Leonotis leonurus: The anticoagulant and antidiabetic activity of
Leonotis leonurus

Submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor in the Faculty of Science at Nelson Mandela Metropolitan University

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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>Al</td>
<td>atherogenic index</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AL</td>
<td>aqueous extract</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>6-AN</td>
<td>6- aminonicotinamide</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>AT-II</td>
<td>angiotensin-II</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATP III</td>
<td>adult treatment panel III</td>
</tr>
<tr>
<td>AUCg</td>
<td>area under curve for glucose</td>
</tr>
<tr>
<td>AUCi</td>
<td>area under curve for insulin</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BH4</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>B0</td>
<td>maximum binding</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>untreated control</td>
</tr>
<tr>
<td>[Ca2+]i</td>
<td>intracellular calcium concentration</td>
</tr>
<tr>
<td>[Ca2+]c</td>
<td>cytosolic calcium concentration</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CaMK II</td>
<td>calcium/calmodulin kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CoA</td>
<td>co-enzyme A</td>
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</table>
COX: cyclooxygenase
Cq: the number of cycles required before the fluorescence reaches a threshold
CRP: C-reactive protein
CVD: cardiovascular diseases
Cyclo A: cyclophilin A
DAG: diacylglycerol
DCPIP: dichlorophenolindophenol
DDAH: dimethylaminohydrolase
DIC: disseminated intravascular coagulation
DM: diabetes mellitus
DOC: sodium deoxycholate
DPPIV: dipeptidyl peptidase IV
DVT: deep vein thrombosis
ECM: extracellular matrix
ECs: endothelial cells
EDHF: endothelium-derived hyperpolarization factor
EDRF: endothelium-derived relaxing factors
EDTA: ethylenediaminetetra acetic acid
eNOS: endothelial nitric oxide synthase
ERK: extracellular receptor kinase
ET-1: endothelin-1
F: factor
FBS: fetal bovine serum
FFAs: free fatty acids
FITC: fluorescein isothiocyanate
FpA: fibrinopeptide A
FpB: fibrinopeptide B
FR: flow rate
G6Pase: glucose-6-phosphatase
GABA: gamma aminobutyric acid
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GK: glucokinase
GLP-1: glucagon-like peptide 1
Glucose-6-P: glucose-6-phosphate
GLUT: glucose transporter
GP: glycoprotein
GP IIb/IIIa: glycoprotein IIb/IIIa
GPO: glycerol-3-phosphate oxidase
GPRP: glycyl-L-prolyl-L-arginyll-L-proline
GSIS: glucose-stimulated insulin secretion
GTP: guanosine triphosphate
H₂O₂: hydrogen peroxide
HA: hyaluronic acid
HDL: high density lipoproteins
HIT: heparin-induced thrombocytopenia
HPLC: high-performance liquid chromatography
HS: heparin sulfate
5-HT: 5-hydroxytryptamine
IAPP: islet amyloid polypeptide
ICAM-1: intracellular adhesion molecule-1
IC₅₀: inhibiting concentration of 50%
ID₅₀: inhibiting dosage of 50%
IDF: the international diabetes federation
IEC: ion exchange chromatography
IGT: impaired glucose tolerance
IKK-β: Iκβ kinase
IL: interleukin
IP₃: inositol triphosphate
IPGTT: intraperitoneal glucose tolerance test
IPITT: intraperitoneal insulin tolerance test
IR: insulin resistance
IRS: insulin receptor substrate
IRS-1: insulin receptor substrate-1
IRS-2: insulin receptor substrate-2
Iκβ: I kappa beta alpha
JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carboxyanine iodide
JNK: c-Jun N-terminal kinase
KOH: potassium hydroxide
LDL: low density lipoprotein
LFD: low fat diet
LTP: long term potentiation
M: marrubiin
M_{RT-qPCR}: gene expression stability measure value
MAPKs: mitogen-activated protein kinases
MCP-1: monocyte chemotactic protein-1
MEK: MAPK ERK kinase
MetS: metabolic syndrome
MIC: minimum inhibitory concentration
MP: metalloproteinase
MMP: mitochondrial membrane potential
Na^{+}/Ca^{2+}: sodium-calcium exchanger
NAD(H): nicotinamide adenine dinucleotide
NADPH: nicotinamide adenine dinucleotide phosphate
NC: negative control
NCEP: national cholesterol education programme
NE: norepinephrine
NEFA: non-esterified fatty acids
NF-κB: nuclear transcription factor κB
NMDLA: N-methyl-D-L-aspartic acid
NMMU: Nelson Mandela Metropolitan University
NO: nitric oxide
NOS: nitric oxide synthase
NSB: non-specific binding
O$_2^-$: superoxide anion
OL: organic extract
ONOO$^-$: peroxynitrite
PAF: platelet activating factor
PAI: plasminogen activator inhibitor
PAR: protease-activated receptors
PC: positive control
PCE: prohormone convertase enzymes
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
PDK 1: phosphoinositide-dependent kinase 1
PDX-1: pancreas duodenum homeobox-1
PE: pulmonary embolism
PEPCK: phosphoenoylpoyruvate carboxykinase
PFP: pentafluorophenyl
PG: prostaglandin
PGC-1: PPAR$\gamma$-co-factor-1
PGD$_2$: prostaglandin D$_2$
PGE$_2$: prostaglandin E$_2$
PGF$_{2a}$: prostaglandin F$_{2a}$
PGG$_2$: prostaglandin G$_2$
PGH$_2$: prostaglandin H$_2$
PGI$_2$: prostaglandin I$_2$
PI3$'$K: phosphoinositide-3$'$-kinase
PIP$_2$: phosphatidyl-inositol-3,4-bisphosphate
PIP$_3$: phosphatidyl-inositol-3,4,5-trisphosphate
PKA: protein kinase A
PKB: protein kinase B
PKC: protein kinase C
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PLA₂</td>
<td>phospholipase A₂</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>p-NPP</td>
<td>para-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>PPP</td>
<td>platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PT</td>
<td>prothrombin time</td>
</tr>
<tr>
<td>PTP1B</td>
<td>phosphotyrosine phosphatase IB</td>
</tr>
<tr>
<td>PTZ</td>
<td>pentylenetetrazole</td>
</tr>
<tr>
<td>Pyr</td>
<td>pyruvate</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated activation of normal T cells expressed and secreted</td>
</tr>
<tr>
<td>Rf</td>
<td>retardation factor</td>
</tr>
<tr>
<td>RhoA</td>
<td>ras homolog gene family, member A</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>Rm</td>
<td>relative mobility/migration</td>
</tr>
<tr>
<td>ROIs</td>
<td>reactive oxygen intermediates</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>reversed phase</td>
</tr>
<tr>
<td>R₉</td>
<td>retention time</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>sialic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sec</td>
<td>seconds</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>serine/threonine</td>
</tr>
<tr>
<td>SOCS</td>
<td>suppressor of cytokine signaling</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotin</td>
</tr>
<tr>
<td>SU</td>
<td>sulfonylureas</td>
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</tbody>
</table>
SUR: sulfonyleurea receptor
T1DM: type 1 diabetes mellitus
T2DM: type 2 diabetes mellitus
TA: total activity
TAFI: thrombin-activatable fibrinolysis inhibitor
TAT: thrombin-antithrombin complex
TBA: tetrabutylammonium phosphate
TBARS: thiobarbituric acid reactive substances
TGF-β: transforming growth factor-β
TF: tissue factor
TFPI: tissue factor pathway inhibitor
TLC: thin layer chromatography
TNF-α: tumor necrosis factor-α
t-PA: tissue plasminogen activator
TX: thromboxane
TXA₂: thromboxane A₂
TXB₂: thromboxane B₂
Tyr: tyrosine
TZDs: thiazolidinediones
UCP-2: uncoupling protein-2
u-PA: urinary plasminogen activator
UTR: untranslated region
V: pairwise variation value
VASP: vasodilator-stimulated phosphoprotein
VCAM-1: vascular cell adhesion molecule-1
VDCC: voltage-dependant Ca²⁺ channel
VEGF: vascular endothelial growth factor
VLDL: very low density lipoprotein
VO₂: oxygen consumption
VSCCs: voltage-sensitive Ca²⁺ channels
VSMCs: vascular smooth muscle cells
VTE: venous thromboembolism
vWF: von Willebrand factor
WHO: World Health Organization
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Abstract
Commercial marrubiin, aqueous and organic extracts of *Leonotis leonurus* were tested *in vitro* for their anticoagulant and antiplatelet activities. The aqueous extract inhibited platelet aggregation by 69.5% (100 µg/mL), while the organic extract (100 µg/mL) and marrubiin (5 µg/mL) showed 92.5% and 91.6% inhibition, respectively, by inhibiting the binding of fibrinogen to glycoprotein IIb/IIIa receptor in a concentration dependent manner. The extracts significantly prolonged activated partial thromboplastin time compared to untreated plasma controls. Fibrin and D-Dimer formation were drastically decreased. The extracts and marrubiin concentration-dependently inhibited calcium mobilization induced by collagen and thrombin. The formation of thromboxane A$_2$ was also significantly reduced by both the extracts and marrubiin. Protein secretion and platelet adhesion were significantly reduced by both the extracts and marrubiin. The organic extract and marrubiin showed a more pronounced effect than the aqueous extracts in all the *in vitro* assays. The *ex-vivo* animal model confirmed the results obtained *in vitro*. Similar to the *in vitro* studies, activated partial thromboplastin time clotting time was prolonged by marrubiin and the number of aggregated platelets were significantly reduced relative to aspirin. The findings reflect that marrubiin largely contributes to the organic extract’s anticoagulant and antiplatelet effect *in vitro*.

INS-1 cells were cultured under normo- and hyperglycaemic conditions. Marrubiin and the two *Leonotis leonurus* extracts were screened for anti-diabetic activity *in vitro*. The stimulatory index of INS-1 cells cultured under hyperglycaemic conditions was significantly increased by 60% and 61% (p<0.01; n=5) in cells exposed to the organic extract (10 µg/mL) and marrubiin (500 ng/mL), respectively, relative to the normoglycaemic conditions. The gene expression of insulin was significantly increased by 76.5 and 71%, and of glucose transporter-2 by 93 and 92.5% for marrubiin and the organic extract, respectively, under the same conditions stipulated above (p<0.01; n=4). The extract and marrubiin similarly showed an increase in respiratory rate under hyperglycaemic conditions. Marrubiin increased insulin secretion, HDL-cholesterol, while it decreased total cholesterol, LDL-cholesterol and the atherogenic index in the *in vivo* rat model.
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- Dr D. Schumann for helping with the analysis of glucose-stimulated insulin secretion studies.
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CHAPTER 1
LITERATURE REVIEW
1. Introduction
Diabetes mellitus (DM) is a metabolic disease which has reached epidemic status (Stunvoll et al., 2005; Bakker et al., 2009). The number of people affected by DM has dramatically increased in developing countries such as Africa, Asia and South America (Stunvoll et al., 2005). In 2001, 171 million people were diagnosed with DM, and this number is estimated to increase by 50% in 2010 and 366 million people are predicted to be diagnosed with DM by 2030 (Stunvoll et al., 2005; Bakker et al., 2009). Type 1 DM (T1DM) is characterized by the loss of insulin production due to β-cell failure, while type 2 DM (T2DM) is characterized by the development of insulin resistance. T2DM accounts for 90% of DM cases, hence, it is the most common form of DM (Bakker et al., 2009).

DM is considered to be a hypercoagulable state because patients with this disease have increased risk of thrombosis due to changes that occur in the coagulation cascade. These alterations are attributed to hyperglycaemia. There is an increased rate in the occurrence of atherosclerosis in DM. Eighty percent of T2DM patients will die because of thrombosis, while 75% of these deaths will be caused by cardiovascular conditions (Carr, 2001). Endothelial dysfunction, inflammation and hypercoagulation play a key role in the development of atherothrombotic events (Ajjan and Ariëns, 2009; Nathanson and Nyström, 2009). Changes in the vascular endothelium such as altered vasodilation, angiogenesis, barrier functions, inflammatory activation, and elevated levels of endothelial products occur as a result of endothelial dysfunction. All of these changes are associated with cardiovascular events (Bakker et al., 2009). It is thought that endothelial dysfunction in T1DM is caused by metabolic alterations that occur due to diabetes, specifically hyperglycaemia. However, in T2DM, endothelial dysfunction is caused by both hyperglycaemia and insulin resistance (IR) (Bakker et al., 2009; Nathanson and Nyström, 2009). Endothelial dysfunction leads to an increase in the permeability of endothelial cells (ECs). This results in the influx of lipid particles and the migration of inflammatory molecules in the vessels. As a result lipid laden macrophages are formed and they are called foam cells. The collection of these cells forms fatty streak deposits.
The formation of the fatty streak deposits are the earliest visible signs of the development of atherosclerosis. The occurrence of these fatty streak deposits leads to inflammatory reactions in vessel walls, elevated production of cytokines, and chemo-attractant proteins (Ferroni et al., 2004). All of these reactions amplify or strengthen the atherosclerosis process. Mature atherosclerosis plaques are formed as the years progress due to chronic inflammation and slow conversion of fatty streaks from fibrous tissue deposits. The coagulation cascade is activated when the plaques are unstable and eventually rupture inducing the formation of a thrombus (Ajjan and Grant, 2006; Ajjan and Ariëns, 2009).

Vessel damage in DM can be caused by the formation of advanced glycation end products (AGE), activation of protein kinase C (PKC), and sorbitol formation through the polyol pathway. The formation of reactive oxygen species (ROS) in the mitochondria is associated with the activation of these pathways. Hyperglycaemia indirectly causes the activation of nuclear transcription factor-κβ (NFκβ) in endothelial and vascular smooth cells via elevated oxidative stress and receptor for advanced glycation end products (RAGE) activation. NFκB regulates the expression of genes encoding for leukocyte-cell adhesion molecules and chemoattractant proteins, and other pro-inflammatory mediators such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α). Therefore, NFκB encodes for mediators of atherogenesis. Activation of the NFκB pathway leads to endothelial dysfunction and changes in platelet function. These changes in intraplatelet signalling contribute to the development of atherothrombotic complications in DM (figure 1.1) (Ferroni et al., 2004).

The changes that occur in the metabolic state as a result of DM lead to the development of atherosclerosis. Hyperglycaemia alters the functional properties of a number of cells, including the endothelium and platelets. The proteins which are involved in the coagulation cascade are glycated, leading to a hypercoagulation state in DM patients and an imbalance in the fibrinolytic system. The sections below will discuss these parameters in more detail. The drugs which are used to control blood sugar levels and prevent thromboembolism have certain limitations, therefore, a need exists for the development of new drugs from a natural source which would combat these limitations. Hence this
study focussed on the antidiabetic, antiplatelet and anticoagulation properties of *Leonotis leonurus*.

![Diabetes Mellitus diagram](image)

Figure 1.1: Metabolic dysfunction occurring in DM is possible, through increased oxidative stress, PKC and RAGE activation, of inducing activation of ECs and platelets, which causes a switch toward a prothrombotic, pro-inflammatory condition and contributes to the pathogenesis of atherosclerosis. NO, nitric oxide; NFκβ, AP-1, activator protein-1; PGI2, prostaglandin I2; PAI-1, plasminogen activator inhibitor-1 (Ferroni *et al.*, 2004).

1.1 Normal endothelial functions
The endothelium of various vessels has different specialized functions but the endothelium has general functions which are important for proper functioning of an organism. The function of the endothelium can change to adapt to sensitization by various stimuli, including mechanical, oxidative and metabolic stress, inflammation, hypoxia, and other stresses (Bakker *et al.*, 2009; Hinsbergh, 2001).
1.1.1 General functions of the endothelium cells
The ECs are made up of a single cell layer. They line the entire circulatory system and act as a barrier system by regulating the passing of nutrients, hormones, and macromolecules into the surrounding tissue (Nathason and Nyström, 2009; Bakker et al., 2009). The glycocalyx which is the membrane macromolecules produced by ECs; covers the endothelium and contributes to its active barrier function (Bakker et al., 2009; Kumagai and Kassab, 2009). The glycocalyx is made up of hyaluronic acid (HA) glycosaminoglycans, heparin sulfate (HS) proteoglycans, sialic acid (SA) glycosaminoglycans, thrombomodulin, and others. HA, HS, and SA have been found to be involved in nitric oxide (NO) production by the endothelium when it is exposed to wall shear stress (Kumagai and Kassab, 2009).

Acetylcholine (ACh), bradykinin, serotonin, and prostaglandin (PGI₂) are known agonists of NO release. The interaction between blood and the ECs enables the endothelium to adapt to shear stress through sensing of mechanical forces. This is called chronic adaptation. NO, PGI₂, and endothelium derived hyperpolarisation factor (EDHF) are the main endothelium-derived relaxing factors (EDRF). The vasoregulation function is attained by the production of NO, EDHF and prostaglandins (Nathason and Nyström, 2009; Grover-Pâez and Zavalza-Gómez, 2009; Bakker et al., 2009; Delgado et al., 1999). The endothelium is also involved in the production of various vasoactive agents in the lung (Bakker et al., 2009).

The endothelium also plays a role in homeostasis by maintaining the fluidity of blood. It does this function by limiting blood coagulation, platelet activity and the production of fibrinolysis regulators. The secretion of NO limits blood coagulation and platelet activity by inhibiting the expression of pro-inflammatory cytokines, leukocyte adhesion molecules, and chemokines. EC also releases anticoagulant factors such as tissue plasminogen activator (tPA), thrombomodulin, tissue factor pathway inhibitor (TFPI), protein C and S. However, ECs play a role in blood coagulation by releasing a number of prothrombotic factors such as von Willebrand factor (vWF), factor (F) V, PAI-1 and tissue factor (TF). The release of NO also inhibits the proliferation of vascular smooth
muscle cells (VSMCs), an early sign of angiogenesis (Nathason and Nyström, 2009; Bakker et al., 2009; Ajjan and Ariëns, 2009; Ajjan and Grant, 2006; Balakumar et al., 2009).

The most potent vasoconstrictor, endothelin-1 (ET-1), is produced by the endothelium. Insulin acts as a vasoregulator by inducing the production of NO and ET-1. The endothelium plays an important role in inflammation by recruiting the leukocytes in the site of injury. Local haemorrhage or insufficient blood supply could occur if angiogenesis is impaired (Bakker et al., 2009; Nathason and Nyström, 2009; Grover-Páez and Zavalza-Gómez, 2009).

1.1.2 Endothelial function in glucose metabolism and insulin action
Endothelium is involved in metabolism and protein synthesis. The ECs use fatty acids and glucose as a fuel source. In the ECs, glucose transporter-1 (Glut-1) transports glucose into the cell. Small molecules such as glucose can pass through the inter-endothelial junctions but the endothelium of brain microvessels have only Glut-1 mediated translocation. Proteins are transported via a shuttling of caveolar vesicles between the luminal and the abluminal side. Insulin induces the pathway that involves insulin receptor substrate (IRS)-1, phosphoinositide-3'-kinase (PI3’K), Akt/PKB endothelial NO synthase (NOS or eNOS). Insulin also leads to the release of ET-1 via the mitogen-activated kinase (MAPK) ERK kinase (MEK) extracellular signal-regulated kinases receptor kinase (ERK)-1/2 and endothelin converting enzyme. Phosphorylation of IRS-1 and IRS-2 is required for the production of NO and ET-1 (figure 1.2). NO production and the release of ET-1 can be impaired by a deficiency in IRS-1 and IRS-2; respectively (Bakker et al., 2009; Barron, 2002).
1.1.3 Endothelial dysfunction in diabetes
The damage of the endothelium leads to the development of atherosclerosis and vascular complications in diabetes. Endothelial dysfunction has been associated with a number of risk factors such as, coronary artery disease (CAD), hypertension, hypercholesterolemia, DM, IR, obesity and systemic inflammatory disease (Bakker et al., 2009; Nathason and Nyström, 2009; Ajjan and Ariëns, 2009; Ajjan and Grant, 2006; Ferroni et al., 2004, Kobayashi and Kamata, 2001). In an ideal situation there is a balance in the secretion of NO, anticoagulant factors, and pro-coagulant factors, refer to section 1.1.1. However, endothelial damage or dysfunction creates an imbalance in these factors, leading to the progression of atherosclerosis (Nathason and Nyström, 2009; Ajjan and Ariëns, 2009; Ajjan and Grant, 2009; Adams et al., 2008; Ferroni et al., 2004). As it has been mentioned earlier, NO, EDHF, ET-1 and PGI₂ are potent vasoconstrictors. ET-1 is the main vasoconstrictor that alters the vascular action of other vasoactive factors such as angiotensin-II (AT-II), thromboxane A₂ (TXA₂), prostaglandin H₂ (PGH₂), and norepinephrine (NE). Therefore the chain of reactions that occur leading to the progression of atherosclerosis from endothelial dysfunction is caused by the imbalance
between these mediators (figure 1.3) (Nathason and Nyström 2009; Grover-Pàez and Zavalza-Gómez, 2009; Adams et al., 2008).

Figure 1.3: Imbalance in vasoregulating factors induces endothelial dysfunction. The term endothelial dysfunction refers to an imbalance in the production of vasodilators, e.g., NO, EDHF, PGI₂, and vasoconstrictors, e.g., ET-1, AT-II and PGH₂. This imbalance may affect endothelial homeostasis and predisposing the endothelium towards a pro-thrombotic and pro-atherogenic milieu. Endothelial dysfunction is a key factor in all stages of atherosclerosis development (Nathason and Nyström, 2009).

1.1.3.1 NO availability and dysfunctional vasoregulation
NO is the key EDRF that plays a primary role in the maintenance of vascular tone and reactivity (Grover-Pàez and Zavalza-Gómez, 2009). The bioavailability of NO is determined by the balance between NOS activity and the reduction of NO through quenching by reactive oxygen intermediates (ROIs). NOS activity may be inhibited by substrate (L-arginine) availability. In hyperglycaemic conditions, NOS activity is hindered and ROS levels are elevated (Bakker et al., 2009; Ajjan and Grant, 2006; Ferroni et al., 2004). NOS can become uncoupled in the presence of sub-optimal concentrations of L-arginine or co-factors such as tetrahydrobiopterin (BH₄). This leads to oxidative stress because of ROS generated. Normally, ROS is cleared by multiple intra- and extracellular mechanisms. These mechanisms are disabled in high levels of ROS. This leads to the formation of peroxynitrite which is a product formed when the
superoxide radical reacts with NO. Peroxinitrile intensifies reduction in NO production by bringing itself to NOS uncoupling (figure 1.4) (Bakker et al., 2009; Nathason and Nyström, 2009; Grover-Pàez and Zavalza-Gómez, 2009). ROI inhibits NO production by inhibiting dimethylaminohydrolase (DDAH). The inhibition of DDAH by oxidative stress results in plasma accumulation of asymmetric dimethylarginine (ADMA). ADMA is a NOS inhibitor and an increase in its concentration is a sign of cardiovascular events and diabetic kidney disease in T1DM and T2DM. Superoxide generation is also elevated by arginase which is an enzyme that converts L-arginine to urea and ornithine. Increased activity of arginase leads to a reduction in bioavailability of NO. Vascular dysregulation in diabetes has been linked to elevated activity of arginase. It has been postulated that hyperactivity of arginase activity in diabetes is caused by activation of the Ras homolog gene family, member A (RhoA), by elevated glucose levels. Insulin contains the expression of arginase, however, insulin signalling is disturbed in DM. ET-1 and EDHF may be altered in DM (Bakker et al., 2009). Therefore, downregulation of NO production leads to impairment of vessel dilation, elevating shear stress on EC, thereby playing a role in the development of endothelial dysfunction (Ajjan and Grant, 2006).

