87**, 95-103

Wang Z. and J. Spadoro. (1989). Determination of target copy number of quantitative standards used in PCR-based diagnostic assays. Birkhäuser. Boston. 31-43

Westermark P., Engström U., Johnson K.H., Westermark G.T., and Betsholtz C. (1990). Islet amyloid polypeptide - pinpointing amino acid residues linked to amyloid fibril formation. *Proceedings of the National Academy of Science*. **87**, 5036-5040

Weyer C., Bogardus C., and Mott D.M. (1999). The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Journal of Clinical Investigation*. **104**, 787-794

White M.F. (2002). IRS proteins and the common path to diabetes. *American Journal of Physiological Endocrinology Metabolism*. **283**, 413-422

Whithers D.J., Burks D.J., Towery H.H., Altamuro S.L.,. Flint C.L., and White M.F. (1999). Irs-2 coordinates Igf-1 receptor-mediated β-cell development and peripheral insulin signaling. *Nature Genetics.* **23**, 32 - 40

Wiman B. (1995). Plasminogen activator inhibitor-1 (PAI-1) in plasma: Its role in thrombotic disease. *Thrombosis Haemostasis*. **74**, 71-76

Winocour P.D., Watala C., and Kinlough-Rathbone R.L. (1990). Membrane fluidity is related to the extent of glycation of proteins, but not to alterations in the cholesterol to phospholipids ratio in isolated platelet membranes from diabetic and control subjects. *Thrombosis and Haemostasis*. **67**, 571-575

Winocour P.D., Lopes-Virella M., and Laimins M. (1985). Effect of insulin treatment in streptozotocin-induced diabetic rats on *in vitro* platelet function and plasma von Willebrand factor activity and factor VIII-related antigen. *Journal of Laboratory Clinical Medicine*. 6, 319-325

Winocour P.D.,Colwell J.A., and Laimins M. (1984). Platelet survival in streptozotocin-induced diabetic rats. *Thrombosis Haemostasis*. **51**, 307-312

Winocour P.D., Lopes-Virella M., and Laimins M. (1983). Time course of changes *in vitro* platelet function and plasma von Willebrand factor activity (VIIIR:WF) and factor VIII-related antigen (VIII:Ag) in the diabetic rat. *Journal of Laboratory Clinical Medicine*. **102**, 795-804

Wolfrum C., Besser D., and Luca E. (2003). Insulin regulates the activity of forkhead transcription factor Hnf-3β/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. *Proceedings of the National Academy of Sciences*. **100**, 11624-11629

Wong M.L., and Medrano J.F. (2005). Real-Time PCR for mRNA quantification. *Biotechniques*. **39**, 1-11

Xie L., Helmerhorst, E., Taddel, K., Plewright, B., van Bronswijk, W. and Martins, R. (2002). Alzheimer's β-amyloid peptides compete for insulin binding to the insulin receptor. *Journal of Neuroscience*. **22,** 1–5

Yagui K., Yamaguchi T., Kanatsuka A., Shimada F., Huang C.I., Tokuyama Y., Ohsawa H., Yamamura K.I., Miyazaki J.I., Mikata A., Yoshida S., and Makino H. (1995). Formation of islet amyloid fibrils in β-secretory granules of transgenic mice expressing human islet amyloid polypeptide (amylin). *European Journal of Endocrinology*. **132**, 487-496

Yamada K., Naemura A., Sawashita N., Noguchi Y., and Yamamoto J. (2004). An onion variety has natural antithrombotic effect as assessed by thrombosis/thrombolysis models in rodents. *Thrombosis Research*. **114**, 213-220

Yudkin, J. S. (1999). Abnormalities of coagulation and fibrinolysis in insulin resistance: evidence for a common antecedent? *Diabetes Care*. **22**, 25-30

Zhande R., Mitchell J.J., and Wu J. (2002). Molecular mechanism of insulin-induced degradation of insulin receptor substrate 1. *Molecular Cell Biology*. **22**, 1016-1026

Zhang B.B. (2002). Insulin signalling and action. Wiley Publishers. London. 67-69

Internet References

URL:http://www.plantexusa.com/products/commercial/gemfibrozil.htm, 20 May
2005

URL:http://www.esculape.com/ drugsoft/plavix.html, 20 May 2005

URL:http://www.moldb.nihs.go.jp/jp/scripts/name_exact_e.asp?name_e= Ticlopidine
Hydrochloride, 20 May 2005

URL:http://www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/
RNeasyMini.aspx - 36k, 3 August 2006

URL:http://www.who.int/diabetes/BOOKLET_HTML/en/index4.html, 3 January
2006

Appendix

Nelson Mandela Metropolitan University

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f o r   t o m o r r o w

Chairperson of the Research Ethics Committee (Human)
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Ref: N 01/11/03/07 [H06SB-003/Approval]

Contact person: Mrs U Spies

12 September 2006

Dr C Frost
NMMU
Department of Biochemistry and Microbiology
Faculty of Sciences

Dear Dr Frost

TO INVESTIGATE MULTI-THERAPEUTIC ANTICOAGULANT/ANTI-PLATELET OR ANTI-FIBRINOLYTIC AGENTS WHICH CAN PROVIDE INSIGHT FOR THE PRODUCTION OF DRUGS WHICH WOULD BE USEFUL IN TROMBOSIS

Your above-entitled re-application for ethics approval served at the August 2006 ordinary meeting of the Research Ethics Committee (Human).

The Committee approved the above-mentioned application.

Please inform your co-investigators of the outcome. We wish you well with the project.

Yours sincerely

Prof R du Randt
Chairperson: Research Ethics Committee (Human)

cc: Department of Research Management
        Faculty Officer, Faculty of Health Sciences

Nelson Mandela Metropolitan University

for tomorrow

Chairperson of the Research Ethics Committee (Animal)
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Ref: N 01/11/03/07 [H06-1/A]

Contact person: Mrs U Spies

8 March 2006

Ms R-A Levendal
Department of Biochemistry and Microbiology
Faculty of Science
NMMU

Dear Ms Levendal

MEDICINAL PLANTS AS ANTI-DIABETIC AGENTS IN ISOLATED RAT ISLET AND RATS, IN VIVO

The proposal entitled "**Medicinal plants as anti-diabetic agents in isolated rat islet and rats, in vivo**" that was resubmitted for ethics approval refers.

The Committee approved the proposal.

Please inform your co-investigators of the outcome. We wish you well with the project.

Yours sincerely

Dr PED Winter
Chairperson: Research Ethics Committee (Animal)

cc: Members of the REC-A Committee
        Department of Research Management
        Faculty Officer, Faculty of Science

/us

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