![Coupled and uncoupled endothelial nitric oxide synthase](image)

**Figure 1.4:** Coupled and uncoupled endothelial nitric oxide synthase. In the presence of sub-optimal concentrations of L-arginine or BH₄, substrate and cofactor, respectively, for the synthesis of NO, eNOS may become uncoupled. These results in the production of reactive oxygen species, *e.g.*, superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻), decrease the bioavailability of NO which may ultimately lead to endothelial dysfunction (Nathason and Nyström, 2009).
1.1.3.2 Leukocyte adhesion and inflammation
ROS generation activates transcription factors NFκβ and activator protein (AP-1). Activation of these transcription factors leads to the expression of atherogenic molecules with pro-inflammatory properties. These include IL-β, IL-6, IL-1, C-reactive protein (CRP) and TNF-α. TNF-α has been associated with IR and DM, and its levels can decrease by dietary restrictions and weight loss. The levels of IL-6 and CRP are elevated in T2DM. Endothelial dysfunction leads to the expression of adhesion molecules (vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and E-selectin) that bind to leukocytes. The leukocytes subsequently migrate into the subendothelial space with the help of chemoattractant cytokines. The enhancement of the inflammatory response leads to the formation of foam cells from lipids and proliferation of VSMCs leading to the development of thrombosis. CD40 ligand (CD40L) is a transmembrane protein produced by inflammatory cells, VSMCs, ECs and platelets. The interaction of CD40L with its receptor CD40 results in the expression and production of cytokines (Bakker et al., 2009; Ajjan and Grant, 2006; Ferroni et al., 2004).

1.1.3.3 Decreased thromboresistance
The pro-inflammatory cytokines that are activated in endothelial dysfunction alter the coagulation cascade and fibrinolytic factors. These cytokines lead to the expression of TF in ECs. NO is an antiplatelet agent, but its production is inhibited by endothelial dysfunction (section 1.1.3.1). These factors create a prothrombotic environment. In addition to the above mentioned factors, CRP, elevates the levels of fibrinogen while suppressing fibrinolysis through PAI-1. The expression of anticoagulant protein C is also suppressed by thrombomodulin that is produced by ECs. Fibrin D-Dimer represents intravascular fibrin. Activation of coagulation and fibrinolysis leads to elevated levels of D-Dimer. Elevated levels of D-Dimer correlate with CRP and IL-6 levels and are markers of coronary artery risk. The levels of vWF are also found to be increased in inflammation and can lead to the development of cardiovascular complications and DM. Therefore inflammation can lead to coagulation activation, platelet activation, adhesion, platelet aggregation and changes in platelet signalling. Platelets secret inflammatory chemokines
such as platelet factor-4 and are regulated upon activation of normal T cells expressed and secreted (RANTES), and they also secrete inflammatory cytokines such as IL-1β and CD40L. These inflammatory agents are released subsequent to platelet activation leading to an inflammatory response and atherogenesis. The interaction of P-selectin (a glycoprotein found on the surface of platelets) with platelet activating factor (PAF) and/or RANTES can stimulate the production of IL-8, monocyte chemotactic protein-1 (MCP-1), TNF-α and TF by macrophages. The combination of activity of these inflammatory agents leads to the production of thrombin which results in coagulation and platelet activity (Bakker et al., 2009; Ajjan and Ariëns, 2009; Adams et al., 2008; Ajjan and Grant, 2006; Ferroni et al., 2004; Constans et al., 2000).

1.1.3.4. Structural changes in endothelial extracellular matrix and barrier dysfunction
ECs are polar and made up of extracellular matrix (ECM), a glycocalyx at its luminal side and the abluminal side has a basement membrane. The constituents of the basement membrane are changed in DM and the membrane is thickened. These changes are caused by an increase in activity of transforming growth factor-β (TGF-β), resulting in loss of counter regulation of matrix proteins to maintain the endothelial structure due to the defective matrix. The HS proteoglycans are reduced in DM while the chondroitin sulfate proteoglycans and dermatan sulfate proteoglycans are increased. The reduction in the HS proteoglycans leads to loss of glycocalyx. This in turn leads to a number of vascular abnormalities including changes in NO bioavailability, adhesion of mononuclear cells and platelets to the endothelial surface, and loss of macromolecules through the endothelium (Berg et al., 2006). In studies conducted by Yuan et al. (2007) and Simionescu (2007), hyperglycaemia has been shown to be the cause of vascular injury in both in vitro and in vivo models. In hyperglycaemia, modification of matrix proteins by glyco-oxidation and formation of AGEs cause changes in the synthesis of matrix proteins (Bakker et al., 2009).

1.1.3.5 Microalbuminuria
Microalbuminuria is a condition involving the excretion of albumin in urine, amounting to 30-300 mg/dL in both females and males. It is used as an indicator of kidney damage.
and atherosclerosis in DM. Microalbuminuria is also an indicator of vascular leakage resulting from endothelial dysfunction preceding change in insulin sensitivity. Stehouwer et al. (2002) observed that in T2DM patients, microalbuminuria, endothelial dysfunction, and low grade inflammation are tightly associated, and these are independently associated with cardiovascular death. However, in contrast, Stehouwer et al. (2004) observed that in elderly people with or without DM, microalbuminuria is directly associated with impaired endothelium-dependent-flow-mediated vasodilation. These observations support the hypothesis that reduced levels of NO synthesis play a role in microalbuminuria associated with cardiovascular disease risk. The hydrostatic pressure and glomerular leakage are believed to contribute in microalbuminuria. The changes in proteoglycans of the glomerulus, along with the availability of vascular endothelial growth factor (VEGF), TGF-β and hyperglycaemia, alter the permeability/selectivity of the glomerulus (Bakker et al., 2009; Solbu et al., 2009a; Solbu et al., 2009b).

1.1.3.6 Altered angiogenesis and tissue repair
In hyperglycaemic conditions and DM, the regeneration function of ECs by angiogenesis is lost. As a result, wound healing is poor; myocardial infarction and high risk of rejection of transplanted organs is evident in DM patients. Diabetic retinopathy is also observed due to the poor blood supply. An unstable vessel grows in the vitreous fluid over the retina, which causes bleeding into the eye due to the increase in risk of vascular leakage. VEGF levels are found to be elevated in the eye fluid in diabetic retinopathy patients. Angiotensin converting enzyme (ACE) inhibitors cause a decrease in VEGF levels, suggesting that AT-II contributes to altered angiogenesis. The production of ROS, and accumulation of glycolysis intermediates due to hyperglycaemia and the AGE formation contributes to vessel damage in diabetic retinopathy. Andreoli and Miller (2007) have shown that anti-VEGF can alleviate vessel damage in diabetic retinopathy (Bakker et al., 2009; Wirostko et al., 2008).
1.1.3.7 Hyperglycaemia-induced endothelial dysfunction in diabetes
Endothelial dysfunction is very common in T2DM patients and in people who are IR. There is evidence that endothelial dysfunction occurs before DM as vascular damage has been observed in non-DM people and those who are predisposed to DM. There are a number of independent factors that have been associated with endothelial dysfunction in T2DM, and these include hypertension, dyslipidemia, obesity, microalbuminuria, low grade inflammation, hyperglycaemia and IR on the endothelium (Nathanson and Nyström, 2009; Bakker et al. 2009; Adams et al., 2008; Kobayashi and Kamata 2001).

The activity of NOS in ECs that have been experimentally exposed to diabetic conditions both in vivo and in vitro has been found to be attenuated (Cosentino and Luscher, 1998; Huvers et al., 1999; Huszka et al., 1997; Lambert et al., 1996). In T1DM and T2DM, endothelial dysfunction is closely associated with atherosclerosis and microangiopathy. T1DM patients suffer from either early or late microalbuminuria nephropathy. As it has been discussed earlier, DM patients with endothelial dysfunction have poor EC-dependent vasodilation, elevated blood levels of vWF, thrombomodulin, selectin, PAI-1 and type IV collagen. When EC dysfunction is fixed, it stimulates changes in vessels that would worsen the vasculopathy and result in DM progress. A decrease in the levels of nicotinamide adenine dinucleotide phosphate (NADPH) pools occurs as a result of hyperglycaemia. Oxidative stress also occurs in DM. Oxidation of proteins and phospholipids occur as a result of hyperglycaemia and elevated levels of fatty acids (Grover-Pàez and Zavalza-Gómez, 2009).

Hyperglycaemia-induced endothelial dysfunction is through the activation of PKC, polylol pathway, oxidative stress, and non-enzymatic glycation (Schneider et al., 2002; Quehenberger et al., 2000; O’Brien et al., 1997). Insulin and glucose play an important role in the secretion of ET-1. The synthesis of ET-1 by ECs is induced by insulin, while hyperglycaemia leads to the formation of AGEs and the synthesis of ET-1 through the activation of NFκβ. AGEs induce vascular inflammation and endothelial dysfunction. The interaction between AGE and its receptor, RAGE, leads to the alteration of the production of pro-inflammatory cytokines and other inflammatory pathways in ECs.
Beyond hyperglycaemia, oxidative stress is induced by a diabetic state (Grover-Pâez and Zavalza-Gómez, 2009; Bakker et al., 2009).

1.2 Normal actions of insulin
Under physiological conditions, glucose is the fuel source for the brain. However, glucose and free fatty acids can be used as a fuel source by other organs (Jellinger and Mace, 2007). Throughout the whole body, glucose homeostasis is regulated by an endocrine hormone called insulin (Meshkani and Adeli, 2009). The glucose levels are controlled within a strict range, normally between 60 and 150 mg/dL (3.3 and 8.3 mmol/L). GLUT proteins are needed to transport the hydrophilic glucose across the membrane. There are five GLUT proteins, of these GLUT-4 is found on the cell membranes of muscle cells and adipocytes. Translocation of GLUT-4 from vesicles in the cytosol occurs when insulin is present. This facilitates the entry of glucose into the cell (Jellinger and Mace, 2007). GLUT-2 facilitates the entry of glucose in the pancreatic β-cells (Stunvoll et al., 2005).

Insulin is a hormone with multiple effects on metabolism and various cellular processes in different tissues and organs. It plays a key role in maintaining a balance in glucose utilization and production. The main function of insulin is to lower blood glucose levels by increasing the transport of glucose into the cell. It promotes glycogenesis, once glucose is in the cell, it is converted to glycogen. It decreases glycogenolysis and gluconeogenesis, while it increase lipogenesis and stimulates protein synthesis (Tortora and Grabowski, 2006). Insulin is also involved in anti-atherogenic processes, by promoting the release of the vasodilating agent, NO. It inhibits platelet aggregation by inhibiting the action of PAI-1. It promotes vascular cell growth and production of matrix proteins (Jellinger and Mace, 2007).

1.2.1 Inducible mechanism of insulin secretion
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 Gavrilo, 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, which 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 (Meshkani and Adeli, 2009; 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 PKC. Akt (also known as PKB) is a serine/threonine (Ser/Thr) kinase. The Akt pathway mediates the effects of insulin on glycogen and protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis. The Akt pathway regulates both glucose uptake via 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 1.5 (Meshkani and Adeli, 2009; LeRoith and Gavrilova, 2005; Schinner et al., 2005).

Figure 1.5: Signalling pathways of the insulin receptor. After autophosphorylation of insulin receptor, Tyr (tyrosine) phosphorylation in 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 MAPK pathway that results in cell proliferation and differentiation (LeRoith and Gavrilova, 2005).
1.2.2 Biphasic insulin secretion
There are two phases of insulin secretion. The first phase also known as the triggering phase starts within seconds of an increase in levels of a secretagogue that makes contact with the β-cells. In this phase, an increase in cellular calcium causes a sharp peak of insulin release. The second phase of insulin release or the amplification phase is longer and requires ATP and calcium (Henquin et al., 2003). In the development of T2DM, the first phase is reduced (Yki-Järvinen, 1992) and this may be predictive of this disease (Bouchard, 1995). Cross-sectional studies of individuals with varying degrees of glucose tolerance showed that first-phase insulin was reduced in individuals with high plasma glucose levels and in people with fasting hyperglycaemia (Brunzell et al., 1976, Kahn et al., 1969). These results were further supported by a study which showed that people with impaired glucose tolerance (IGT), a precursor of T2DM, had a reduced plasma insulin level at 30 minutes after the ingestion of glucose, whereas the plasma insulin levels were normal at 120 minutes (Gerich, 1997). The first 30 minutes reflect the first phase, while the 120 minutes reflect the second phase of insulin release. It has been accepted that, the reduction in the insulin release in the first-phase is responsible for the development of IGT (LeRoith and Gavrilova, 2005).

1.2.3 Regulation of insulin secretion
Nutrients, neurotransmitter, and hormones control insulin secretion. There is more information available concerning the mechanisms underlying the first phase of glucose-stimulated insulin secretion (GSIS) than there is for the second phase. The ATP-sensitive K\(^+\) channel is largely responsible for glucose-induced depolarization of the β-cell. The most critical stimulator of insulin release is glucose (Choi et al., 2007). A variety of modulatory pathways control insulin secretion from the pancreatic β-cell (Lee et al., 2003; Kindmark et al., 1995). The mechanism by which insulin secretion is mediated involves the closure of ATP-regulated K\(^+\) channels, depolarization of β-cells, followed by an opening of voltage-sensitive Ca\(^{2+}\) channels (VSCCs), causing an increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) (Choi et al., 2007; Choi et al., 2006; Lee et al., 2003; Kindmark et al., 1995). Two ion channel families are involved in the release of Ca\(^{2+}\) from intracellular calcium stores, inositol triphosphate (IP\(_3\)) receptors and ryanodine receptors. Plasma membrane Ca\(^{2+}\) ATPase or sarco-endoplasmic Ca\(^{2+}\) ATPase maintain
low levels of $[\text{Ca}^{2+}]_i$ by extrusion of $\text{Ca}^{2+}$ or by pumping $\text{Ca}^{2+}$ into the lumen of the calcium stores, respectively. Active $\text{Ca}^{2+}$ transport is also provided by sodium-calcium (Na$^+$/Ca$^{2+}$) exchanger (Choi et al., 2006).

Although an increase in $[\text{Ca}^{2+}]_i$ is necessary and sufficient to trigger insulin release, its elevation alone is not very effective. Several metabolites act as amplifying signals by mediating the potentiation of insulin secretion at suitable $[\text{Ca}^{2+}]_i$ levels. These metabolites include adenosine triphosphate (ATP), guanosine triphosphate (GTP), cyclic adenosine monophosphate (cAMP), NADPH, and malonyl-CoA. Several protein kinases also act as amplifying signals, these include calcium/calmodulin kinase II (CaMK II), protein kinase A (PKA) and PKC (Choi et al., 2007).

The $\text{K}_{\text{ATP}}$ channel-independent amplification pathway is the second glucose signalling pathway in $\beta$-cells (Best et al., 1992; Gembal et al., 1992). Glucose metabolism causes the closure of $\text{K}_{\text{ATP}}$ channel, thus, triggering the exocytosis of insulin by increased $[\text{Ca}^{2+}]_i$, and the $\text{K}_{\text{ATP}}$ channel-independent amplification pathway augments that response (Aizawa et al., 1998; Aizawa et al., 1994; Best et al., 1992; Gembal et al., 1992). The mechanisms underlying this pathway are not known (Henquin, 2000; Straub and Sharp, 2002; Liu et al., 2003). The second $\text{K}_{\text{ATP}}$ channel-independent amplification pathway does not have any increase in $[\text{Ca}^{2+}]_i$. This occurs in the presence of agonists that increase intracellular diacylglycerol (DAG) levels and an increase in cAMP levels amplifies this response (Liu et al., 2003). The only difference between these two pathways is the requirement for GTP. The reducing levels of GTP in islets blocks the augmentation of the $\text{Ca}^{2+}$-independent pathway, while it has no effect on the $\text{Ca}^{2+}$-dependent pathway (Komatsu et al., 1998). Figure 1.6 illustrates the glucose signalling pathway and insulin secretion in $\beta$-cells.
Figure 1.6: A schematic representation of coupling glucose metabolism to insulin secretion in pancreatic β-cells. GLUT, glucose transporter; GK, glucokinase; glucose-6-P, glucose-6-phosphate; Pyr, pyruvate; VDCC, voltage-dependent Ca\(^{2+}\) channel (Fujimoto et al., 2007).

ATP is not the only energy metabolite which plays a role in insulin secretion, NADPH also plays a role. The mitochondrial nicotinamide adenine dinucleotide (NAD(H)) shuttle activity is very high in β-cells, indicating a role played by NADPH in insulin secretion (Brown et al., 2002). Rats that were given 6-aminonicotinamide (6-AN), which forms a metabolically inactive analog of NADPH in vivo, caused hyperglycaemia in these rats (Ammon and Steinke, 1972). Rat islets that were treated with 6-AN, were isolated and showed glucose-induced and amino acid-induced inhibition of insulin release (MacDonald et al., 1974). Watkins and Moore (1977) showed that insulin release was stimulated in toadfish islets that had taken up NADPH. NADPH is a substrate for stearoyl-CoA desaturases, which may increase unsaturated fatty acids in membranes and facilitate insulin granule fusion with the plasma membrane (Flamez et al., 2002). Ramanadham et al., (2002) identified products of these desaturases in INS-1 cells and rat pancreatic islets.

1.3 Hyperglycaemia in relation to insulin resistance
Hyperglycaemia is defined as a condition that is responsible for a reduction in responsiveness to glucose stimulation in β-cells, resulting in impairment in response of
these cells and changes in insulin secretion (Giaccari et al., 2009). The balance between insulin action and secretion regulates glycaemic levels. In normal conditions, the β-cells are able to compensate for the high glucose levels by increasing insulin secretion. However, some cells like the capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, neurons, and Schwann cells in the peripheral nerves, lose the ability to quickly decrease the rates of glucose transport. This leads to visual and renal impairment and neuropathy, respectively (Jellinger and Mace, 2007).

Hyperglycaemia induces endothelial dysfunction and stimulates inflammatory cytokines. The release of cell adhesion molecules due to hyperglycaemia inhibits leukocyte function. Macrovascular damage is further enhanced by the production of ROS. Therefore, hyperglycaemia might play a role in the development and progression of atherosclerosis (Nathanson and Nyström, 2009).

1.4 Insulin resistance
IR is a defect that occurs when normal insulin levels are insufficient to regulate glucose absorption. During the initial stages of IR, the pancreatic β-cells are able to compensate for the decrease in insulin sensitivity by increasing its biosynthesis and secretion. However, β-cells are unable to meet the metabolic load, and eventually they fail to function. Glucose intolerance and T2DM develops after IR. Changes in the metabolic state can induce IR. Hyperglycaemia, hyperlipidemia, hypertension, and low-grade inflammation can lead to the development of IR (figure 1.7). IR is further aggravated by a lack of regulation of carbohydrate and lipid metabolism caused by obesity, dyslipidemia, and hypertension (Nathanson and Nyström, 2009; Jellinger and Mace, 2007; Meshkani and Adeli, 2009; Stunvoll et al., 2005).
Figure 1.7: Pathophysiology of T2DM due to hyperglycaemia and increased circulating fatty acids. Insulin secretion from the pancreas normally reduces glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid release from fat tissue. The various factors shown contribute to the pathogenesis of T2DM by affecting both insulin secretion and action. Decreased insulin secretion will reduce insulin signalling in its target tissues. Insulin resistance pathways affect the action of insulin in each of the major target tissues, leading to increased circulating fatty acids and the hyperglycaemia of DM. In turn, the raised concentrations of glucose and fatty acids in the bloodstream will feed back to worsen both insulin secretion and IR (Stunvoll et al., 2005).

Lavau et al. (1979) reported that rats on a high fat diet (HFD) showed a decrease in the ability of insulin to stimulate glucose metabolism when compared to rats on a low fat diet (LFD). From the study, it was concluded that IR in adipose tissue of rats on a HFD is caused by the decrease in intracellular capacity to use glucose for lipogenesis. Many studies have proved that normal rats fed HFD ad libitum develop obesity (Kim and Park, 2008, Flanagan et al., 2008; Ramadan et al., 2006). The development of obesity was accompanied by an increase in energy intake, body fat content, and IR. Flanagan et al. (2008) had shown that TNF-α is a key mediator of IR in HFD-induced obesity in Wistar rats. This cytokine was highly expressed in the liver and decreased in adipose tissue. In
another study conducted by Borst and Conover (2005), TNF-α was highly expressed in skeletal muscle and adipose tissue although total calorie intake was not increased. In this study, although there was no weight gain for the rats that were on HFD, these rats were glucose intolerant and insulin insensitive. Wistar rats with a HFD had been shown to have an increase in body fat content, impaired glucose tolerance and develop IR (Jang et al., 2009; Flanagan et al. 2008; Borst and Conover 2005). The cellular cause of the increase in TNF-α gene expression in obesity and IR is unknown (Weisberg et al., 2003).

1.4.1 Molecular mechanism of insulin resistance
The molecular mechanisms that lead to IR are poorly understood. Several mechanisms have been proposed, including abnormal insulin production, mutations in insulin receptor and its substrates, and insulin antagonists. Evidence from human and animal studies suggests that, defects in post-receptor or insulin signalling in target tissues lead to IR (Meshkani and Adeli, 2009). Knockouts of insulin receptors have been created to understand the mechanism of the development of IR in a particular tissue or organ. An experiment was completed where insulin receptor knockouts of muscle, liver, fat tissue, neurons and pancreatic β-cells were created. From this experiment, only the liver and the pancreatic β-cells became glucose intolerant. From these findings, the authors concluded that hepatic IR plays a role in the pathogenesis of T2DM (Stunvoll et al., 2005).

T2DM patients have a reduction in autoactivation of the insulin receptors in muscle and adipose tissue; while the skeletal muscle of lean and obese patients have a reduction in the expression of PI3’-K and PKB. In the IR state, the IRS proteins are dephosphorylated by protein-tyrosine phosphatases. Phosphotyrosine phosphatase IB (PTP1B) plays a key role in the negative regulation of insulin signalling (Meshkani and Adeli, 2009; Stunvoll et al., 2005). PTP1B-deficient mice that were fed a HFD remained insulin sensitive and did not gain weight (Elchebly et al., 1999). High expression of PTP1B is observed in the liver and muscle tissue of obese, IR, and T2DM patients (Ahmad et al., 1997). The serine/threonine phosphorylation of IRS-1 inhibits the downstream signalling process. Several IRS serine kinases have been identified including the stress activated protein kinase, c-Jun N-terminal kinases (JNK) and PKC. The members of the suppressor of
cytokine signalling (SOCS) family block insulin signalling by competing with IRS-1 and protein degradation of IRS through the ubiquitin-proteosomal pathway (Meshkani and Adeli, 2009; Stunvoll et al., 2005). The SOCS proteins that alter insulin signalling are SOCS-1, SOCS-3, and SOCS-6. They block insulin signalling by interfering with IRS-1 and IRS-2 tyrosine phosphorylation and proteosomal degradation of IRS proteins (Meshkani and Adeli, 2009).

1.5 Obesity
Various factors including the genetic determinants, lifestyle, ageing, environmental and nutritional factors characterize IR. A lack of physical activity and obesity has been strongly associated with IR. IR impairs the normal release of FFAs from adipose tissue leading to the development of dyslipidemia involving elevated levels of triglycerides, low serum levels of HDL cholesterol and high levels of circulating FFAs. The accumulation of FFAs leads to the overproduction of very-low-density lipoprotein (VLDL) in IR and T2DM patients. The function of insulin is regulated by circulating hormones, cytokines and non-esterified free fatty acids (NEFA) that are produced in the adipose tissue. Insulin is an anti-lipolytic hormone. In obesity, due to the elevated adipose tissue deposits, adipose tissue becomes resistant to insulin. As a result lipolysis is impaired. This leads to an increase in the concentration of NEFA and glycerol, thus worsening IR in skeletal muscle and liver. Excess FFAs promote oxidative stress by leading to the formation of ROS and promoting endothelial dysfunction. IR and T2DM are associated with elevated levels of TNF-α, IL-6, PAI-1, ET-1, and CRP. TNF-α is over-expressed in adipose tissue and augments insulin signalling in the liver. It does this by activating JNK-1 and SOCS, hence inhibiting this signalling pathway. TNF-α activates lipase thereby increasing FFAs levels. FFAs lead to an increase in lipogenesis in hepatic tissue, leading to IR. Similar to TNF-α, IL-6 impairs insulin signalling by inducing serine phosphorylation of IRS-1 and activating SOCS proteins (Meshkani and Adeli, 2009; Lorenzo et al., 2008; Stunvoll et al., 2005; Pirola et al., 2004; Senn et al., 2003).

Insulin sensitivity in the liver and muscle tissue is reduced because of the decrease in the concentration of adiponectin in visceral obesity. Adiponectin exerts its function as an
insulin-sensitizing adipokine by inhibiting liver gluconeogenesis and promoting fatty acid oxidation in skeletal muscle. Adiponectin activates AMP kinase, which then leads to the decrease in the expression of enzymes involved in gluconeogenesis in the liver. Resistin is another adipocyte-specific protein which is involved in the pathogenesis of obesity-associated IR and T2DM. The function of resistin is to increase hepatic glucose concentration and fasting glucose levels. In visceral obesity, resistin induces the expression of SOCS-3 (Meshkani and Adeli, 2009; Guerre-Millo, 2008; Stunvoll et al., 2005).

As it has been stated earlier, there is a close association between IR and inflammatory signalling pathways. In a resting state NFκβ binds to its inhibitor I kappa beta alpha (Iκβ). However, phosphorylation of Iκβ by Iκβ kinase (IKK-β) leads to free NFκB that can mediate IR induced by inflammatory factors released by adipose tissue. PKC which is activated by fatty acid metabolites increase serine phosphorylation of IRS-1, leading to its degradation by SOCS proteins and inflammatory response (Meshkani and Adeli, 2009; Stunvoll et al., 2005).

Mitochondrial metabolism is dysregulated in T2DM. This is evident by the increase in triglycerides levels due to the defect in lipid oxidation that occurs in the mitochondria. The levels of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) co-activator-1 (PGC-1) are decreased in young, lean, IR children who have parents with T2DM. PGC-1 takes part in mitochondrial fatty acid oxidation and ATP synthesis. Therefore, lipid accumulation could be caused by the defective PGC-1 (Stunvoll et al., 2005).

1.6 Glucose toxicity
The gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are inhibited by insulin to stop glucose production. On the other hand, glucagon can increase glucose production in fasting hyperglycaemia in T2DM. The lack of inhibition of glucagon activity in a hyperglycaemic state in T2DM leads to an overproduction of glucose through gluconeogenesis and glycogenolysis (Meshkani and
Glucose toxicity refers to the chronic exposure of cells to high levels of glucose (hyperglycaemia) leading to cellular dysfunction that is irreversible over time. In β-cells ROS are generated from glucose metabolism. The ROS are normally detoxified by catalase, superoxide dismutase and a redox-regulating enzyme called glutathione peroxidase. Hyperglycaemia does not only inhibit the action of these enzymes but it also inhibits pancreas duodenum homeobox-1 (PDX-1), which is an important regulator of insulin promoter activity. The hindrance in PDX-1 activity leads to β-cell dysfunction and the generation of ROS, which stimulates NFκβ activity that causes β-cell apoptosis (Stunvoll et al., 2005).

1.7 Lipotoxicity
Insulin is involved in lipid metabolism by promoting lipid synthesis and inhibiting lipase activity. By inhibiting lipolysis, insulin reduces the circulatory levels of FFAs and VLDL production. In the IR state, lipid and lipoprotein metabolism is altered (Meshkani and Adeli et al., 2009). IR in non-diabetic and diabetic obese patients leads to elevated levels of NEFA because of the stimulation of adipose tissue lipolysis. In the presence of glucose, the levels of long-chain acyl co-enzyme A (Co-A) increase because fatty acid oxidation is inhibited in β-cells. This mechanism forms part of the normal insulin secretion process. However, accumulation of Co-A, can inhibit insulin secretion by opening potassium channels in the β-cells (Stunvell et al., 2005). Secondly, lipotoxicity can inhibit insulin secretion by increasing the expression of uncoupling protein-2 (UCP-2) which leads to reduced levels of ATP (Stunvell et al., 2005). Thirdly, lipotoxicity leads to β-cell apoptosis by leading to the generation of NO or fatty acid or triglyceride-induced ceramide production (Stunvell et al., 2005).

1.8 Islet amyloid
Islet amyloid deposits are a characteristic pathological feature of T2DM (Kahn et al., 1999). Islet amyloid consists of an amyloid polypeptide, also called amylin. Amylin is co-secreted with insulin but at a lower rate. Amylin also known as islet amyloid polypeptide (IAPP) is found in most T2DM patients and it is thought to be cytotoxic to β-
cells possibly by leading to ROS formation (Stunvoll et al., 2005; Leahy et al., 2005). Amylin is a 37 amino acid peptide (Westermark, 1994). It is produced primarily by β-cells as a 67 amino acid peptide, called pro-IAPP (precursor of IAPP). Pro-IAPP is processed within the β-cell secretory granules by the action of prohormone convertase enzymes (PCE) 2 and 3 (Wang et al., 2001; Kahn et al., 1999). Islet amyloid is believed to play a role in the loss of β-cells and the decrease in insulin secretion in T2DM. In an autopsy that was conducted in T2DM patients, it was discovered that 90% of the patients had islet amyloid deposits (Kahn et al., 1999). The degree of the islet amyloid deposits correlates with the severity of T2DM in humans (Westermark, 1994). The presence of islet amyloid deposits results in a reduction in β-cell mass both in humans and primates (Clark et al., 1994; de Koning et al., 1993). In a study conducted by de Koning (1993), it was shown that the development of islet amyloid deposits occurs before the development of the hyperglycaemic state in monkeys. Amylin has been reported to cause β-cell death in vitro (Hiddinga and Eberhardt, 1999). Lorenzo et al. (1994) exposed human and rat β-cells to amylin fibrils, resulting in β-cells death in both species.

1.9 Anti-diabetic drugs
When selecting a drug for the treatment of T2DM, one must consider the long-term complications of the disease in order to protect the patient. Since IR plays a key role in the pathogenesis of T2DM and the development of cardiovascular complications, interventions must be aimed at improving tissue insulin sensitivity. These interventions include changes in lifestyle, exercise and losing weight. These would reduce the progression of the impairement of glucose tolerance which leads to DM (Diabetes Prevention Program Research Group, 2002; Tuomilehto et al., 2001). By improving glucose tolerance through exercise and diet, one can impove many of the cardiovascular risk parameters of the metabolic syndrome (MetS). Figure 1.8 depicts therapeutic choices for the management of hyperglycaemia.
1.9.1 Thiazolidinediones
The thiazolidinediones (TZDs) enhance insulin sensitivity, thereby, reducing glucose levels (figure 1.9).

Figure 1.8: Pharmacological treatment of hyperglycaemia according to site of action (Stunvoll et al., 2005).

Figure 1.9: The chemical structure of thiazolidinediones, 
The TZDs also improve inflammatory and endothelial function, dyslipidemia, and fibrinolysis. The TZDs have a high affinity for a specific PPAR subtype (Nathason and Nyström, 2009; Jellinger and Mace, 2007; Stunvoll et al., 2005). There are three PPAR subtypes, (i) PPARα which is expressed abundantly in the liver, heart, muscle, and vascular wall; (ii) PPARδ/β which is abundantly expressed in skin, brain, and adipose tissue; (iii) PPARγ which is predominantly expressed in adipose tissue but also expressed in pancreatic β-cells, endothelium, and macrophages (Nathason and Nyström, 2009). TZDs bind to PPARγ, hence it is thought to act primarily in adipose tissue. TZDs improve insulin sensitivity by redistributing tissue triglycerides or fat from visceral stores to subcutaneous adipose tissue. The action of TZDs not only reduces NEFA’s circulating levels but it also reduces plasma levels of FFAs while increasing the levels of adiponectin. TZDs also improve renal function by improving albumin excretion (Jellinger and Mace, 2007; Stunvoll et al., 2005). TZDs can be used in patients with reduced renal function because they show insignificant gastrointestinal side-effect unlike metformin. The side effects of using TZDs are weight gain due to water retention. In some patients, water retention can lead to edema and mild hemodilution (Stunvoll et al., 2005). Troglitazone was the first drug belonging to the TZD class to be used for the treatment of T2DM. However, it was removed from the market because it increased the risk of idiosyncratic hepatotoxicity. The TZDs that are currently available on the market are pioglitazone and rosiglitazone which do not cause the problems encountered with troglitazone. They also reduce hepatic lipid levels in non-alcoholic steatohepatitis, while rosiglitazone also reduces plasma levels of CRP in T2DM patients. However, rosiglitazone can cause cardiovascular death and myocardial infarction (Jellinger and Mace, 2007). TZDs can lower the risk of thrombosis by increasing the rate of fibrinolysis through the inhibition of PAI-1 (Ajjan and Ariëns, 2009).

1.9.2 Metformin
Metformin (figure 1.10) is the first line of treatment of T2DM in combination with exercise and diet. It improves insulin sensitivity in tissues by decreasing hepatic glucose production and increasing glucose uptake in skeletal muscle and adipose tissues with
minimum risk of hypoglycaemia. It also reduces appetite thereby reducing calorie intake in the stomach and may improve insulin secretion by pancreatic β-cells.

![Metformin chemical structure](image)

**Figure 1.10:** The chemical structure of metformin (Amini et al., 2005).

Metformin reduces the levels of circulating triglycerides, FFAs and LDL cholesterol, while increasing the levels of HDL cholesterol. It also improves endothelial function (Nathason and Nyström, 2009; Jellinger and Mace, 2007; Stunvoll et al., 2005). The weight gain that is observed in treatment with TZDs is limited in the treatment with metformin. The mechanism of action of metformin is thought to be via the activation of 5' adenosine monophosphate-activated protein kinase (AMPK). Metformin indirectly activates AMPK by activating complex-1 in the respiration chain. The activation of AMPK down-regulates gluconeogenesis, cholesterol biosynthesis, and fatty acid synthesis pathways. AMPK activation in the endothelium and myocardial tissue is also associated with angiogenesis regulation (Nathason and Nyström, 2009). Metformin possesses anti-thrombotic effects by lowering the *in vivo* levels of factor (F) VII and fibrinogen. It also increases fibrinolysis by lowering the levels of PAI-1. The side effects associated with the use of metformin are lactic acidosis caused by the activation of complex-1 and gastrointestinal symptoms (Nathason and Nyström, 2009; Jellinger and Mace, 2007).

### 1.9.3 α-Glucosidase inhibitors
There are three drugs that belong to the α-glucosidase class; acarbose, migitol, and voglibose. Of these three drugs, acarbose is the most extensively studied. Acarbose reduces the blood glucose levels. It mediates this action by inhibiting α-glucosidase found in the small intestine. α-Glucosidase hydrolyzes oligosaccharides to monosaccharides.
The side effects shown by the use of acarbose are gastrointestinal symptoms (Nathason and Nyström, 2009; Jellinger and Mace, 2007).

1.9.4 Sulfonylurea derivatives
Sulfonylureas (SUs), figure 1.11, increase insulin secretion by closing ATP-dependent K⁺ channels that are found in a number of cells, including pancreatic β-cells, skeletal muscle tissue, VSMC, endothelium, kidney, and central nervous system. The ATP-dependent K⁺ channel is made up of two SU proteins, sulfonylurea receptor (SUR) 1 and SUR2, and Kir 6.2. SUR1 is expressed in β-cells, while SUR2 is expressed in myocardial. Kir 6.2 is a pore-forming unit K⁺-inward rectifier channel. The SUs close the K⁺ channels by binding to the pancreatic β-cell SUR1/Kir 6.2 protein. The influx of Ca²⁺, because of the depolarization of the membrane, leads to the exocytosis of insulin vesicles. They also improve endothelial function. Gliclazide which belongs to the second generation of SUs has a structure which is similar to that of hydrazine (known to possess anti-oxidant properties) (Ridker et al., 1997, Shimabukuro et al. 2006; Vallejo et al., 2000). Gliclazide is effective against lipid peroxidation (Renier et al., 2000; Desfaits et al., 1997). It also reduces markers of endothelium inflammation (Mamputu and Renier, 2002; Rakel et al., 2007; Renier et al., 2003). SUs can lead to hypoglycaemia because they can also act in low blood glucose levels. Tolbutamide, gliclazide, and glipizide have a short duration of action, while gimepiride, glibenclamide (glyburide) have a longer duration of action that can last up to 24 hours. The risk of hypoglycaemia is lower in SUs that have a short duration of action, and gliclazide has an added advantage as it can be used in patients with reduced renal function. The SUs also decrease the levels of glycosylated haemoglobin. Nateglinide is a drug which belongs to the meglitinides class and it binds to SUR1 like SUs. Another drug that belongs to the meglitinides class is repaglinide which binds to a different site of the receptor, thereby stimulating insulin secretion. The advantage of using meglitinides is that they do not cause hypoglycaemia but they have a short duration of action compared with SUs. They can also be used in patients with a reduced renal function (Nathason and Nyström, 2009; Stunvoll et al., 2005).
1.9.5 Exogenous Insulin
Insulin improves insulin sensitivity and endothelial function in T2DM and supports the action of TZDs and SUs which would be ineffective without proper levels of insulin. However, the use of the combination of insulin and TZDs causes an increased risk of edema and cardiac failure and its use has been stopped in European countries (Nathason and Nyström, 2009; Stunvoll et al., 2005).

1.9.6 Glucagon-like peptide 1
Glucagon-like peptide 1 (GLP-1) is formed from the partial proteolysis of preproglucagon that is produced in the small and large intestine by the enteroendocrine L-cells. GLP-1 causes high insulin responses that are glucose dependent, increase the mass of β-cells, and inhibit intestinal motility. However, the half-life of GLP-1 is between 1 and 2 minutes because it is degraded by dipeptidyl peptidase IV (DPPIV). As a result, GLP analogues with a longer half-life, such as exenatide and liraglutide and inhibitors of DPPIV have been developed. GLP-1 has also been reported to improve vascular integrity and protects against endothelial dysfunction (Nathason and Nyström, 2009; Stunvoll et al., 2005).
1.10 The coagulation cascade
The blood coagulation cascade is divided into two independent pathways, the intrinsic and the extrinsic pathway. The two pathways merge and form FX and V which forms part of the prothrombinase complex that leads to FIIa (thrombin) formation. The extrinsic pathway which is responsible for haemostatic regulation and response to vascular injury involves FVII which is activated by TF. The physiological function of the intrinsic pathway is not thoroughly understood but it is believed to cause poor haemocompatibility of cardiovascular biomaterials since it is triggered by the activation of FXII on contact with artificial materials. The intrinsic pathway involves FXII, FXI, FIX, and FVIII (Vogler and Siedlecki, 2009; Ajjan and Ariëns, 2009; Ajjan and Grant, 2006).

The blood coagulation cascade involves a series of interconnected reactions with the resulting pathway of one enzyme activating the next enzyme. The cascade is a self-amplifier with zymogens being activated to enzymes that lead to the formation of thrombin. The final step of the blood coagulation cascade is the conversion of fibrinogen to fibrin by thrombin. The fibrin clot is formed after polymerization (figure 1.12). The fibrin clot formation does not only involve the interaction between the coagulation proteins, but the platelets also play a role, hence it is a complex process. When a break occurs in a vessel wall, TF is brought into contact with the plasma. In the initiation phase, TF binds and activates FVII. The TF/FVII complex activates FIX and FX, with FX activating FV. Prothrombin is cleaved to active thrombin by FX and FV. The thrombin that is released is not enough to sustain the thrombus but enough to maintain platelet activation (Vogler and Siedlecki, 2009; Ajjan and Ariëns, 2009; Ajjan and Grant, 2006).

The adhesion of platelets to the site of injury is mediated by the interaction of vWF with collagen. The activation of platelets by exposed collagen and thrombin brings about full platelet activation and degranulation leading to the release of FV. The coagulation cascade is enhanced, because FV is activated by FXa and thrombin which activates FVIII that forms an active complex with FIXa. The generation of sufficient thrombin from the platelet surface FXa/Va complex leads to the conversion of fibrinogen to fibrin. A stable haemostatic plug is generated after FXIII, a transglutaminase that is activated by
thrombin, forms cross-links in the fibrin clot (Ajjan and Ariëns, 2009; Ajjan and Grant, 2006).

Figure 1.12: A simplified line diagram of the plasma-coagulation cascade showing intersection of the intrinsic and extrinsic pathways (many mediators and cofactors involved in haemostasis are not shown and the interaction with platelets has been ignored for simplicity). Activated forms of the zymogens are denoted by an “a” suffix. Calcium-dependent reactions are suspended in the presence of calcium-chelating anticoagulants such as citrate used to prepare platelet-poor plasma for coagulation studies (Vogler and Siedlecki, 2009).

1.10.1 Antithrombotic mechanisms
Natural anticoagulants act as a defence mechanism against thrombosis. These defence mechanisms include protein C, tissue factor pathway inhibitor (TFPI), heparin-antithrombin pathway, thrombomodulin, and thrombin-activatable fibrinolysis inhibitor (TAFI). Protein C inhibits FVa and FVIIIa. TFPI inhibits the initiation phase of blood coagulation by blocking TF-FVII complex. The heparin-antithrombin complex inhibits thrombin and also inactivates FIXa, FXa and FXIa. Thrombomodulin which is expressed on EC also inhibits thrombin. Protein C can also be activated by thrombin-thrombomodulin complex. TFPI is activated by thrombomodulin and it inhibits FXa and thrombin (Ajjan and Ariëns, 2009; Ajjan and Grant, 2006).
1.10.2 Conversion of fibrinogen into fibrin

Fibrinogen is a multifunctional protein essential for haemostasis. It is a 450 Å, 340 kDa glycoprotein (Wolberg, 2007; Vadseth et al., 2004). The concentration of fibrinogen circulating in human plasma produced by hepatocytes is 2-4 mg/mL (Wolberg, 2007; Southan et al., 1985). Fibrinogen consists of two sets of non-identical polypeptide chains: Aα (66 062 kDa), Bβ (54 358 kDa) and γ (48 529 kDa), crosslinked by 29 disulfide bonds (Ajjan and Ariëns, 2009; Wolberg, 2007; Vadseth et al., 2004). All six polypeptide chains of fibrinogen are assembled with their N-terminal converged in a central ‘E’ nodule of the molecule. The C-terminal of the Bβ and γ chains extended outward to form a ‘D’ nodule (figure 1.13) (Wolberg, 2007).

![Diagram](image)

Figure 1.13: Thrombin-mediated conversion of fibrinogen to fibrin involves the sequential release of fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from the central E nodule of fibrinogen. Fibrinopeptide release permits the protofibril formation by lateral aggregation of fibrin fibres. Factor XIIIa which is generated by thrombin crosslinks self-associated fibrin to form a covalently stabilized matrix (Wolberg, 2007; Dugan et al., 2006; Vadseth et al., 2004).

Studies in which a specific amount of thrombin is added to fibrinogen have helped in the elucidation of the mechanisms of fibrin production and clot assembly. Thrombin initiates the fibrin clot formation by binding to the E nodule of fibrinogen and removes the N-
termini of the Aα and Bβ chains. Fibrinopeptide cleavage and fibrin polymerization can be represented in a two-step transition mechanism (Southan et al., 1985). FpA is hydrolyzed first, revealing a polymerization domain called the ‘A’ site. The A site permits the non-covalent interaction between the E nodule and the exposed ‘a’ pocket in the γ chain of the D nodule of another fibrinogen molecule. Prototibrils result from the E: D interaction (Wolberg, 2007; Reber et al., 1985). The initial polymerization step is accompanied by a conformational change which enhances the hydrolysis of FpB. An additional polymerization step occurs exposing the N-terminus of the Bβ chain. These N-terminal polymerization domains also require complementary domains at the C-terminus of another fibrinogen molecule before fibrin polymerization and clot formation could occur. The removal of the N-terminal of Bβ chains exposes the B site. Similar to the A: a interaction, the ‘B’ site interacts with the ‘b’ pocket in the Bβ chain of the D nodule. The transglutaminase reaction which is catalyzed by FXIII involves glutamine and lysine residues. FXIII forms multiple cross-links within the γ and α-chains. The formation of this stable haemostatic plug renders the clot resistant to fibrinolysis (Ajjan and Ariëns, 2009; Wolberg, 2007; Southan et al., 1985).

FpA is required for normal protofibril formation because delayed FpA release leads to delayed polymerization (Rooney et al., 1998). FpB promotes the lateral aggregation of existing fibrin protofibrils (Blomback et al., 1978). FpA and FpB are similar in length and amino acid composition and both contain a large number of acidic residues (Martinelli and Scheraga, 1979). In studying the mechanism of action of thrombin on fibrinogen, sensitive and rapid high performance liquid chromatography (HPLC) methods separating small amounts of FpA and FpB that have been developed.

1.10.3 Fibrin clot lysis
Plasmin, the main enzyme involved in fibrinolysis, is formed by the action of two plasminogen activators namely, tissue plasminogen activator (t-PA) and urinary plasminogen activator (u-PA) (Castañon et al., 2007; Vadseth et al., 2004). t-PA is primarily active in the vascular system. It is the main agent for the dissolution of thrombi via the activation of plasminogen to plasmin (Castañon et al., 2007; Bachmann, 2001). u-
PA is found in connective tissue, and plays a role in tissue remodeling and cell migration (Castañon et al., 2007). The presence of fibrin enhances the activation of plasminogen to plasmin by t-PA (figure 1.14), whereas, no influence is observed in the case of u-PA (Castañon et al., 2007; Bachmann, 2001).

The αC domain in fibrin is the initial cleavage site for plasmin. This step is followed by multiple cleavages between the D and E regions of fibrin by plasmin. Fibrin degradation products of various sizes are a product of these multiple cleavages by plasmin. The inhibitors of fibrinolysis are α2-plasmin inhibitor (or α2-antiplasmin) which forms an irreversible stable complex when bound to plasmin and PAI-1 thus inhibiting plasminogen activation. TAFI inhibits fibrinolysis by inhibiting plasminogen activation through cleavage of C-terminal lysine residues on fibrin (Ajjan and Ariëns, 2009).

1.10.4 Platelets in haemostasis
The physiological role of platelets is in haemostasis, which is the maintenance of vessel integrity and stopping bleeding upon vessel injury. However, in pathophysiological conditions, platelet activity can lead to various responses that play a role in atherosclerosis (Xiang et al., 2008). Platelets play a role in the pathogenesis of cardiovascular, cerebrovascular, and peripheral vascular diseases (Jin et al., 2007). They
also play a role in thrombosis and bleeding via platelet adhesion, activation and aggregation (Jeng et al., 2007; Jin et al., 2005). When platelets are activated, they change shape, aggregate, and secrete contents of their intracellular granules via various intracellular biochemical pathways (Kim et al., 2008a and b). Platelet aggregation is induced by various agonists, such as adenosine diphosphate (ADP), collagen and thrombin and is dependent on the release of ADP and prostaglandin (PG)H\textsubscript{2}/thromboxane TXA\textsubscript{2}. When platelets are activated, they change shape and secrete pro-activatory substances that activate and recruit more platelets to the site of injury. The central component in the platelet response is TXA\textsubscript{2}. TXA\textsubscript{2} is an eicosanoid which is formed via a cyclooxygenase-TXA\textsubscript{2} synthase pathway. It is considered as a powerful agonist for platelet activation and greatly contributes to thrombus formation. The binding of TXA\textsubscript{2} to a G-protein-coupled receptor induces phospholipase C\beta activation. An increase in [Ca\textsuperscript{2+}]\textsubscript{i} and PKC activation occurs due to the activation of phospholipase C\beta. The change in platelet shape and eventually the adhering of platelets to the site of injury is caused by this increase in [Ca\textsuperscript{2+}]\textsubscript{i} and PKC activation (Jin et al., 2007; Jin et al., 2005). The increase in [Ca\textsuperscript{2+}]\textsubscript{i} as a result in either influx of Ca\textsuperscript{2+} or secretion from intracellular stores play a major role in the response of platelets to various agonists (Jin et al., 2005). [Ca\textsuperscript{2+}]\textsubscript{i} release is through the activation of G protein-coupled receptor, the protease-activated receptors [PARs] and an integrin-type receptor, α2β1 (Kim et al., 2008a). Therefore, the inhibition of cyclooxygenase-1 (COX-1) enzyme activity or TXA\textsubscript{2} and/or the inhibition of cystolic Ca\textsuperscript{2+} mobilization in platelets may inhibit platelet aggregation (Jin et al., 2007, Jin et al., 2005; and Nurtjahja-Tjendraputra et al., 2003).

1.10.5 Platelet signalling and platelet-fibrinogen interaction
The activation of platelets involves a number of steps that includes distinct receptors and ligands. The coagulation factors bind to platelets via their glycoprotein (GP) receptors or via phospholipids that are exposed after platelet activation. Figure 1.15 represents a signalling pathway of platelet activation with main agonists, respective receptors, G proteins, and relative effectors (Xiang et al., 2008; Ajjan and Grant, 2006).
The signalling pathway of platelets is an intricate process as most platelet agonists can act synergistically. Agonists can bind more than one receptor; vWF can bind to GPIb and αIIbβ3, collagen binds to GPVI and α2β1, thrombin binds PAR receptors and GPIb, while ADP binds to two ADP receptors on platelets. TXA2, ADP, 5-hydroxytryptamine (5-HT) and ATP act as positive-feedback mediators by activating platelets upon their release, therefore, amplifying the platelet activation signal (Xiang et al., 2008).

Procoagulant phospholipids are exposed upon vessel injury. Platelets are activated by thrombin and/or collagen causing a change in their shape. The platelets become circular in shape upon activation and they form membrane blebs. The tenase (a complex of Ca\textsuperscript{2+}, activated FX, FIX and FVIII) and prothrombinase reactions are enhanced by phosphatidylserine-containing membranes. The membranes bind FV, FVIII, FIX, and FX, bringing them into close proximity, resulting in conformational changes in proteins necessary for platelet activation. The binding of platelets to its adhesive ligands and the
synthesis and secretion of mediators involved in platelet activation initiate the platelet aggregation process. Platelet aggregation involves the binding of fibrinogen to platelets via GPIIb-IIIa. This integrin has a high affinity for fibrinogen only in activated platelets. The interaction between fibrinogen and GPIIb-IIIa promotes aggregation. Platelet aggregation results in the synthesis of TXA2 and the release of ADP and serotonin from dense bodies and fibrinogen, FV, P-selectin and PAI-1 from α-granules (Anfossi et al., 2009; Ajjan and Grant, 2006; Ohaeri and Adoga, 2006).

1.1 Hypercoagulation-related to diabetes mellitus

Hypercoagulability plays a role in the pathogenesis of thrombosis in DM patients. There is an altered haemostatic balance in DM patients, i.e. changes in blood coagulability, fibrinolytic system and platelet function. DM is characterized by an increase in plasma levels and fluctuations in blood glucose levels. There is a close relationship between hyperglycaemia and the development of thrombosis. This relationship between the two diseases or disorders explains why there is a high rate of atherothrombotic cases in DM patients. DM patients have been found to have elevated levels of TF (Khechai et al., 1997). Chronic hyperglycaemia causes the glycation of albumin, which stimulates the monocytes to produce TF mRNA. Hyperglycaemia is associated with elevated levels of FVII. This elevation is thought to be caused by high blood triglyceride levels which are found in diabetic dyslipidemia. Other factors with elevated levels are increased in hyperglycaemic conditions are FXII, FXI, FVIII, and vWF. Drastic changes in blood glucose levels (hypo- or hyperglycaemia) cause structural changes in fibrinogen. Fibrinogen is glycated in DM, this leads to the formation of more compact clots. Fibrin clots formed in DM patients have been shown to have reduced susceptibility to plasmin. All these changes contribute to the prothrombotic effect and cause a hypercoagulable state in DM (Ajjan and Ariëns, 2009; Meshkani and Adeli, 2009; Carr, 2001).

The levels of protein C which is a vitamin K-dependent protein are decreased in T1DM, while the levels of TFPI are elevated in nephropathy associated with T1DM (Carr, 2001). Fibrinolysis is regulated by the balance between its activators (primarily t-PA) and inhibitors like PAI-1. PAI-1 is a serine protease inhibitor and a marker of impaired
fibrinolysis. Fibrinolysis is required to maintain haemostasis because low-grade coagulation consistently occurs in the blood. Extreme inhibition of fibrinolysis leads to hypercoagulation and thrombosis. IR leads to elevated levels of PAI-1 resulting in an impaired fibrinolytic system (Meshkani and Adeli, 2009; Jellinger and Mace, 2007; Carr, 2001).

1.12 Platelet hyperactivation in diabetes

In vivo platelet activation and hyperactivation in DM patients is caused by chronic hyperglycaemia (figure 1.16). TX biosynthesis and TXB₂ release is elevated in DM patients. In vitro and in vivo experiments conducted by Gresele et al., (2003) showed that hyperglycaemia induces elevated platelet activation when the platelets are exposed to high shear stress conditions. Platelet sensitivity was increased as a result of impaired calcium homeostasis, PKC activation, decreased synthesis of NO in platelets, and ROS production. As a result of hyperglycaemia, non-enzymatic glycation of glycoproteins (GPs) occurs. The glycation of GPs leads to alteration in their structure, conformation, and lipid dynamics, and these changes cause an increase in expression of P-selectin and GPIIb/IIIa, availing them to their ligands (Ferroni et al., 2004).

![Figure 1.16: An illustration summarising the effects of altered glycaemic control on platelet function](Ferroni et al., 2004).
In addition, hyperglycaemia causes LDL non-enzymatic glycation which in turn leads to platelet dysfunction by increasing $[\text{Ca}^{2+}]_{i}$. Indeed, $[\text{Ca}^{2+}]_{i}$ levels are found to be high in DM patients compared to normal healthy individuals, stimulating TXA$_2$ biosynthesis and increasing phosphotyrosine content in platelets. Tyrosine phosphorylation is associated with platelet activation, aggregation, Ca$^{2+}$ mobilization and thromboxane synthesis. In addition to increased $[\text{Ca}^{2+}]_{i}$ levels, DM patients have reduced magnesium levels which enhance platelet hyperaggregation and adhesiveness (Haouari and Rosado, 2008; Haouari et al., 2007; Ferroni et al., 2004).

T2DM patients have elevated small dense LDL and triglyceride levels while HDL levels are reduced. This change in lipid profile alters membrane fluidity. High ROS levels in T2DM cause lipid peroxidation which enhances platelet activation, adhesion, and aggregation. Platelet activation also increases the formation of microparticles in DM patients (Haouari and Rosado, 2008; Ferroni et al., 2004).

Insulin receptors are expressed in human platelets. The insulin signalling pathway is activated by the platelet receptors but it does not lead to glucose uptake, instead it plays an anti-aggregating role. When platelets are activated, many cellular proteins are tyrosine phosphorylated (Clark et al., 1994). Platelets from T2DM exhibit a significantly higher p38 MAPK phosphorylation. P38 MAPK phosphorylates and activates phospholipase A$_2$ (PLA$_2$) in turn results in arachidonic acid (AA) synthesis. Tyrosine phosphorylation in platelets leads to platelet aggregation, Ca$^{2+}$ mobilization and TX synthesis (Haouari and Rosado, 2009). Insulin plays an antiaggregating role by reducing platelet sensitivity to aggregation agonists which include ADP, thrombin, platelet activating factor, collagen, sodium arachidonate and catecholamines. Insulin inhibits calcium mobilization and platelet sensitivity. IR associated with T2DM and obesity reduces platelet sensitivity to the anti-aggregating action of insulin. Therefore, a prothrombotic state is observed in obesity which could lead to a high risk of cardiovascular events (Anfossi et al., 2009; Ferroni et al., 2004).
1.13 Current antiplatelet therapy in DM and non-DM patients
There are various antiplatelet agents that are used in order to reduce thrombotic complications, table 1.1. There are four strategies that have been used to inhibit platelet function (i) inhibition of agonist generation, (ii) inhibition of receptors, (iii) inhibition of G-protein, and (iv) inhibition of the enzymatic cascade (Xiang et al., 2008).

1.13.1 Aspirin
Aspirin (figure 1.17) is a COX inhibitor. It prevents vascular complications in DM patients. Aspirin irreversibly inhibits the activity of COX-1 by acetylation. Inhibition of COX-1 inhibits the production of TXA₂. It is also able to acetylate fibrinogen to yield a more porous fibrin gel which has a higher affinity for plasminogen and is easier to lyse. In vivo studies conducted by Undas et al. (2003) have shown that aspirin modulates FXIII activity. Based on a clinical trial conducted by the United State Physician’s Health Study and the Hypertension Optimal Treatment, it was concluded that aspirin is more beneficial in DM patients than in non-DM patients (Steering Committee of the Physician’s Health Study Research Group, 1989). It is prescribed for the DM patients who are high risk for cardiovascular complications (Colwell, 1997). However, a reduced response to aspirin, ‘aspirin resistance’ and gastro-intestinal problems have been observed with its use (Ajjan and Ariëns, 2009; Anfossi et al., 2009; Xiang et al., 2008; Ferroni et al., 2004).

![Figure 1.17: The chemical structure of aspirin (Kauffman, 2000).](image-url)
Table 1.1: Clinical situations of established antiplatelet agents based on systematic reviews (Xiang et al., 2008).

<table>
<thead>
<tr>
<th>Agents</th>
<th>Targets</th>
<th>Efficacy and indication</th>
<th>Maintenance dose</th>
<th>Adverse effects</th>
<th>Cost</th>
<th>Current problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>COX-1 irreversible inhibition</td>
<td>Worldwide established cardiocerebrovascular disease</td>
<td>75-81 mg/day</td>
<td>Gastrointestinal bleeding</td>
<td>Lowest</td>
<td>Resistance (about 25%)</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>P2Y12 irreversible inhibition</td>
<td>Stroke, transient ischemia, etc.</td>
<td>250 mg Bid</td>
<td>Diarrhea, nausea, vomiting, bone marrow suppression, thrombotic thrombocytopenic purpura, hepatotoxicity</td>
<td>Higher</td>
<td>Delayed onset of action, high individual variability</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>P2Y12 irreversible inhibition</td>
<td>Superior to ticlopidine, stroke, myocardial infarction, peripheral arterial disease, etc.</td>
<td>75 mg/day</td>
<td>Less toxic than ticlopidine, aplastic anaemia and thrombotic thrombocytopenic purpura, bone marrow suppression</td>
<td>High</td>
<td>High individual variability, resistance (6-58%)</td>
</tr>
<tr>
<td>Prasugrel</td>
<td>P2Y12 irreversible inhibition</td>
<td>Superior to clopidogrel, undergoing phase III trials</td>
<td>10 mg/day</td>
<td>Major bleeding</td>
<td>Low</td>
<td>Not reported</td>
</tr>
<tr>
<td>Cangrelor</td>
<td>P2Y12 irreversible inhibition</td>
<td>Undergoing phase II trials, acute coronary syndromes, percutaneous coronary intervention</td>
<td>2 µg/kg/min</td>
<td>Trivial bleeding</td>
<td>Uncertain</td>
<td>Intravenous administration</td>
</tr>
<tr>
<td>AZD6140</td>
<td>P2Y12 irreversible inhibition</td>
<td>Undergoing phase II trials</td>
<td>100 mg/day</td>
<td>Major nonfatal haemorrhage</td>
<td>Uncertain</td>
<td>Not reported</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>Phosphodiesterase enzyme 5 cAMP</td>
<td>Stroke, transient ischemia, etc.</td>
<td>300 mg/day</td>
<td>Modest bleeding</td>
<td>Low</td>
<td>More efficacious when with aspirin but less when alone</td>
</tr>
<tr>
<td>Clistazol</td>
<td>Phosphodiesterase enzyme 5 cAMP</td>
<td>peripheral arterial disease, stroke, percutaneous coronary intervention</td>
<td>100 mg/day</td>
<td>Modest bleeding</td>
<td>Low</td>
<td>To be carefully evaluated</td>
</tr>
<tr>
<td>Abciximab</td>
<td>GPIIb/IIIa irreversible inhibition</td>
<td>Myocardial infarction, percutaneous coronary intervention</td>
<td>0.125 µg/kg/min</td>
<td>Major bleeding, thrombocytopenia</td>
<td>Higher</td>
<td>Intravenous administration, Resistance</td>
</tr>
<tr>
<td>Eptifibatide</td>
<td>GPIIb/IIIa reversible inhibition</td>
<td>Myocardial infarction, percutaneous coronary intervention</td>
<td>2 µg/kg/min</td>
<td>Major bleeding, thrombocytopenia</td>
<td>High</td>
<td>Intravenous administration</td>
</tr>
<tr>
<td>Tirofiban</td>
<td>GPIIb/IIIa reversible inhibition</td>
<td>Inferior to abciximab, myocardial infarction, percutaneous coronary intervention</td>
<td>0.15 µg/kg/min</td>
<td>Major bleeding, thrombocytopenia</td>
<td>Low</td>
<td>Intravenous administration</td>
</tr>
<tr>
<td>5CH530348</td>
<td>PAR-1</td>
<td>Undergoing phase III trials, acute coronary syndromes</td>
<td>1.0 mg/day</td>
<td>Trivial bleeding</td>
<td>Uncertain</td>
<td>To be further evaluated</td>
</tr>
</tbody>
</table>
1.13.2 Thienopyridines
Alternatives to aspirin-intolerant patients are agents which are used to inhibit platelet aggregation; these are thienopyridine (ticlopidine and clopidogrel), figure 1.18. Thienopyridines irreversibly inhibit the activity of ADP receptors P2Y<sub>12</sub>. The inhibition of these receptors prevents GPIIb/IIIa from interaction with fibrinogen. Ticlopidine, a precursor of clopidogrel is associated with a reduction of fibrinogen levels. Clopidogrel does not cause this effect but drug resistance has been observed with its use (Ajjan and Ariëns, 2009; Anfossi et al., 2009; Xiang et al., 2008; Ferroni et al., 2004). In a study conducted by Bhatt et al. (2002), clopidogrel was found to be more effective than aspirin in reducing ischemic events in DM patients with a history of thrombosis. Other drugs that inhibit ADP receptors P2Y<sub>12</sub> are prasugrel, cangrelor and AZD6140 (Xiang et al., 2008).

![Chemical structures of thienopyridines](image)

Figure 1.18: The chemical structure of thienopyridines and thienopyrimidinones (Jakubowski et al., 2005).

1.13.3 Dipyridamole
Dipyridamole (figure 1.19) is a drug that is used in combination with aspirin. Dipyridamole blocks the platelets response to ADP by increasing cAMP levels through inhibition of phosphodiesterase. The combination of aspirin and dipyridamole reduce proteinuria levels in T2DM. However, dipyridamole is not effective on its own. Cilastazol is another drug that targets increased cAMP levels (Xiang et al., 2008).
1.13.4 Abciximab
Abciximab is a monoclonal antibody against GPIIb/IIIa (Ferroni et al., 2004). This reduces the number of deaths and myocardial infarction incidents in DM and non-DM patient (Kleiman et al., 1998). The adverse effects caused by the use of this drug are major bleeding, thrombocytopenia, and resistance to its use. Eptifibatide and Tirofiban are other drugs that are used to inhibit GPIIb/IIIa activity, however, their inhibition is reversible (Xiang et al., 2008; Ferroni et al., 2004).

1.14 New anticoagulant drugs
New anticoagulant drugs that target specific steps on the coagulation cascade have been developed. The drugs that are discussed below are still on clinical trials, some have reached phase III.

1.14.1 Indirect inhibitors of FXa
This class of inhibitors includes synthetic drugs fondaparinux and idraparinux, which indirectly inhibit FX activation by inhibiting antithrombin. Since these drugs do not bind to platelet factor IV, they do not cause heparin-induced thrombocytopenia (HIT), therefore they can be used to treat thrombotic complications that are associated with HIT. However, the disadvantage associated with these drugs is that they do not have a specific antidote (Franchini and Mannucci, 2009).
1.14.1.1 Fondaparinux
This drug (figure 1.20) is licensed to treat venous thromboembolism (VTE), deep vein thrombosis (DVT), and pulmonary embolism (PE). It is administered subcutaneously and has a half-life of 17 hours and is excreted in the urine unchanged, however, severe bleeding was observed in treatment groups (Franchini and Mannucci, 2009; Bauer, 2004; Cheng, 2002).

![Figure 1.20: The anticoagulant pentasaccharide Fondaparinux (Chen and Yu, 2009).](image)

1.14.1.2 Idraparinux
Idraparinux (figure 1.21) is derived from the hypermethylation of fondaparinux. It is administered subcutaneously and has a longer plasma half-life as compared to fondaparinux, i.e. 80-130 hours. Similar to fondaparinux, it can treat VTE, DVT, and PE. However, the disadvantage of idraparinux treatment is delayed excretion from the body after prolonged administration. Bleeding complications might be caused by the prolonged elimination from the body. Secondly, there is no antidote that could neutralize its actions (Franchini and Mannucci, 2009; Chen and Yu, 2009; The amadeurs investigators, 2008; Spyropoulos, 2008).

![Figure 1.21: The anticoagulant pentasaccharide Idraparinux (Chen and Yu, 2009).](image)
1.14.2 Direct FXa inhibitors
1.14.2.1 Rivaroxaban
Rivaroxaban (figure 1.21) is given orally and absorbed by the gastro-intestinal tract. It inhibits the active site of FXa that is free or bound to the prothrombinase complex. It is excreted via renal (two thirds) and fecal (one third) routes. It has a relatively long half-life, 5.7-9.2 hours. Rivaroxaban reduces the risk of VTE, PE, and DVT (Franchini and Mannucci, 2009, Remko 2009; Spyropoulos, 2008).

1.14.2.2 Apixaban
Apixaban (figure 1.22) is an FXa inhibitor that is given orally. Like rivaroxaban, apixaban inhibits the active site of free or bound FXa. It is also eliminated via renal and fecal routes. Apixaban reduces the risk of VTE, with a low number of cases that reported severe bleeding (Franchini and Mannucci, 2009; Remko 2009; Spyropoulos, 2008).

Figure 1.22: The chemical structures of rivaroxaban and apixaban (Remko, 2009).

1.14.3 Direct thrombin inhibitors
1.14.3.1 Dabigatran etexilate
Dabigatran etexilate (figure 1.23) is given orally and once it is absorbed by the gastro-intestinal tract, it is converted to its active form, dabigatran. Its plasma half-life is 14-17 hours and it is eliminated via the kidneys. It is used to treat VTE, and so far a low number of cases have shown major bleeding (Franchini and Mannucci, 2009; Remko 2009; Autar, 2009, Van Huis et al., 2009).
1.14.3.2 Ximelagatran
Ximelagatran (figure 1.24) is given orally and once it is absorbed it is bioconverted to melagatran. Melagatran inhibits the active site of thrombin. It has a shorter plasma half-life of 4-5 hours and is eliminated via the kidneys. Studies which have been conducted on ximelagatran suggest that it can be used to treat VTE and prevention of strokes in patients with atrial fibrillation and myocardial infarction in patients who had ischemia. However, ximelagatran causes hepatotoxicity (Franchini and Mannucci, 2009; Autar, 2009).

1.15 Medicinal plants
Medicinal plants have been used to treat various ailments since ancient times (Scorzoni et al, 2007). The use of traditional health practices is very common in Africa (Scorzoni et al, 2007; Shai et al., 2008). The World Health Organization (WHO) has estimated that about 80% of developing countries use traditional medicine exclusively (Eloff, 1998), and 20 000 plant species are used for medical purposes (Scorzoni et al, 2007). South Africa has a unique and diverse botanical inheritance, which is mostly endemic. Of the 30
000 South African plant species, 3000 of them are used for therapeutic purposes (van Vuuren, 2008). Sixty to 80% of South Africans rely primarily on traditional medicine to treat animal ailments or for personal health purposes (Shai et al., 2008). The principal component of traditional medicines could be therapeutic, therefore the interest in studying plants has increased due to their multipurpose applications (Eloff, 1998; Ncube et al., 2008). There is a need to conserve such plants, where some of the indigenous South African plants can become extinct before their potential as a source of pharmaceutical drugs could be studied (Shai et al., 2008). Traditional medicine has been recognized as part of primary health by the South African government, as a result government is promoting its integration into the official health care system under the Reconstruction and Development Plan (Stafford et al., 2005).

Scientific validation of the medicinal plants is required to investigate their safety, efficacy, quality and the correct dosage of the plant (Shai et al., 2008; Scorzoni et al, 2007; Ncube et al., 2008). Traditional knowledge can be used to assist scientists to target plants that are known to be medically useful. An example of this is where 122 drugs have been developed from 94 plant species having been discovered through ethnobotanical leads (Fennell et al., 2004a and b).

1.15.1 Leonotis leonurus

*Leonotis leonurus* (Lamiaceae) is an indigenous plant to South Africa, figure 1.25 (A) (Van Wyk et al., 2000). It is commonly known as wilde dagga, umunyane, and umficane in Afrikaans, Sotho, and Xhosa; respectively (Van Wyk et al., 2000; Watt and Breyer-Brandwijk, 1967). It is widely disturbed in South Africa, figure 1.25 (B). It is a shrub that grows to be 2 to 5 meters tall. It has a thick wooden base and pale brown branches. This plant is often called lion’s ears because of its bright hairy orange flowers that look similar to the lion’s ears. The long narrow leaves lie opposite each other on the stem, (Van Wyk et al., 2000). It contains volatile oils, reducing sugars, cardiac glycosides, tannins, quinones, saponins, alkaloids, triterpene steroids and a diterpenoid called marrubiin (M) which is derived from premarrubiin, figure 1.25 (C) (Bienvenu et al., 2002; Van Wyk et al., 2000). Recently, a new diterpenoid ester 1,2,3-trihydroxy-3,7,11,15-
tetramethylhexadecan-1-yl-palmitate, figure 1.25 (D) has been isolated from ethanol/chloroform leaf extracts of *Leonotis leonurus*. This compound was tested for antimalarial, cytotoxic and antibacterial activity, but it was found to have no effect on the above mentioned activities (Agnihotri *et al.*, 2009).

Figure 1.25: An illustration of (A) *Leonotis leonurus*, (B) geographical distribution of *Leonotis leonurus*, and (C) the chemical structures of premarrubiin and M (Van Wyk *et al.*, 2000) and (D) 1,2,3-trihydroxy-3,7,11,15-tetramethylhexadecan-1-yl-palmitate (Agnihotri *et al.*, 2009).

1.15.1.1 Traditional uses and biological activity of *Leonotis Leonurus*

*Leonotis leonurus* has been traditionally used for various ailments. The persons of European descent in the Transvaal (Gauteng) smoked the leaves of this plant to alleviate epileptic seizures (Watt and Breyer-Brandwijk, 1967). The aqueous leaf extract was evaluated for its anticonvulsant activity against epileptic seizures in mice produced by pentylenetetrazole (PTZ, 90 mg/kg), picrotoxin (8 mg/kg), bicuculline (20 mg/kg), N-
methyl-D-L-aspartic acid (NMDLA, 400 mg/kg) (Bienvenu et al., 2002). Doses of 200 and 400 mg/kg of the extract significantly delayed the onset of seizures in all models except in a bicuculline induced model. The results suggested that the aqueous extract acted by a non-specific mechanism. These results confirmed that the plant is effective against epilepsy (Bienvenu et al., 2002). A study conducted by Risa et al. (2004) showed that the aqueous extract (10 mg/mL) did not exhibit an activity against the γ-aminobutyric acid (GABA) receptor and the ethanol extract had a weak binding affinity, suggesting that the anti-epileptic activity is not via this receptor.

*Leonotis leonurus* has been reported to treat snake-bites and the decoctions have been externally used to treat boils, eczema, skin diseases, itching and muscular cramps (Van Wyk et al., 2000). Jäger et al. (1996) screened plants that have anti-inflammatory effects for possible inhibition of prostaglandin synthesis. Prostaglandins sensitize neurons to pain and are involved in the activation of inflammation. The aqueous (2.5 mg/mL) and the organic (ethanol, 20 mg/mL) extracts inhibited COX activity by 2% and 90%, respectively. Persons of European descent in the Western Province used *Salvia* species in combination with *Leonotis leonurus* for influenza, chest inflammation, pulmonary tuberculosis, rheumatic conditions, gynaecological complaints and skin diseases (Watt and Breyer-Brandwijk, 1967). Kamatou et al. (2006) reported a synergistic relationship between *Salvia chamelaegnea* and *Leonotis leonurus*. Various ratios of leaf acetone extracts of the two plants were shown to be more effective against Gram-positive bacteria than when they were used separately (Kamatou et al., 2006). Antimicrobial activity of *Leonotis leonurus* was again tested by Steenkamp et al. (2004) against both Gram-negative and -positive bacteria. The methanol and aqueous extracts inhibited microbial growth (Gram-negative and -positive bacteria) with a minimum inhibitory concentration (MIC) $\geq 4$mg/mL. The age and time of harvest of the plant can influence the effect it exerts against bacterial growth and COX-1 activity (Fennell et al., 2004a). Stafford et al. (2005) evaluated the effect of storage on the chemical composition and biological activity of *Leonotis leonurus*. The aqueous and organic (ethanol or hexane) extract (0.5 mg) was applied on thin layer chromatography (TLC) to identify antibacterial compounds. The antibacterial compounds were found to be stable, there was no change in composition
between fresh, 1 and 5 year old plant material. The organic extract yielded the highest antibacterial property as compared to the aqueous extract. The ethanol extract of *Leonotis leonurus* had an increase in antibacterial activity as it aged. Fresh plant extract inhibited *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* growth with a MIC of 1.56 mg/mL, however, the 5 year old plant material was more potent with a MIC of 0.78 mg/mL. Similar to the antibacterial assays, the ethanol extract showed the highest COX inhibition. The fresh plant ethanol extract totally inhibited COX activity (100% inhibition), while a small decrease in the plant activity was observed for the year old plant material (95±4%). TLC fingerprinting of the extract did change overtime, but the major antibacterial compounds were stable. A second group of antibacterial compounds which were not visible in a fresh extract or after a year of storage appeared after a 5 year storage period. It was concluded that these compounds increased their quantities as a result of storage. This study provides evidence that *Leonotis leonurus* retains its efficacy after storage. In a study conducted by Low Ah Kee (2008), the aqueous extract of *Leonotis leonurus* decreased the calcium chloride-induced clotting time. The cytotoxic effect of the methanol and the aqueous extracts was evaluated against 4 cancer cell lines. Only the methanol extract had cytotoxic effects against HL-60, an acute promyelocytic leukaemia cell line.

*Marrubium vulgare* (Lamiaceae) is a plant found in North and South America and Western Asia and has been found to possess antibacterial activity against Gram-negative and -positive bacteria. The range of the methanolic extract of *Marrubiim vulgare* that was tested against the various bacteria was 50–600 mg/mL (Masoodi et al., 2008). Similar to *Leonotis leonurus; Marrubium vulgare* contains the diterpenoid M. M isolated from *Marrubium vulgare* significantly increases tolerance of pain induced in mice and inhibited abdominal constriction induced by 0.6% acetic acid, with an inhibitory dose of 50% (ID$_{50}$) value of 2.2 μmol/kg. Pain induced by formalin (0.92% formaldehyde) was significantly inhibited by M with an ID$_{50}$ value of 6.6 and 6.3 μmol/kg for the first and second phase, respectively. Capsaicin-induced pain (1.6 μg/paw) was inhibited with an ID$_{50}$ value of 28.8 μmol/kg (De Jesus et al., 1999). The antinoceptive property of M isolated from *Marrubium vulgare* was reported by Meyre-Silva et al. (2005). M (10
mg/mL) inhibited pain sensation by 92% using the writhing test, with an ID$_{50}$ value of 2.2 µmol/kg. Formalin-induced pain was inhibited by 28.3% and 46.7% for the first and second phase, respectively. Similar to De Jesus et al. (1999), Meyre-Silva et al. (2005) obtained data that suggested that M exhibited central and peripheral effects. M also inhibited capsaicin-induced pain by 37.3%. Stulzer et al. (2006) showed that M had an anti-edematogenic effect on different inflammatory-induced drugs. M inhibited histamine-induced inflammation (3µg/ear) by 73.3% with an ID$_{50}$ value of 13.84 mg/kg. Bradykinin-induced inflammation (3µg/ear) was inhibited by 70% with an ID$_{50}$ value of 18.82 mg/kg. Lastly, carrageenan-induced inflammation (400 µg/ear) was inhibited by 63% with an ID$_{50}$ value of 13.61 mg/kg. Ovalbumin-induced allergic edema was significantly inhibited by 67.6%. M exhibited non-specific anti-inflammatory properties and the results confirm and justify the use of Marrubium vulgare by traditionalists to treat inflammatory diseases (Stulzer et al., 2006).

Pallant and Steenkamp (2008) conducted a study in which they tested 10 medicinal plants that are traditionally used for respiratory tract infections to evaluate their antimycobacterial, antibacterial and antifungal properties. The aqueous and methanol extracts (1mg/mL) of Leonotis leonurus were found not to have any effect on the growth of the causative agents of respiratory tract infections, i.e. Streptococcus pneumonia, Klebsiella pneumonia, Helicobacter influenza, Staphylococcus aureus, Mycobacterium smegmatis and Candida albicans.

A decoction of leaves had been used by the Hottentots as a strong laxative and treatment for women’s menstrual disorders (Watt and Breyer-Brandwijk, 1967; Ososki et al., 2002). Leaves, twigs, and stem extracts of Leonotis leonurus were bioassayed for their in vitro antiplasmodial properties. The aqueous extracts had low antiplasmodial activity (inhibiting concentration at 50% (IC$_{50}$) ≥ 100 µg/mL) compared to the organic extracts (dichloromethane: methanol, 1:1). The organic extracts of the leaves, twigs, and stems were more potent than the aqueous extracts; they inhibited plasmodial activity with IC$_{50}$ of 5.4, 5.4 and 15 µg/mL; respectively (Clarkson et al., 2004). Therefore, Leonotis leonurus has a potential of being developed as an antimalaria drug.
Ojewole (2005) investigated the potential antinoceptive, anti-inflammatory, and antidiabetic properties of an aqueous leaf extract of *Leonotis leonurus* (50-800 mg/kg) in mice and rats. The thermal (hot plate method) and chemical (acetic acid) methods were used to evaluate the antinoceptive activity of the extract. A 3% solution of acetic acid was used to induce pain in the animal models. A significant dose dependent protection from both heat-induced pain and acetic-acid induced writhings was observed (p<0.05-0.001). The leaf extract (800 mg/kg) delayed the reaction time of the mice from heat-induced pain and acetic-acid induced writhings by 77.18 and 82.34%, respectively. The results obtained from the analgesic study suggested that the extract had centrally and peripherally mediated analgesic properties. It was concluded that the extract possibly inhibited COXs and lipoxygenases for the peripheral analgesic action, while the centrally mediated analgesic properties may possibly be mediated through the inhibition of central pain receptors. Fresh egg albumin (0.5 mL/kg) was injected into the subplantar surface of the right hind paw of the rats to induce acute inflammation (edema). A significant (p<0.05-0.001) dose-dependent reduction in inflammation was observed in the rat paw. It was postulated that the *Leonotis leonurus* leaf extract reduces inflammation through a mechanism similar to diclofenac, a commercial drug which is used to reduce inflammation and as an analgesic that reduces pain in a condition such as rheumatoid arthritis. It exerts its function by inhibiting prostaglandin synthesis. The drug has also been shown to reduce chemotaxis, superoxide toxic radical formation, oxygen-free radical formation, and neutral protease production by affecting the polymorphonuclear leukocyte function. DM was induced with streptozotin (STZ, 90 mg/kg). The extract showed significant (p<0.05-0.001) dose-dependent reduction in glucose levels of the diabetic rats (Ojewole, 2005).

There have been reports that *Leonotis leonurus* has been used traditionally to treat hypertension (Van Wyk *et al.*, 2000). Ojewole (2003) evaluated the aqueous extract for its cardiovascular and hypotensive effects on rats. The arterial blood pressures and heart rates of normal, anaesthetized spontaneously hypertensive rats was significantly reduced in a dose-dependent manner by the aqueous extract (25–800 mg/kg), p<0.05–0.001. The hypotensive effect of the extract was more pronounced in hypertensive than in normal
rats. The hypotensive effect of the extract was also observed in conscious, normal and spontaneously hypertensive rats, with their arterial blood pressures and heart rates being reduced in a dose-dependent manner. The aqueous extract (25–800 µg/mL) was found to act as vasorelaxant in aortic strips and portal veins of rats suspended in buffer under physiological conditions (Ojewole, 2003). Kenechukwu (2004) reported that *Leonotis leonurus* is a cardioactive plant. The cardiovascular parameters of the anaesthetized normotensive Wistar rats were modified by the aqueous and methanol extract. The aqueous extract showed a positive chronotropic effect in a dose dependent manner (0.5 and 1.0 mg) and a vasoconstrictive effect at higher doses (2.0-7.0 mg). The methanol extract showed a positive chronotropic and inotropic effect on the rats and on isolated working hearts. The aqueous extracts of the aerial parts of *Marrubium vulgare* has been reported to possess anti-hypotensive activity (El Bardai *et al.*, 2001; El Bardai *et al.*, 2003). The aqueous extract of *Marrubium vulgare* significantly reduced the systolic blood pressure in spontaneously hypertensive rats (p<0.05) (El Bardai *et al.*, 2001). The cyclohexane fraction of the aqueous extract (0.016–0.064 mg/mL) of this plant inhibited KCl-induced contraction in a dose-dependent manner in rat aorta incubated in a buffer under physiological conditions (El Bardai *et al.*, 2003). M which was isolated from *Marrubium vulgare* (log concentration, -6 to -4 M) significantly inhibited aorta contractions in a dose-dependent manner in isolated rat aorta with an IC₅₀ value of 24 ± 2.3 µM, p<0.05 (El Bardai *et al.*, 2003).

*Marrubium vulgare* was evaluated for its hypoglycaemic properties in rabbits (Ramos *et al.*, 1992). A solution of 50% dextrose (4 mL/kg) was used to induce hyperglycaemia. A decoction of the plant was prepared (132 g was boiled in a litre of water). The plant decoction (4 mL/kg) was administered (gastric administration) to the animals through a gastric tube and a glucose tolerance test was performed. *Marrubium vulgare* significantly reduced hyperglycaemia by 30.4% (p<0.05) and reduced the area under the glucose curve by 25.8%. *Marrubium vulgare* was again evaluated for its hypoglycaemic property and the ability to reduce serum lipids in T2DM patients (Herrera-Arellano *et al.*, 2004). Dried leaves were packed in a 1 gram filter paper envelop. An infusion of the packed dried leaves was prepared by boiling it in a cup of water for 5 minutes. The diabetic patients
were administered (oral administration) with this decoction 3 times a day for 21 days. *Marrubium vulgare* exhibited minimum hypoglycaemic effects, 0.64%. Cholesterol levels were reduced by 4.18%, while an insignificant reduction in triglycerides was observed, 5.78%. The aqueous leaf extract of *Marrubium vulgare* was found to be beneficial in cardiovascular diseases (CVD) by protecting human-LDL against lipid peroxidation and promoting HDL-mediated cholesterol efflux (Berrougui et al., 2006). The extract (25-100 µg/mL) significantly decreased lipid peroxidation, p<0.05. The extract was also found to scavenge free radicals and increase HDL-mediated efflux in a dose-dependent manner in THP-1 macrophages (Berrougui et al., 2006).

The toxicity of the aqueous *Leonotis leonurus* extract was evaluated in rats (Maphosa et al., 2008). For acute toxicity tests, doses between 200 to 3200 mg/kg were administered in the animals and a series of biochemical parameters were tested. Changes in rat behaviour were observed from 5 to 240 minutes after administration of the extract. The high doses (1600 and 3200 mg/kg) had a negative reaction on the animals. These doses caused a decrease in respiratory rate, motor activity, loss of righting reflex, ataxia, and salivation. Even death occurred as a result of the administration of 3200 mg/kg dose. Respiratory failure, convulsions, paralysis of skeletal muscles, and coma were observed before death. Sub-acute toxicity was tested with doses between 400 to 1600 mg/kg daily for 14 days. At doses of 1600 mg/kg, the extract significantly decreased red blood cells, packed cell volume, haemoglobin concentration, the number of platelets, white blood cells and its differential count (p<0.05) and also significantly decreased the levels of creatinine and alkaline phosphate (p<0.05). Chronic toxicity was evaluated with 200 and 400 mg/kg doses (daily for 35 days). Histological changes in internal organs such as the lungs and the kidneys were observed. Hyperplasia was observed in lungs and changes in the glomerulus in the kidneys. Based on the results obtained from this study, it was concluded that the farmers who use *Leonotis leonurus* for livestock ailments should be cautious when administering high doses of the plant as it can cause mortality or detrimental changes in biochemical parameters (Maphosa et al., 2008).
Since *Leonotis leonurus* has been used traditionally for various ailments, Ma (2006) evaluated the pharmaceutical suitability of an aqueous extract of this plant in order to formulate and produce capsules that would contain the same dosage contained in the decoctions that are used traditionally. Based on this study, it was concluded that it would be difficult to formulate a capsule from the aqueous extract because of its hygroscopic nature, resulting in stability problems.

Based on the current findings and literature on *Leonotis leonurus*, it provides an interesting indigenous South African plant to elucidate the scientific validity of its many traditional uses.
CHAPTER 2
INTRODUCTION TO THE STUDY
The MetS which is also known as IR or syndrome X is a cluster of interrelated risk factors that signal the elevated risk for T2DM and CAD or CVD (Singh et al., 2009; Meshkani and Adeli, 2009; Maury and Brichard, 2010). There are a number of definitions of the MetS. WHO defines MetS as a syndrome which includes IR along with at least two of the following states; hypertension, hyperlipidemia, obesity and microalbuminuria. A different criterion to define the MetS has been proposed by the National Cholesterol Education Programme (NCEP) Adult Treatment Panel (ATP) III. This Programme suggests that the MetS diagnosis requires at least 3 of the following 5 states; obesity, hypertriglyceridemia, decreased HDL cholesterol, hypertension or increased fasting blood glucose levels (Singh et al., 2009; Meshkani and Adeli, 2009). The International Diabetes Federation (IDF) defines the MetS as a syndrome which includes central obesity and the presence of any 2 of the following 4 states; hypertriglyceridemia, decreased levels of HDL-cholesterol, hypertension or impaired fasting glucose. Increased levels of inflammatory and/or abnormalities in the coagulation and fibrinolytic system, TNF-α, IL-6, PAI-1, or reduced levels of anti-inflammatory markers such as adiponectin (Meshkani and Adeli, 2009; Maury and Brichard, 2010) have been included in the IDF criterion.

In a previous study completed by Mnonopi (2007), it was found that the Leonotis leonurus extracts and M increased the rate of fibrinolysis, decreased platelet aggregation, and had potential antidiabetic activity by increasing insulin secretion in primary rat islets. The aim of the project is to elucidate the mechanisms by which the Leonotis leonurus extracts and M exhibit their profibrinolytic, antiplatelet and anti-diabetic activities. Based on the criterion defining MetS, it is possible that Leonotis leonurus could potentially combat this syndrome as it has been shown to increase glucose uptake and decrease obesity. It has anti-inflammatory and hypotensive properties, decreases coagulation and increases fibrinolysis. It is also known as a cardioactive plant.
A broad outline of the objectives of the study is given below.

2.1 Objectives:
(1) Extraction, quantification and standardisation of the extract using TLC and high performance liquid chromatography (HPLC).

(2) Determining the effect of the aqueous extract (AL), organic extract (OL) and marrubiin (M) on (a) coagulation, (b) fibrinolysis and (c) platelets.

(a) Coagulation
   (i) *In vitro, ex vivo and in vivo*
       • Determining the coagulation pathway(s) which is/are affected by the extracts and M by completing prothrombin time (PT) and activated partial thromboplastin time (APTT) tests that measure the integrity of the extrinsic and intrinsic pathways, respectively.
       • Determine the effect of the extracts and M on fibrinogen-C test which evaluates the formation of fibrin from fibrinogen.

(b) Fibrinolysis
   (i) *In vitro*
       Determining the mechanism by which the extracts and M mediate the profibrinolytic activity by evaluating:
       • Fibrinogen degradation which will be investigated by using SDS-PAGE.
       • D-Dimer formation, a fibrin degradation product, formation will be evaluated.
   (ii) *Ex vivo and in vivo*
       • Evaluating the effect of M on D-Dimer formation.
(c) Platelets

(i) *In vitro*
- Flow cytometry will be used to evaluate platelet aggregation using PAC-1, an antibody that only binds activated platelets.
- The anti-aggregatory potential of the extracts and M will be investigated for platelet adhesion and protein secretion. The acid phosphatase and BCA assays will be used to monitor these changes.
- Determining the intraplatelet calcium mobilization.
- Determining the ability of the extracts and M to inhibit TXA$_2$ synthase activity.

(ii) *Ex vivo and in vivo*
- Flow cytometry will be used to evaluate platelet aggregation using PAC-1 which is an antibody that binds only activated platelets.

(3) Determining the effect of the OL extract and M under diabetic conditions

(a) *In vitro*
- Determining the effect of the OL extract and M on insulin secretion. GSIS will be conducted on INS-1 cells to observe whether the results obtained from the rat islets can be reproduced in cultured cells.
- The effect of the OL extract and M on oxygen consumption rate will be investigated both under normoglycaemic and hyperglycaemic conditions.
- The effect of the OL extract and M on mitochondrial membrane potential (MMP) of INS-1 cells will be evaluated.
- The effect of the OL extract and M on gene expression of insulin and Glut-2 will be investigated.

(b) *In vivo* rat model
- Establishing an obese rat model using a cafeteria diet.
- Body weight and fasting blood glucose levels will be measured once a week.
Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) will be conducted to determine glucose intolerance and insulin sensitivity before and after treatment with extracts and M.

Post-experimental fasting plasma insulin levels will be determined.

Post-experimental triglyceride, high density lipoproteins (HDL), low density lipoproteins (LDL) and total cholesterol of the rat plasma will be determined.

The atherogenic index of each group will be determined.
CHAPTER 3
MATERIALS AND METHODS

3.1 Plant material
Leonotis leonurus was collected at Nelson Mandela Metropolitan University (NMMU) (South campus) as identified by Mr C. Thomas (the curator of the Department of Botany, NMMU).

3.2 Plant extraction procedure
The extraction procedure used was described by Light et al. (2002). The plant material was rinsed under running water, and dried at 50°C in an oven. The leaves were extracted either with 20 mL of water per gram of plant material for the AL or 10 mL of acetone per gram of plant material for the OL. Both extracts were sonicated for an hour, and filtered through Whatman No.1 filter paper. For the OL extract, the solvent was removed using a rotary evaporator at 60°C and distilled water was used to dissolve the remaining resin. The extracts were then freeze-dried and stored at 4°C in the dark.

3.3 Quantification of marrubiin
3.3.1 Quantification of marrubiin with 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 (Rf) of a compound is compared with the Rf of a known compound (preferably both run on the same TLC plate). 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). TLC was completed to quantify the M content in the AL and OL extracts. Ten milligrams of the OL and AL extracts were dissolved in 1 mL of toluene, of which 5 µL of this solution was applied on a pre-coated silica plate. Standard commercial M (LGC Promochem) (0.5- 5 µg) was spotted onto plates to prepare calibration curves used for quantification by densitometry (Alphalmager™ 3400); figure 3.1 (Scott et al., 2004).
3.3.2 Quantification of marrubiin with HPLC

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 varies for different compounds, retention time ($R_t$) on 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 (Boyer, 1993; Sheehan, 2009). 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 (e.g. carboxylic group for a weak acid cation, ammonium for a base anion exchanger) 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).

A reverse phase separation was completed using HPLC (128 Gold System, Beckman) to quantify the M content in the OL extract against M standard (LGC Promochem, UK). A kinetex C18 2.6 µm pentafluorophenyl (PFP) (10 x 0.46 cm) column was used (Phenomenex). The mobile phase was a mixture of acetonitrile and water, at a ratio of 50:50, and a flow rate of 1 mL/min.

3.4 In vitro: Coagulation, fibrinolysis and platelet studies

Table 3.1 depicts the range of concentrations used for the coagulation, fibrinolysis and platelet studies.

Table 3.1: A summary of the key used for the in vitro coagulation, fibrinolysis and platelet studies (final concentrations).

<table>
<thead>
<tr>
<th>Treatment (µg/mL)</th>
<th>1</th>
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<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tbody>
<tr>
<td>AL/OL</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>250</td>
<td>500</td>
<td>1000</td>
<td>2000</td>
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<tr>
<td>M</td>
<td>1.25</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
<td>100</td>
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<td>PC</td>
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<td></td>
<td>Heparin/thrombin/collagen/aspirin/plasmin for the respective assays discussed below.</td>
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<td>NC</td>
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<td>C</td>
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<tr>
<td></td>
<td>Untreated platelets or plasma</td>
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</table>
3.4.1 Coagulation studies
The coagulation cascade is divided into two pathways, namely: the extrinsic and the intrinsic pathways. The activation of these two pathways result in the generation of thrombin, the activation of fibrinogen, the release of fibrinopeptides, the formation of soluble fibrin, and finally the formation of F XIII-mediated cross-linked insoluble fibrin (refer to figure 1.12). PT and APTT were completed to evaluate the effect of the extracts and M on the extrinsic and intrinsic pathways, respectively. The changes in plasma levels of glucose or insulin affect the plasma levels of the coagulation factors. Plasma fibrinogen levels correlate with glycaemic regulation in cross-sectional studies in DM, these levels may fall during insulin therapy in humans (Ceriello, 1997). In DM patients, the inhibitors of the coagulation cascade are depressed due to their non-enzymatic glycation. The clots or insoluble fibrin are not dissolved as a result of the elevated levels of PAI. The fibrinogen-C test was completed to evaluate if the extracts and M could possibly inhibit or limit the formation of fibrin from fibrinogen. The prolongation of clot formation and inhibition of fibrin formation would result in less clot formation. This effect could potentially decrease CAD events in DM patients.

Calibration plasma (Beckman) was used to conduct the coagulation studies (PT, APTT), D-Dimer and fibrinogen-C assays. Healthy donors are used to prepare calibrated plasma. It is designed for the calibration of the ELECTRA coagulation systems for the determination of PT, APTT, fibrinogen, vWF, plasminogen, plasmin inhibitor, protein C and protein S (Beckman).

3.4.1.1 Prothrombin time test
The PT test which measures the extrinsic pathway is altered when there are changes in vitamin K-dependent coagulation factors (Azevedo et al., 2007). These factors include FII, FVII, FV and FX. A RecombiPlaStin kit (Beckman) was used to determine the clotting time. The Thromboplastin reagent which is included in the RecombiPlaStin kit is a liposomal preparation which contains a recombinant human tissue factor in a synthetic phospholipid mixture that has calcium chloride, a buffer, and a preservative (Tripodi et al., 1992). Thromboplastin reagent is very sensitive and the recombinant human tissue
factor contains no contaminating coagulation factors. This makes the PT reagent sensitive to deficiencies of FX, FVII, FV, and FII (Quick et al., 1966).

In the PT test, the extrinsic pathway is activated by the calcium ions, resulting in the formation of the fibrin clot from the conversion of fibrinogen to fibrin (Rizza and Walker, 1971; Quick, 1966). The PT test was performed according the manufacturer’s instructions. Recombiplastin (200 µL) was incubated at 37˚C for 180 sec on the CL Analyser (IVD, Beckman Coulter), while a premix of 100 µL of plasma was incubated for 180 sec at 37˚C with either the diluent factor (C), AL (1-4), OL (1-4), M (1-4) or heparin (0.1 U/mL, PC) (Bodene). To evaluate the PT, 150 µL of plasma premix solutions (as described above) were added to the recombiplastin (200 µL) and the clotting time was measured.

3.4.1.2 Activated partial thromboplastin time (APTT)
Alteration in the levels of factors VIII, IX, X, XI, and XII has an impact on APTT which measures the integrity of the intrinsic pathway (Azevedo et al., 2007). The SynthASil kit (Beckman) which is used to measure APTT contains the APTT buffered reagent. The APTT reagent contains synthetic phospholipids (which are required for optimal platelet-like activity) and highly defined non-settling colloidal silica. SynthASil is sensitive to reduced levels of contact factors (which are involved in the intrinsic and common pathways), the anticoagulant effect of heparin and the presence of inhibitors. Prolonged clotting time is observed in conditions such as: a deficiency of FXII, FXI, FX, FIX, FVIII, FV, FII, or fibrinogen, liver diseases, vitamin K deficiency, and the presence of heparin, lupus anticoagulant or other inhibitors (Van den Basselaar et al., 1993).

To evaluate the effect of the extracts and M on APTT, 100 µL of the APTT reagent was added to tubes containing 100 µL of plasma and either the diluent factor (C), AL (AL 1-3) OL (OL 1-3), M (M 1-3), or heparin (0.1 U/mL, PC). The mixtures were incubated at 37˚C for 180 sec on the CL Analyser, after which 100 µL of 0.025 M CaCl₂ solution was added to initiate the activation of the intrinsic pathway. The clotting time was measured, and the anticoagulatory activity of test samples evaluated relative to the untreated control (C).
3.4.1.3 Fibrinogen-C assay
Thrombin converts fibrinogen into fibrin in plasma. Under these conditions i.e. high thrombin levels and low fibrinogen levels, the rate of the reaction is a direct function of the fibrinogen concentration.

A fibrinogen-C kit (Beckman) was used to determine the effect of the extracts and M on the conversion of fibrinogen to fibrin by comparing the protein levels of the treated plasma with that of untreated plasma control (C). Impairment in the conversion of fibrinogen to fibrin occurs during the coagulation process when there are abnormalities in fibrinogen (Davis et al., 1969). Fibrinogen levels are used as a diagnostic marker to determine disease states that include disseminated intravascular coagulation (DIC), liver disease, inflammatory diseases and malignancies (Grannis, 1970). An increased risk in cardiovascular disease is directly associated with high levels of fibrinogen (Stone and Thorp, 1985; Kannel et al., 1987; Krobot et al., 1992). Thrombolytic therapy reduces the fibrinogen levels (Grannis, 1970).

The fibrinogen-C assay was performed according to the manufacturer’s instructions. Diluted plasma (10 x dilution; 200 μL) was incubated with 50 μL of either the diluent factor (C), AL (AL 1-4), OL (OL 1-4), M (M 1-4), or heparin (0.1 U/mL, PC) at 37°C on the CL Analyser for 180 sec. After the incubation period, a thrombin (Beckman) solution (35 U/mL; 100 μL) was added and the effect on the conversion of fibrinogen to fibrin was evaluated by comparing the fibrin levels of the treated plasma with that of the untreated plasma control (C).

3.4.2 Fibrin(ogeno)lysis
3.4.2.1 Fibrinogen degradation
Fibrinogen and fibrin degradation products are formed by the action of plasmin (Khavkina et al., 1995). Plasmin is specific to the Aα, Bβ, and γ chains of fibrinogen at regions binding the central E fragment with the two C-terminal D fragments (Odriljin et al., 1996). Fibrinogenolysis (fibrinogen cleavage by plasmin) yields similar fragments to those generated by fibrin. The physiochemical nature of fibrinogen differs from that of fibrin (Ekert and Muntz, 1972). Thrombin cleaves fibrinogen to fibrin, and in this
reaction FpA and FpB are released from the N-terminal of Aα and Bβ chains, respectively, refer to figure 1.13 (Wolberg, 2007). When fibrinogen is digested by plasmin, 3 fragment D species are formed, D1 (92 000 Da), D2 (86 000 Da) and D3 (82 000 Da). Fibrin digestion by plasmin results in 3 fragment D species which have a sum of 184 000 Da when molecular weights are combined (Fernson et al., 1975). Under physiological conditions, several pathways modulate the fibrinolytic activity of the endothelium. Two of the pathways are found to be altered in DM patients (Juhan-Vague et al., 1989). One pathway is the thrombomodulin-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 FV and FVIII, 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 DM patients (Kwaan, 1992; Matsuda et al., 1996). The profibrinolytic activity of the extracts and M was evaluated by assessing their effects on D-Dimer formation and fibrinogen degradation.

The effect of the extracts and M on fibrinogenolysis was evaluated. The untreated plasma, OL, AL, and M degradation products were analysed by using SDS-PAGE. SDS-PAGE is an electrophoretic method that can be used to determine the molecular mass of proteins. This method offers a high resolution-separation pattern of protein subunits. Most proteins bind to the detergent (SDS) in a constant weight ratio. The SDS masks the charge of the polypeptide chains, therefore, the protein subunits migrate on the polyacrylamide gels based on their molecular size. Separation of the protein subunits is achieved by a molecular sieve through the gel matrix. For reducing conditions; 2-mercaptoethanol or dithiothreitol is added to the gel matrix mix in order to cleave disulphide bonds on the polypeptide chains. This enables optimum binding of SDS to the denatured polypeptide. The molecular mass of the protein subunits can be estimated from
SDS-PAGE because there is a linear relationship between the logarithm of the molecular mass and relative mobility (Chiou and Wu, 1999; Laemmlli, 1970).

![Figure 3.2: A typical calibration curve depicting the relative mobility/migration (Rm) of the wide range molecular markers (Sigma).](image)

Fibrinogen (1 mg/mL) and plasmin (10 μg/mL) were incubated separately at 37°C for 3 min. After the incubation period, fibrinogen with plasmin (PC, 10 μg/mL), or M (M 6), or the plant extracts (AL 6 and OL 6) were incubated at room temperature (RT) for different time intervals (1.5 and 3 hours). The reactions were stopped by the addition of a denaturing buffer containing 2% sodium dodecyl sulfate (SDS) and 10% β-mercaptoethanol (Laemmlli, 1970). The reaction products were analysed by SDS-PAGE (figure 3.2) (Estêvão-Costa et al., 2000).

### 3.4.2.2 D-Dimer

When plasmin digests the insoluble fibrin, it yields a variety of soluble derivatives. These derivatives contain a D-Dimer domain which was not present on the original fibrinogen molecule, its degradation products, or on the insoluble fibrin (Gaffney, 1983; Palareti, 1993). The D-Dimer test is used widely as a diagnostic tool for thrombosis and
monitoring thrombolytic therapy (Bounameaux et al., 1994). High levels of D-Dimer are found in conditions such as deep vein thrombosis, PE and DIC (Gaffney et al., 1988).

The D-Dimer Latex reagent (Beckman) has a suspension of polystyrene latex particles that are coated with a monoclonal antibody that is highly specific for the D-Dimer domain. The coated latex particles (latex reagent) agglutinate when plasma contains the D-Dimer domain. The degree of agglutination is directly proportional to the D-Dimer concentration, which is determined by a turbidimetric immunoassay that measures the decrease of the transmitted light at 405 nm. The decrease of the transmitted light is caused by the formation of the aggregates (Newman et al., 1992).

The D-Dimer assay (Beckman) was performed according to the manufacturer’s instructions. The buffer (150 μL) was added to 42 μL of plasma containing 8 μL of either the diluent factor (C), AL (AL 1-3), OL (OL 1-3), or M (M 1-3). The mixture was then incubated at 37˚C in the CL Analyser for 15 minutes. After the incubation period the Latex D-Dimer was subsequently added. The effect of the extracts and M on the rate of fibrinolysis via D-Dimer formation was evaluated by comparing the protein levels of the treated plasma with that of the untreated plasma control. PC for this assay was heparin (0.1 U/mL).

3.4.3 Platelet aggregation/adhesion studies
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 researchers to mimic one or several aspects of these three phases to obtain reliable data (Reininger, 2006).
Platelet aggregation and adhesion studies were completed to evaluate the effect of the extracts and M on platelet activity.

3.4.3.1 Isolation of platelets
Blood was drawn by venipuncture from healthy human subjects. An anticoagulant solution consisting of sodium citrate (0.105 M, 10 µL) was used to prevent clotting. Healthy individuals were defined as people who have not been sick for 3 months prior to the experiment and were not using any medication. Platelet rich plasma (PRP) was obtained by centrifugation at 300 x g for 10 minutes, platelet poor plasma (PPP) was obtained by centrifugation at 900 x g for 15 minutes. The platelets were gently suspended in a buffer composed of 145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 0.5 mmol/L Na₂HPO₄, 6 mmol/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 at 37°C (Bellavite et al., 1994).

3.4.3.2 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. Platelet suspensions (200 µL) were placed in a 96-well microtiter plate for measurement of turbidity (BioTek Power Wave XS) (600 nm). Each sample was also used to perform triplicate platelet counts using a haemocytometer chamber. A standard curve was constructed correlating to the number of platelets at an A₆₀₀nm (figure 3.3).

![Figure 3.3: A standard curve showing the platelet number (20-120 x10⁶) using a haemocytometer chamber and a microtiter plate reader (A₆₀₀nm), the error bars represent SD of triplicate results (R² = 0.984).](image-url)
3.4.3.3 Platelet aggregation (microscopic method)
This method was described by Shahriyary and Yadzanparast (2007). A suspension of human platelets (5 x 10^6 platelets/mL, 1 mL) was preincubated with 10 μL of either buffer A (C) AL (AL 1-3), OL (OL 1-3), or M (M 1-3) extracts for 1 hour at 37˚C. After the incubation period, the platelets were activated with 0.5 U/mL thrombin (10 μL), and subsequently incubated for 20 minutes at 37˚C. The platelet suspensions were transferred from eppendorfs to 24 well-uncoated plates, for platelet aggregation and viewed under the microscope for platelet aggregation. ADP and collagen-induced platelet aggregation was not investigated. According to Shahriyary and Yazdanparast (2007), platelets were presumed to be aggregated when they formed clumps which were referred to as aggregates.

3.4.3.4 Platelet aggregation (flow-cytometry method)
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 0.5-40 μm 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 given sample volume. 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 is prone to standardization problems (Michelson, 2000). 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 (Michelson, 2000). Flow cytometry can be used to determine both
the activation state of circulating platelets and the reactivity of circulating platelets (Kasuya et al., 2006; Michelson et al., 2000; Shattil et al., 1987; Karlheinz et al., 1998; Heijnen et al., 1999; Michelson et al., 1991). The test monoclonal antibody used was PAC1. PAC1 (BD-Bioscience) 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 3.4) (Michelson, 2000).

![Figure 3.4: The effect of platelet activation on monoclonal antibody binding (Michelson et al., 2000).](image)

This method was adapted from Michelson et al. (1991). A PRP sample containing a platelet count of 30 x10⁶ platelets/mL was used for all flow cytometry experiments. A volume of 40 μL of PRP was aliquoted into tubes containing a saturated concentration of fluorescein isothiocyanate (FITC)-PAC1 (BD Biosciences) (10 μL) added. To inhibit fibrin polymerization, 2 μL of 2.5 mmol/L (final concentration) of peptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) (Sigma) was added. For a PC, 100 μL of thrombin stock (50 U/mL) was added to make a final concentration of 10 U/mL, the same volume of buffer was added for an untreated control (C), AL (AL 1-3); OL (OL 1-3) or M (M 1-3) were added. Unstained cells were used to determine background staining. 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 Beckman flow cytometer (Beckman Coulter, Cytomics FC 500). Twenty thousand
events were counted to measure fluorescent light scattering within each sample. All parameters were collected in list mode files and then analysed.

3.4.3.5 Platelet adhesion (microscopic method)
The extracellular matrix (ECM) is a network of proteins and proteoglycans that have various effects, such as proliferation, differentiation, survival, migration, and cell adhesion on the cells (Soucy and Romer, 2009; Young et al., 2009). ECM proteins include fibronectin, collagen, and laminin (Young et al., 2009). When a tissue is injured, platelets migrate and adhere to the site of injury. The platelets do not only adhere to the site of injury, but they also adhere to each other to form an aggregate which then amplifies the platelet aggregation signal. In this experiment, ECM coated plates (Novamed) were used as a biomimetic material in order to determine the effect of the extracts and M on platelet adhesion.

This method was described by Shahriyary and Yadzdanparast (2007). A suspension of human platelets (5 x10^6 platelets/mL, 1 mL) was preincubated with 10 µL of either buffer A (C), AL (AL 1-3), OL (OL 1-3), or M (M 1-3) extracts for 1 hour at 37°C. After the incubation period, the platelets were activated with 10 µL of 0.5 U/mL thrombin, and subsequently incubated for 20 minutes at 37°C. The platelet suspensions were transferred from eppendorfs to 24 well ECM plates and viewed under the microscope for platelet adhesion. ADP and collagen-induced platelet adhesion was not investigated. Flattening of the platelets on the plate surface with less platelets floating in solution was the criteria used by Shahriyary and Yadzdanparast (2007) to render platelet adhesion on ECM plates.

3.4.3.6 Platelet adhesion (spectrophotometric method)
The effect of the extracts and M on platelet adhesion was quantified using a spectrophotometric-based method. This method was described by Shahriyary and Yadzdanparast (2007). The activity of the enzyme acid phosphatase (an enzyme found in platelets) was used to measure the number of platelets. The activity of this enzyme is stable, independent of platelet stimulation, since it is not released. Since this enzyme is
not released, the number of resting and activated platelets can be determined using a standard curve with a known number of unstimulated platelets. \( p \)-Nitrophenol was used to determine the activity of acid phosphatase (Bellavite et al., 1994).

A suspension of human platelets (5 x10\(^6\) platelets/mL, 1 mL) was preincubated with 10 μL of either buffer A (C), AL (AL 1-3), OL (OL 1-3), or M (M 1-3) extracts 1 hour at 37°C. After the incubation period, the platelets were activated with 10 μL thrombin (0.5 U/mL), and subsequently incubated for 20 minutes at 37°C. Aliquots (1 mL) of the platelet suspensions were added to the ECM plates. A solution of 1 mg/mL \( p \)-nitrophenyl phosphate (\( p \)-NPP) in citrate buffer (140 μL) [0.1 M sodium citrate, 0.1 M acetic acid, and 0.1% (w/v) Triton-X 100, pH 5] was added to the plate. Fifty microlitres of PRP or PPP was used for estimation of total platelet count or as a blank, respectively. The plate was incubated at RT for 1 hour with gentle motion. After incubation, the reaction was stopped and colour development was initiated with the addition of 100 μL of NaOH (2 M). The formation of the product \( p \)-nitrophenol was measured at 412 nm on a microtitre plate reader (BioTek Power Wave XS). The percentage of platelet adhesion was determined according to the following formula: (sample-blank/total-blank) x 100, figure 3.5 (Eriksson and Whiss, 2005).

3.4.3.7 Platelet acid phosphatase assay

Human platelet suspensions (15 to 60 x10\(^6\) platelets/mL, 1 mL), containing known platelet numbers were incubated with \( p \)-nitrophenyl phosphate (140 μL) [0.1 M sodium citrate, 0.1 M acetic acid, and 0.1% (w/v) Triton-X 100, pH 5]. The formation of the product \( p \)-nitrophenol was measured at 412 nm on a microtitre plate reader (BioTek Power Wave XS) and this was used as a function of platelet number. The standard curve that was obtained was used to determine the number of platelets attached to the ECM plates, figure 3.5 (Shahriyary and Yadzdanparast, 2007).
Figure 3.5: A standard curve showing the absorbance at 412 nm by the reaction product $p$-nitrophenol as a function of platelet number (40-120 x10^6/mL), the error bars represent SD of three independent experiments in triplicate ($R^2 = 0.95$).

3.4.3.8 Protein secretion assay
Activated platelets secrete granule contents such as α-granule proteins, β-thromboglobulin, platelet factor 4 and platelet-derived growth factor (PDGF) as well as ADP from the platelet dense granules. ADP amplifies the platelet response induced by other agonists and stimulates the generation of TXA$_2$ (Clemetson, 1999; Cassar et al., 2003; Rand et al., 2003; Clutton et al., 2001; Coller, 1995). The effect of the extracts and M on protein secretion was evaluated using the bicinchoninic acid (BCA) assay.

The principle of the BCA assay is similar to the Lowry procedure. Both methods rely on the formation of Cu$^{2+}$-protein complex under alkaline conditions. The formation of the complex is followed by the reduction of Cu$^{2+}$ to Cu$^+$. The amount of reduction is proportional to the amount of protein present. The formation of the purple-blue complex of BCA with Cu$^+$ in alkaline conditions is monitored at a maximum absorbance of 540 nm. The advantages of using the BCA method are (i) it is more sensitive and applicable than either Biuret and Lowry procedures, (ii) it has less variability than the Bradford assay, (iii) the colour complex that is formed is stable, (iv) it is less susceptible to
detergents, and (v) it is applicable over a broad range of protein concentrations (www.sigma.com).

A suspension of human platelets (5 x10^6 platelets/mL, 1 mL) was pre-incubated with 10 µL of either buffer A (C), AL (AL 2-4), OL (OL 2-4), or M (M 2-4) extracts for 1 hour at 37°C. After the incubation period, the platelets were activated with 10 µL of thrombin (0.5 U/mL), and subsequently incubated for 20 minutes at 37°C (Shahriyary and Yadzdanparast, 2007). The platelets were then centrifuged at 1100 x g for 20 minutes. The levels of protein released into the supernatant were quantified using the BCA method, figure 3.6 (Smith et al., 1985).

![BSA standard curve](image)

Figure 3.6: An example of a BSA standard curve (0.0625-2 mg/mL) used during experimentation. The error bars represent SD of triplicate standards per experiment, R² = 0.972.

### 3.4.3.9 Thromboxane B₂ assay

The Invitrogen human TXB₂ ELISA kit is a competitive immunoassay which is used to determine the quantity of TXB₂ in biological samples. The assay is based on the competition between TXB₂ and TXB₂-alkaline phosphatase tracer for a limited amount of TXB₂-specific antiserum. The signal obtained with the assay is inversely proportional to the amount of TXB₂ in each sample. The microtitre plate was pre-coated with mouse anti-rabbit IgG, which binds to all TXB₂ antiserum that were added to the well. After the
incubation step, the plate was thoroughly washed and a solution of a substrate p-NPP for alkaline phosphatase was added. A yellow product was formed and absorbance was read at 412 nm in a microtitre plate reader (BioTek Power Wave XS) to quantify TXB₂ in each sample (www.invitrogen.com).

TXA₂ is a strong platelet aggregation agonist. It induces Ca²⁺ mobilization and PKC activation via the activation of phospholipase Cβ. The change in platelet shape and eventually the adhering of platelets to the site of injury is caused by this increase in [Ca²⁺]ᵢ and PKC activation (Jin et al., 2007; Jin et al., 2005).

PRP (5 x 10⁶ platelets/mL, 1 mL) was incubated with either buffer A (C), AL (AL 2-4); OL (OL 2-4); M (M 2-4) or 10 µL thrombin (0.1 U/mL) at 37˚C for 180 sec. The amount of TXB₂ was assayed by the Invitrogen human TXB₂ ELISA kit according to the manufacturer’s instructions, table 3.2 (Invitrogen).

Table 3.2: The TXB₂ protocol for standard and test sample preparation (www.invitrogen.com).

<table>
<thead>
<tr>
<th>Well</th>
<th>Tris buffer (µL)</th>
<th>Standard/sample (µL)</th>
<th>Tracer (µL)</th>
<th>Antibody (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td></td>
<td></td>
<td>50 (at development)</td>
<td></td>
</tr>
<tr>
<td>NSB</td>
<td>150</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>B₀</td>
<td>100</td>
<td></td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Standard/sample</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The amount of TXB₂ reflects the activity of TXA₂ synthase which catalyses the reaction that results in TXB₂ formation.

Corrected B₀ (maximum binding) was calculated by subtracting the average NSB (non-specific binding) from average B₀. TA is the total activity of the tracer.

%B/B₀ = (Sample or standard – average NSB)/corrected B₀ x 100
A log-log standard curve was constructed by plotting the % $B/B_0$ for each standard on the $y$–axis and the known concentrations on the $x$–axis. TXB$_2$ concentrations for samples were then calculated from the standard curve, figure 3.7. The amount of TXB$_2$ reflects the activity of TXA$_2$ synthase (www.invitrogen.com).

![Figure 3.7: An example of an immunoassay standard curve of plasma TXB$_2$ standards (0.016-2 ng/mL) used during experimentation. The error bars represent SD of triplicate standards per experiment, $R^2 = 0.953$.]

### 3.4.3.10 Calcium mobilization assay

The fluorescent Ca$^{2+}$ indicators are the most commonly used tools to measure [Ca$^{2+}$]. The introduction of Fura-2 has been a major advance in the field of Ca$^{2+}$ research. Fura-2 is a fluorescent dye which is a ratiometric indicator of Ca$^{2+}$ which forms a stable complex with Ca$^{2+}$ ($K_d$ of 0.14 M, at pH of 7.2 and temperature of 22°C). Fura-2 is available commercially in its ester form (Fura-2/AM, figure 3.8) and salt (Fura-2 salt) form. An advantage of the ester form is that it is membrane permeable, which allows for easy loading into the cells. In its ester form Fura-2 cannot bind Ca$^{2+}$ but once it is inside the cell; it is de-esterified by intracellular esterases. The de-esterification process allows it to be trapped in the cell, thus allowing the binding of Ca$^{2+}$ (Gulaboski et al., 2008).
Calcium mobilization plays a key role in platelet activation and aggregation because it is required in the release of granules. As it has been stated in section 3.4.3.9, an increase in 
\([\text{Ca}^{2+}]_i\) results in changes in platelet shape and adhesion.

Cytosolic \(\text{Ca}^{2+}\) measurements were obtained by using the fluorescent dye Fura 2-AM. PRP was resuspended in Tyrode’s buffer containing no calcium at a density of \(3 \times 10^8\) platelets/mL (1 mL). Platelets were loaded with Fura-2/AM (1 µL) (4 µM final concentration) and incubated at 37°C for 30 minutes. The unloaded dye was removed through a washing step with the buffer. PRP was resuspended in 1 mM CaCl\(_2\) Tyrode’s buffer (1 mL) with either 10 µL of Tyrode’s buffer (C), AL (2-4); OL (2-4); or M (M 2-4) and 10 µL of thrombin (0.1 U/mL) or collagen (2 µg/mL). Two agonists were used, thrombin and collagen, in order to investigate the specificity/non-specificity of the extracts and M. The suspensions were incubated at 37°C for 5 minutes. Measurement of the cytosolic \(\text{Ca}^{2+}\) concentration was performed using black microtitre plates on a Fluroskan Ascent FL spectrophotometer to determine the relative fluorescence (excitation 340 nm, 380 nm and emission, 510 nm). Figure 3.9 shows the standard curve which was used to determine \(\text{Ca}^{2+}\) mobilization.
Figure 3.9: An example of Ca²⁺ standard curve (25-400 ng/mL). The error bars represent SD of triplicate standards per experiment, R² = 0.98.

3.5 *Ex vivo* rat model for coagulation studies

Table 3.3 summarises the experimental groups that were used for the *ex vivo* rat model.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Key</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control rats (1% Tween in saline)</td>
<td>C</td>
</tr>
<tr>
<td>Aspirin-treated rats (50 mg/kg)</td>
<td>PC for platelet inhibition</td>
</tr>
<tr>
<td>M-treated rats (50 mg/kg)</td>
<td>M</td>
</tr>
</tbody>
</table>

Animals were obtained from the University of Cape Town Animal Unit and housed in pairs/triplicates in standard animal cages. The animals weighing 250-350 g were exposed to a 12 hour day/night cycle. The rats were fed rat chow and water *ad libitum* (Gadi *et al.*, 2009). They were allowed to acclimatize for 1 week and thereafter were randomly divided into 3 groups of 6 rats per group (table 3.3). The untreated control group (C), the group treated with aspirin (PC) to inhibit platelet aggregation and the group treated with M, the experimental group. When treatment was terminated, blood was extracted from the heart ventricles and collected using a 1 mL syringe that contained 100 µL of 0.105 M sodium citrate. The blood was then centrifuged at 300 x g for 15 min. The PRP was
assayed for the coagulation parameters (PT, APTT, fibrin formation, D-Dimer and platelet aggregation (section 3.4.1.1, 3.4.1.2, 3.4.1.3 and 3.4.2.1, respectively). The plasma collected was stored at -20°C until the coagulation parameters were assayed. Platelet aggregation was conducted on fresh PRP (see section 3.4.3.4).

3.6. In vitro diabetic studies on INS-1 cells
Based on the coagulation and platelet studies, the most promising findings were obtained for the OL extract. Numerous studies have been completed on the AL extract. M is only present in the OL extract and one of the aims of the study was to establish whether the anticoagulant, anti-platelet and anti-diabetic effects of the OL extract can be attributed to M. Based on these reasons, it was decided to continue only with the OL extract for the diabetes studies. The effect of the OL extract and M on glucose-stimulated insulin secretion (GSIS) was evaluated on INS-1 cells. Insulin levels of chronic, basal and stimulated samples were determined. Oligomycin and camptothecin were the NC in the oxygen consumption and MMP studies, respectively.

3.6.1 Maintenance of INS-1 cells
INS-1 cells are the most widely used insulin-secreting cells lines. The behaviour of INS-1 cells do not perfectly mimic the physiology of primary β-cells, however, they are extremely useful in studying the molecular events underlying β-cell function (Poitout et al., 1996). The rat INS-1 cells secrete insulin in response to glucose concentrations in the physiological range (Hohmeier et al., 2000). INS-1 cells were kindly donated by Professor M. Donath (University of Zurich, Switzerland). The cells were maintained at 37°C in a humidified environment with 10% CO₂. They were supplemented with RPMI 1640 media containing glutamax, 5% fetal bovine serum (FBS), 10 mM HEPES, 50 mM 2-mercaptoethanol and 1 mM sodium pyruvate. When the cells reached 70% confluency, they were subcultured using a 1:3 split ratio. For all the in vitro diabetic studies, the INS-1 cells were cultured in both normoglycaemic (11.1 mM glucose) and hyperglycaemic conditions (33.3 mM glucose).
3.6.1.1 Glucose-stimulated insulin secretion in INS-1 cells

The glucose-stimulated insulin secretion (GSIS) is controlled by glucose-derived signals in the pancreatic β-cells (Špaček et al., 2008). During hyperglycaemic conditions, insulin enhances glucose uptake and utilization (Jellinger and Mace, 2007). GSIS was performed to determine whether the OL extract or M had an effect on insulin secretion in INS-1 cells under normo- and hyperglycaemic conditions. Table 3.4 summarises the treatments used for GSIS studies.

Table 3.4: A summary of the key used for the GSIS studies.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL (µg/mL)</td>
<td>2.5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>M (ng/mL)</td>
<td>125</td>
<td>250</td>
<td>500</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td>untreated cells</td>
</tr>
</tbody>
</table>

The method that was used was described by Maedler (2001). Cells were seeded at a density of 30 000 cells/mL in 24 well plates and left overnight to attach. After 48 hours of exposure of the INS-1 cells to a range of OL extract (OL 1-3) or M (M 1-3) in normo- (11.1 mM glucose) and hyperglycaemic (33.3 mM glucose) conditions, GSIS was performed as follows:

- 1 mL of media was transferred into an eppendorf tube (chronic sample, equivalent to 48 hour insulin secretion).
- The remaining RPMI media was removed from the 35 mm culture plates.
- 1 mL of 1 x Krebs 3.3 mM glucose solution was added to the plates.
- The INS-1 cells were incubated at 37°C for 30 minutes.
- Subsequent to the incubation period, the solution was aspirated off.
- 1 mL of 1 x Krebs 3.3 mM glucose solution was added to each culture plate.
- The INS-1 cells were incubated at 37°C for 1 hour.
- This solution was transferred into an eppendorf (basal sample, insulin secretion after stimulation with low glucose concentration).
- 1 mL of 1 x Krebs 16.7 mM glucose solution was added to each plate.
• The INS-1 cells were incubated at 37˚C for 1 hour.
• This solution was transferred into an eppendorf tube (stimulated sample, insulin secretion after stimulation with a high glucose concentration).
• 1 mL of 0.18 M HCl in 70% ethanol was added to the dishes.
• The INS-1 cells were incubated at 4˚C for 1 hour.
• This solution was transferred into an eppendorf tube (insulin content sample, insulin content of the INS-1 cells).
• All samples were stored at -20˚C, until analyzed using a radioimmunoassay (RIA) to determine insulin levels.
• The stimulatory index was determined using the following equation: Stimulatory index = glucose stimulated insulin secretion ÷ basal insulin secretion.

3.6.1.2 Rat insulin radioimmunoassay
A Linco rat insulin RIA kit was used to determine insulin levels according to the manufacturer’s instructions (table 3.5). In a RIA, 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 unlabelled antigen can be generated, and the concentration of the antigen in the unknown sample can be determined using the standard curve (figure 3.10). Therefore the most important entities in a RIA 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).
Table 3.5: Protocol for the determination of insulin content (www.millipore.com).

<table>
<thead>
<tr>
<th>Tube number</th>
<th>Tube number</th>
<th>Assay buffer (µL)</th>
<th>Standards and samples</th>
<th>[¹²⁵I] insulin tracer (µL)</th>
<th>Rat insulin antibody (µL)</th>
<th>Vortex, cover and incubate at 4°C for 24 hours</th>
<th>Precipitating reagent (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2</td>
<td>3,4 (NSB)</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5,6 (B₀)</td>
<td>-</td>
<td>100 µL</td>
<td>0.1 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>7,8</td>
<td>-</td>
<td>100 µL</td>
<td>0.2 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>9,10</td>
<td>-</td>
<td>100 µL</td>
<td>0.5 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>11,12</td>
<td>-</td>
<td>100 µL</td>
<td>1 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>13,14</td>
<td>-</td>
<td>100 µL</td>
<td>2 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>15,16</td>
<td>-</td>
<td>100 µL</td>
<td>5 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>17,18</td>
<td>-</td>
<td>100 µL</td>
<td>10 ng/mL</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>19-∞</td>
<td>-</td>
<td>Samples</td>
<td>100</td>
<td>100</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

After the addition of the precipitating reagent, the samples were vortexed and incubated for 20 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1 mL Beckman Ready Gel® liquid scintillation cocktail (Beckman).

The average of the NSB, tubes 3–4, was subtracted from the average of each tube, except the total counts. The percent of tracer bound was calculated as follows:

\[
\%B = \left( \frac{\text{Total binding counts (tubes 5–6)}}{\text{Total counts (tubes 1–2)}} \right) \times 100
\]
The percent of total binding (% B/B₀) for each standard and sample was calculated as follows: % B/B₀ = (Sample or standard/Total binding) x 100

A log-log plot was constructed by using the % B/B₀ for each standard concentration. Insulin concentrations for samples were extrapolated from the standard curve, figure 3.10 (Morgan and Lazarow, 1963; Thorell and Lanner, 1973; Feldman and Rodbard, 1971; Westgard, 1981).

Figure 3.10: An example of a radioimmunoassay standard curve of rat insulin standards (0.2-10 ng/mL) used during experimentation. The error bars represent SD of triplicate standards per experiment, R² = 0.973, n= 5.

The samples from GSIS (section 3.6.1.1) were incubated with ¹²⁵I 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.

3.6.1.3 Oxygen consumption
A Rank Brother Oxygen Electrode, an electrochemical oxygen sensor, was used to determine the oxygen consumption of INS-1 cells. It is capable of detecting 10⁻⁴ atm to
1atm oxygen, and detects the oxygen through the use of two electrodes, namely a platinum (cathode) and silver (anode) electrode which is separated from the test medium via an oxygen permeable Teflon membrane. A 3M KCl solution connects the two electrodes. The platinum is reduced to form water when it comes into contact with oxygen, while the silver electrode is oxidised to form AgCl. The current that is produced from the oxidation-reduction reactions is related to the partial pressure of oxygen using the following formula (Hitchman, 1978):

\[ I_d = 4.0F.0P_m.0A.0P(0_2)/0b \]

Where \( F \) = Faraday’s constant,

\( P_m \) = oxygen permeability of the Teflon membrane

\( A \) is the surface area of the Pt electrode, and

\( b \) = the thickness of the Teflon membrane

Table 3.6 summarises the treatments used for oxygen consumption studies.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL (µg/mL)</td>
<td>10</td>
</tr>
<tr>
<td>M (ng/mL)</td>
<td>500</td>
</tr>
<tr>
<td>C</td>
<td>untreated cells</td>
</tr>
<tr>
<td>NC</td>
<td>Oligomycin-treated cells (100 µM)</td>
</tr>
</tbody>
</table>

Table 3.6: A summary of the key used for the oxygen consumption studies.

Oligomycin is an ATP synthase activity blocker, and it was used as NC. It inhibits ATP synthase activity by suppressing the proton channel which is necessary for oxidative phosphorylation of ADP to ATP. INS-1 cells cultured in 10 cm dishes were allowed to reach confluence. Once the cells were confluent, they were treated with the OL extract (OL 3) and M (M 3) in RPMI media for 48 hours (under both normo- and hyperglycaemic conditions). After the treatment period, a cell count was conducted using Trypan Blue and a haemocytometer. RPMI media (1 mL) was used to set the oxygen limit. Cell aliquots (1 mL, \( 10^6 \) cells/mL) in fresh media were placed within the incubation
chamber set at 37˚C. The oxygen consumption of these cells was then continuously measured over 500 seconds (sec). LabChart 6 was used to capture and analyse data. Respiration rates were calculated from the initial steady state of the oxygen uptake traces and expressed per 10^6 viable cells. (Affourtit and Brand, 2008). Experiments were performed using RPMI media (control) as described in 3.6.1, the OL extract, M, C, and oligomycin (100 µM).

3.6.1.4 Mitochondrial membrane potential
The effect of camptothecin (NC), OL (OL 3) and M (M 3) on the MMP of INS-1 cells was evaluated using a fluorescent dye called 5,5΄,6,6΄-tetrachloro-1,1΄,3,3΄-tetraethylbenzimidazol-carbocyanine iodide (JC-1). JC-1 is a lipophilic cationic probe which exists in monomeric form with an emission wavelength of 527 nm and excitation wavelength of 490 nm. The emission wavelength shifts to 590 nm when hyperpolarisation of the mitochondrial membrane occurs. At this stage, JC-1 forms JC aggregates. The advantages of staining the mitochondria with JC-1 are (1) it is a quantitative and qualitative method, (2) it is possible to identify populations that have different mitochondrial content, (3) it has been extensively studied to observe the behaviour of the mitochondria in a variety of conditions, and (4) has been validated by analysing the MMP at a level of single mitochondria (Salvioli et al., 1997).

Table 3.7 summarises the treatments used for oxygen consumption studies.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL (µg/mL)</td>
<td>10</td>
</tr>
<tr>
<td>M (ng/mL)</td>
<td>500</td>
</tr>
<tr>
<td>C</td>
<td>untreated cells</td>
</tr>
<tr>
<td>NC</td>
<td>Camptothecin-treated cells (100 µM)</td>
</tr>
</tbody>
</table>

The MMP in INS-1 cells was determined by using the JC-1 flow cytometry Mitochondrial Membrane Potential Detection kit (BD Bioscience). Camptothecin was
used as an NC. Camptothecin is an anticancer drug which causes cytotoxic effects in cancerous cells. It binds and inhibits topoisomerase I resulting in a decrease in MMP and apoptosis of the infected cells (Rang *et al.*, 1999). Cells which had been exposed to experimental conditions (OL 3, M 3, camptothecin (100 µM) and C) for 48 hours were detached from using a standard trypsin treatment and resuspended in 1 mL of the culture media. Cells from each experimental population (20 000 cells/mL) were stained with JC-1 dye (according to the manufacturer’s instructions) then exposed to the flow cytometry for the quantification of the effect of the experimental conditions to MMP. The treated cells were washed with 0.1 M phosphate buffered saline (PBS, pH 7.4) and then gently resuspended in 0.5 mL of 1 x JC-1 reagent solution. Thereafter, the cells were incubated at 37°C in a 5% CO₂ incubator for 15 minutes. The cells were then centrifuged for 5 minutes at 400 x g and the supernatant was removed. The pellet was washed twice with a 1 x assay buffer (2 mL) and centrifuged at 400 x g for 5 minutes. The pellet was resuspended in 500 µL of 1 x assay buffer and the cells were analyzed by flow cytometry.

3.6.1.5 Total RNA isolation and quantification
The INS-1 cells which were cultured in 10 cm dishes were exposed to OL 3 or M 3. RNA was isolated for quantitative real-time polymerase chain reaction (RT-qPCR) using an RNeasy Mini kit (Qiagen) for extraction, using the selective binding properties of a silica-gel-based membrane combined with the speed of microspin technology. A highly denaturing buffer containing guanidine isothiocyanate was 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 was eluted with RNase-free water (http://www.qiagen.com). RNA extraction was performed in 10 cm dishes and was performed as follows:

- A 100 x dilution of β-mercaptoethanol was prepared in RLT buffer supplied by Qiagen to lyse the islets (350 µL of the lysis buffer is required per plate).
- Cells were scraped with a cell scraper.
• 350 µL of 70% ethanol was added to the homogenized lysate, and mixed well with a pipette.
• 700 µL of the sample was added to an RNeasy spin column placed in a 2 mL collection tube.
• The sample was centrifuged at 8000 rpm (eppendorf mini spin, F-45-12-11, rotor size: 6.1 cm) for 15 sec, and the flow through was discarded.
• 700 µL of buffer RW1 was added to the RNeasy spin column, and this was centrifuged at 8000 rpm for 15 sec, the flow through and the collection tube was discarded.
• The RNeasy column was transferred into a new 2 mL collection tube.
• 500 µL of Buffer RPE was added to the column, and centrifuged at 8000 rpm for 15 sec.
• The flow through was discarded, and 500 µL of Buffer RPE was added again, and the column was centrifuged at 8000 rpm for 15 sec.
• The flow through was discarded, and the column was transferred into a new 2 mL collection tube, and centrifuged at 8000 rpm for 1 minute.
• To elute, the column was transferred into a new 1.5 mL collection tube, and 40 µL of RNase-free water was added directly to the silica-gel membrane.
• This was centrifuged at 8000 rpm for 1 minute.

To concentrate the RNA, the flow through was eluted through the column once again, and centrifuged at 8000 rpm for 1 minute.

RNA quantification is an essential step prior to gene expression analyses (Aranda et al., 2009). Quartz cuvettes are used to quantify RNA at 260 nm. A reading of 1 unit corresponds to 40 µg of RNA per mL. The A260/A280 ratio was used to determine the purity of RNA, using Tris-HCl buffer (pH 7.0) to prepare a 50 x dilution of each sample.
A QuantiTect Reverse Transcription kit (Qiagen) was used according to the manufacturer’s instructions, to generate cDNA for each sample, in order to perform RT-qPCR.

### 3.6.1.6 Reverse transcriptase assay
The reaction mixtures were prepared as shown in table 3.8 and 3.9.

#### Table 3.8: Genomic DNA elimination reaction components (www.qiagen.co.za).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (1 reaction)</th>
<th>Volume (2 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Wipeout buffer, 7x (µL)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Template RNA</td>
<td>up to 1 µg</td>
<td>up to 2 µg</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Total volume (µL)</td>
<td>14</td>
<td>28</td>
</tr>
</tbody>
</table>

#### Table 3.9: Reverse transcription reaction components (www.qiagen.co.za).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription mastermix</td>
<td></td>
</tr>
<tr>
<td>Quantiscript Reverse Transcriptase</td>
<td>2</td>
</tr>
<tr>
<td>Quantiscript RT Buffer, 5 x</td>
<td>8</td>
</tr>
<tr>
<td>RT Primer mix</td>
<td>2</td>
</tr>
<tr>
<td>Entire genomic DNA elimination reaction (Table 3.8)</td>
<td>28</td>
</tr>
<tr>
<td>Total Volume</td>
<td>40</td>
</tr>
</tbody>
</table>

### 3.6.1.7 Real-time quantitative polymerase chain reaction
This technique is very sensitive, as a result, very low levels of cDNA can be detected. RT-qPCR is frequently used for primary validation of gene expression, bacterial species identification, and single nucleotide polymorphism genotyping (Dvoràk et al., 2003).

The most commonly used fluorescent DNA binding dye is Sybr green. Sybr green is monitored at an excitation and emission wavelengths of 494 and 521, respectively.
Detection is monitored during the extension phase of RT-qPCR (www.qiagen.com). As in the case of classical polymerase chain reaction (PCR), one reaction of RT-qPCR consists of strand separation, annealing and elongation steps. Fluorescence is monitored for each sample on each cycle of RT-qPCR. The increase in the number of copies of cDNA of interest is observed by an increase in fluorescence intensity, the fluorescence linearly increases until a plateau is reached (Dvorák et al., 2003). Table 3.10 has a list of gene primers which were used as reference genes and the genes of interest with their accession numbers and amplification temperatures.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>5'-3'</th>
<th>3'-5'</th>
<th>Amplification T°C</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclo A</td>
<td>XM541363</td>
<td>ACGTGGTCAAGACCTGAGTGG</td>
<td>CGTGCTCCCCACACGACCC</td>
<td>58</td>
<td>104.8</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_017008</td>
<td>TCCTTGCCCTCAGACACATG</td>
<td>ACTTGAGTTCCTCTCTTAC</td>
<td>60</td>
<td>104.6</td>
</tr>
<tr>
<td>β-Tubulin</td>
<td>NM_175102</td>
<td>AAACCCAGCCCCAGTTCTAAG</td>
<td>GCCATCCTCGTGCTCAAATG</td>
<td>58</td>
<td>104.8</td>
</tr>
<tr>
<td>Glut-2</td>
<td>NM_012879</td>
<td>ATGACATCAATGGCCACAGACAC</td>
<td>GGACACAGACAGACAGACAG</td>
<td>60</td>
<td>98.2</td>
</tr>
<tr>
<td>Insulin</td>
<td>NM_019129</td>
<td>TGCCCCAGGCTTTGTCAAA</td>
<td>TCCACCTTCAGGGAGCTT</td>
<td>60</td>
<td>95.6</td>
</tr>
</tbody>
</table>

The reference genes were used to normalize data obtained from RT-qPCR. Three reference genes, viz cyclophilin A (cyclo A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-tubulin, were tested to observe their stability across all the experimental conditions. The Cq value denotes the number of cycles required before the fluorescence reaches a threshold. A higher target DNA level in a sample correlates to a lower Cq value (Fujikawa et al., 2006). Insulin and Glut-2 were the genes of interest.
GeNorm was used to determine the stability of the reference genes and all the reference genes used had a gene expression stability measure ($M_{RT-qPCR}$) and the pairwise variation (V) values were less than 1.5 (Hellemans et al., 2007). Efficiency was determined for β-tubulin, cyclo A, GAPDH, Glut-2 and insulin by setting up standard curves for each of these genes using the BIO-RAD iQ detection system. IQ software calculated the gene efficiency of each gene. Figure 3.11 shows a typical standard curve for tubulin.

Figure 3.11: A typical standard curve for tubulin (10-30 ng/mL), the RT-qPCR amplification efficiency was 104.8%.

3.7 *In vivo* diabetic studies
Three PCs were used for the *in vivo* studies: metformin, sulfonylurea and aspirin. Metformin is a drug which enhances glucose uptake, sulfonylurea induces insulin secretion and aspirin is a cardioprotective drug that inhibits platelet aggregation. Table 3.11 summarises the treatment used for these diabetic studies.
3.7.1 Experimental animals

Table 3.11 summarises the key used for the *in vivo* animal studies.

**Table 3.11: A summary of the key that was used for the *in vivo* animal model.**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Key</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean control rats (1% Tween in saline)</td>
<td>LC</td>
</tr>
<tr>
<td>Obese control rats (1% Tween in saline)</td>
<td>OC</td>
</tr>
<tr>
<td>Metformin-treated obese rats (50 mg/kg)</td>
<td>PC MET</td>
</tr>
<tr>
<td>Sulfonylurea-treated obese rats (50 mg/kg)</td>
<td>PC SUL</td>
</tr>
<tr>
<td>Aspirin-treated obese rats (50 mg/kg)</td>
<td>PC ASP</td>
</tr>
<tr>
<td>OL-treated obese rats (50 mg/kg)</td>
<td>OL</td>
</tr>
<tr>
<td>M-treated obese rats (50 mg/kg)</td>
<td>M</td>
</tr>
</tbody>
</table>

Animals were obtained from the University of Cape Town Animal Unit and housed in pairs/triplicates in standard animal cages. The animals were exposed to a 12 hour day and night cycle. Animals were randomly divided into 7 groups with 6 rats per group. The lean control group (LC), the obese control group (OC), the obese metformin PC group (PC MET), the obese sulfonylurea PC group (PC SU), the obese aspirin PC group (PC ASP), the obese OL treatment group (OL) and the obese M treatment group (M) (Ashour *et al*., 2009). When the experiment was designed, equivalent M levels in OL and M were going to be used (1000 mg/kg due to the 5% M in OL and 50 mg/kg, respectively), however, as a result of solubility problems of the OL extract, 50 mg/kg was used for both OL and M. Solubility tests were completed on the OL extract and concentration >50 mg/kg were found to be insoluble in 1% Tween in saline. The lean rats were fed rat chow and the obese rats were fed on a cafeteria diet (18% protein, 41.4% carbohydrates and 4.5% fat content, caloric consumption: 15% protein, 68% carbohydrates and 17% fat) and water *ad libitum* for 8 weeks and treated for 2 weeks. The control group was treated with the vehicle suspension (1% Tween in saline). The body weight and fasting blood glucose levels (extracted from tail vein) were measured weekly.
3.7.2 Biochemical assays completed

3.7.2.1 Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT)

Glucose and insulin tolerance tests were performed after a 15 hour fasting period. Rats were injected intraperitoneally with either glucose (2 g/kg body weight) or insulin (1 unit/kg body weight). Blood glucose levels were determined from the tail vein at 0, 30, 60 and 120 minutes. Blood glucose was determined with a glucometer (OneTouch select system kit, Lifescan) using 10 μL of blood collected from the tip of the tail vein after the insulin or glucose administration. IPGTT and IPITT were performed post-treatment. The One Touch select strips are specific to D-glucose.

Glucose oxidase oxidises glucose according to the following reaction:

\[ \text{D-glucose} + \text{O}_2 \rightarrow \text{D-gluconic acid} + \text{H}_2\text{O}_2 \]

The \( \text{H}_2\text{O}_2 \) formed by the oxidase reaction may be measured colorimetrically through the use of a coupled enzyme. \( \text{H}_2\text{O}_2 \) is coupled via peroxidase to a chromogenic \( \text{O}_2 \) acceptor which undergoes a colour change (www.lifescan.com).

Area under curve (AUC) of glucose (AUC\(_g\)) and insulin (AUC\(_i\)) were calculated by using the following formula: \( \text{AUC} = (\text{concentration}_0 \ (\text{mM}) + \text{concentration}_1 \ (\text{mM}))/2 \times \text{time}_1 - \text{time}_0 \ (\text{min}) \) (Chiou, 1978).

3.7.2.2 Plasma triglyceride, cholesterol levels and coagulation parameters

Triglycerides are formed by an ester bond between 3 fatty acid molecules and glycerol. Determination of triglyceride concentration is used as a diagnostic tool in DM patients. The triglyceride GPO-PAP kit (Roche Diagnostics) is an enzymatic colorimetric assay. Lipoprotein lipase is used to completely hydrolyse triglycerides into its constituents. Glycerol is phosphorylated by glycerol kinase to yield glycerol-3-phosphate, which is oxidized to dihydroxyacetone phosphate and \( \text{H}_2\text{O}_2 \) by glycerol-3-phosphate oxidase (GPO). \( \text{H}_2\text{O}_2 \) reacts with 4-aminophenazone and 4-chlorophenol to yield 4-(p-benzoquinone-monoimino)-phenazone. This reaction is catalysed by peroxidase. The red colour intensity of 4-(p-benzoquinone-monoimino)-phenazone is directly proportional to
the triglyceride concentration. Standard glycerol concentrations were used to construct the standard curve, figure 3.12 (Stein and Myers, 1995).

Figure 3.12: A typical standard curve for glycerol (0.015-0.2 mM). The error bars represent SD of triplicate standards per experiment, R² = 0.993.

At the end of the experiment, blood was drawn from the ventricle of the rat’s heart and collected using a 1 mL syringe that contained 100 µL of 0.105 M sodium citrate. The blood was then centrifuged at 300 x g for 15 min. The triglyceride (table 3.12), HDL, LDL and total cholesterol (table 3.13) of the plasma were determined using enzymatic kits (Roche Diagnostics).

Table 3.12: Determination of triglyceride levels in the rat plasma samples.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol standard/test plasma sample</td>
<td>100</td>
</tr>
<tr>
<td>Triglyceride reagent</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubate at RT for 15 minutes and read absorbance at 540 nm

Cholesterol is a steroid which is synthesised by various tissues, particularly the liver and the intestinal wall. Cholesterol determination is used as a diagnostic tool to assess atherosclerosis risk, lipid and lipoprotein metabolic disorders. The cholesterol CHOD-PAP kit (Roche Diagnostics) was used to determine total cholesterol in the plasma
samples, table 3.13 and figure 3.13. In this enzymatic colorimetric assay, cholesterol esters are cleaved by cholesterol esterase to yield cholesterol, which is oxidised into cholest-4-en-3-one and H₂O₂. H₂O₂ reacts with 4-aminophenazone and 4-chlorophenol to yield 4-(p-benzoquinone-monoimino)-phenazone. This reaction is catalysed by peroxidase. The red colour intensity of 4-(p-benzoquinone-monoimino)-phenazone is directly proportional to the cholesterol concentration (NIH, 1990).

Table 3.13: Determination of cholesterol levels in the rat plasma samples.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol standard/test plasma sample</td>
<td>100</td>
</tr>
<tr>
<td>Cholesterol reagent</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubate at RT for 15 minutes and read absorbance at 540 nm

![Graph](image)

Figure 3.13: A typical standard curve for cholesterol (0.2-1 mmol/L). The error bars represent SD of triplicate standards per experiment, R² = 0.975.

HDL was determined by adding 50 µL of the HDL-cholesterol precipitating reagent (Roche) in the same samples that were used for total cholesterol determination. The difference between total cholesterol and HDL-cholesterol concentrations equates to the
LDL-cholesterol concentration in each sample. The absorbance was read at 540 nm (Lopes-Virella et al., 1977). The atherogenic index (AI) of plasma from each group was determined using the following formula: AI = (total cholesterol (average) – HDL-cholesterol (average)) / HDL-cholesterol (average). The AI reflects the distribution of the particle sizes in lipoprotein subclasses. Its quantity is used to assess the risk factors associated with atherosclerosis (Holmes et al., 2008). Based on the epidemiological data, there are 3 risk categories for AI, low <0.11, intermediate 0.11-0.21 and high >0.21 (Holmes et al., 2008). The plasma collected was stored at -20°C until the coagulation parameters were assayed (sections 3.4.1.1, 3.4.1.2, 3.4.1.3 and 3.4.2.1). Platelet aggregation was conducted on fresh PRP.

3.7.2.3 Plasma glucose and insulin levels
Fasting glucose levels were measured by the glucose oxidase method using glucose strips (OneTouch select system kit, Lifescan) and a glucometer (OneTouch Select, AW 06505401A Lifescan). Fasting plasma insulin concentrations were determined by a radioimmunoassay kit using a double-antibody method (Linco Research), refer to section 3.6.1.2. Figure 3.14 shows the standard curve which was constructed to determine the insulin content of rat plasma samples.

Figure 3.14: An example of a radioimmunoassay standard curve of rat insulin standards (0.2-10ng/mL) used during experimentation. The error bars represent SD of triplicate standards, $R^2 = 0.981$. 

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3.8 Ethical clearance
Ethical clearance for this study was approved by the NMMU HumanEthic Committee and the Animal Ethic Committee (see appendix for a copy).

3.9 Statistical Analysis
Results are expressed as mean ± SEM or SD, unless otherwise indicated. Student t-tests were performed to determine whether the observed results were statistically significant, where values of P<0.05 and P<0.01 were considered to be statistically significant. All groups were compared to relevant C unless otherwise stated.
CHAPTER 4
RESULTS
4.1 Marrubiin Quantification
The quantification of the M content of Leonotis leonurus was completed using TLC and RP-HPLC. The thin-layer chromatogram (figure 4.1) identified the presence of M in the OL extract. The R_f value for the experimental conditions of the commercial M was found to be 0.55 as was that of the spot which corresponded with M 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 M in the TLC chromatogram in figure 4.1. A similar R_f value (0.66) was obtained by Rey et al. (1992), when pure M was identified using TLC. The picture had to be taken immediately because the spots faded with time.

![TLC Chromatogram](image)

Figure 4.1: A typical TLC chromatogram depicting the identification of the marrubiin content from the OL and AL extracts. Lanes 1-6 have decreasing concentrations of the M standard (5000, 4000, 3000, 2000, 1000 and 500 ng, respectively). Lane 7-8, the OL extract (10 mg/mL) and lane 9-10 the AL extract (10 mg/mL). Five microlitres of each extract was loaded.

From the thin-layer chromatogram, a standard curve was constructed (figure 3.1, page 64), and used to determine the concentration of M from OL. This was found to be 2600
ng per 50 μg loaded on the TLC plate, therefore, the percentage of M in the OL extract was 5.2%. The AL extract had no M. In literature only the percentage of premarrubiin, a precursor of M, is known, viz. 0.01567% (Scott et al., 2004).

Ionic RP-HPLC was performed to quantify M content in the OL extract. A standard curve of various M concentrations was constructed to determine the M content in the OL extract, figure 4.2.

![Figure 4.2: A standard curve using commercial marrubiin (0.03-0.25 mg/mL). The error bars represent SD of triplicate results, R = 0.975.](image)

Figure 4.3 illustrates an example of a chromatogram obtained for commercial M applied on the PFP column, using acetonitrile: water (50: 50). The R_t of M was found to be 4.84 minutes. When the OL extract was applied, an example of the RP-HPLC elution profile that was obtained is shown in figure 4.4. Numerous resolved peaks were obtained. At a R_t of 4.83 minutes, a resolved peak was obtained correlating to the commercial M. The M content of the OL extract was found to be 5%, similar to that obtained from TLC.
Figure 4.3: A chromatogram of the PFP (10 x 0.46 cm, Phenomenex) elution profile of commercial M (0.25 mg/mL, 10 µL injection). Samples were eluted from the column using acetonitrile:water (50:50), using a flow rate of 1 mL/min. The absorbance was monitored at 225 nm.

Figure 4.4: A chromatogram of the PFP (10 x 0.46 cm, Phenomenex) elution profile of the OL extract (1 mg/mL, 10 µL injection) M. Samples were eluted from the column using acetonitrile:water (50:50), using a flow rate of 1 mL/min. The absorbance was monitored at 225 nm.
The concentration of M used throughout this study was found to be 5 % within the OL extract. Equivalent concentrations of the M standard were used in order to evaluate if the biological effects of the OL extract could be contributed to M, unless otherwise stated.

4.2 Coagulation assays
The effect of the extracts and M on clotting time was evaluated using the APTT and the PT tests (figure 4.5).

![Figure 4.5](Image)

Figure 4.5: The effect of the extracts and marrubiin on the (A) PT and (B) APTT clotting times. All data represents mean ± SEM, (n=3). * p< 0.05, # p<0.01, relative to the untreated control (C).

The PC (0.1 U/mL heparin) significantly prolonged clotting time to 21.3±0.02 sec, while the OL prolonged the clotting time to 22.2±0.07 sec at 50 µg/mL (OL 2). OL 1 (25 µg/mL), OL 2 (50 µg/mL) and OL 3 (100 µg/mL) prolonged the clotting time by
19.1±0.01, 22.2±0.01 and 18.8±0.05 sec, respectively compared to C 13.3±0.01 sec, p<0.05. No concentration dependent prolongation of the clotting time for the AL extract and M was noted. The AL extract and M prolonged the clotting time to 17.8±0.02 and 18.3±0.01 sec at 50 µg/mL (AL 2) and 1.25 µg/mL (M 1), respectively compared to C (p<0.05) (figure 4.5A).

The PC (0.1 U/mL heparin) significantly prolonged APTT to 70.25±0.03 sec (p<0.01), while the extracts prolonged the clotting time by 51.5± 0.05, 51.6±0.04, and 42.5±0.05 sec at concentrations of 5, 100, and 100 µg/mL for M, the OL, and AL extracts, respectively, compared to an untreated control, 21± 0.5 sec, p<0.05 (figure 4.5B).

The effect of the extracts and M on fibrin formation was evaluated using the fibrinogen-C assay (figure 4.6).

![Figure 4.6: The effect of the extracts and marrubiin on fibrin formation. All data represents mean ± SEM, (n=3). * p< 0.05, # p<0.01, relative to the untreated control (C).](image)

The extracts significantly decreased fibrin formation (in a dose-dependent manner) at almost all concentrations tested (p<0.05) (figure 4.6). At 25 µg/mL the OL 1 extract and M 1 (1.25 µg/mL) both decreased fibrin formation 1.3-fold (p<0.05) while AL 1 (25
µg/mL) increased fibrin formation 1.3-fold when compared to C. At 50 µg/mL, the OL 2 extract and M 2 (2.5 µg/mL) both decreased fibrin formation 2.4-fold (p<0.01) while AL 2 significantly decreased fibrin formation 1.0-fold (p<0.05) when compared to C. At 100 µg/mL the OL 3 extract and M 3 (5 µg/mL) both decreased fibrin formation 4.4-fold (p<0.01) while AL 3 significantly decreased fibrin formation 1.5-fold (p<0.05) when compared to C. M 4 (10 µg/mL) and the OL 4 extract (200 µg/mL) both significantly decreased fibrin formation 6.4-fold (p<0.01), whereas AL 4 extract (200 µg/mL) decreased fibrin formation 2.4-fold (p<0.01). Heparin (0.1 U/mL) significantly decreased fibrin formation 8-fold (p<0.01), relative to C.

High D-Dimer levels in plasma indicate elevated levels of fibrin formation and reflect a state of hypercoagulability (Moresco et al., 2006). The effect of the extracts and M on D-Dimer formation was evaluated (figure 4.7).

![Figure 4.7](image)

Figure 4.7: The effect of the extracts and marrubiin on D-Dimer formation. All data represents mean ± SEM, (n=3). * p< 0.05, # p<0.01, relative to untreated control (C).

The OL 1 extract and M 1 significantly inhibited D-Dimer formation 1.4-fold (p<0.05) at 25 and 1.25 µg/mL, respectively, while AL 1 significantly inhibited D-Dimer formation 1.8-fold at 25 µg/mL, (p<0.01), relative to C. At 50 µg/mL, the OL 2 extract and M 2
(2.5 μg/mL) both significantly inhibited D-Dimer formation 1.9-fold (p<0.01), while AL 2 inhibition was 3-fold (p<0.01). M 3 (5 μg/mL) and the OL 3 extract (100 μg/mL) inhibited D-Dimer formation 2.6-fold, while the AL 3 extract (100 μg/mL) inhibited D-Dimer formation 3.2-fold (p<0.01), relative to C, figure 4.7. The PC (0.1U/mL heparin) reduced D-Dimer formation by 4-fold (p<0.01).

4.3 Fibrino(gen)olysis
Electrophoretic (SDS-PAGE) analysis (figure 4.8) under denaturing and reducing conditions showed that the extracts and M (M 3) degraded fibrinogen.

Figure 4.8: SDS-PAGE (12%) depicting fibrinogen degradation under reducing conditions. Lines: 1) Molecular mass markers (kDa), 2) Fibrinogen incubated without an enzyme or extract for 3 hours, 3) Fibrinogen incubated with plasmin, 4 and 5) Fibrinogen incubated with M (5 μg/mL) for 1.5 and 3 hours; respectively, 6 and 7) Fibrinogen incubated with the OL extract (100 μg/mL) for 1.5 and 3 hours; respectively, 8 and 9) Fibrinogen incubated with the AL extract (100 μg/mL) for 1.5 and 3 hours; respectively.

Plasmin (PC, 10 μg/mL) in lane 3 completely degraded the Aα chain and Bβ chains while the γ chain was partially degraded compared to the untreated fibrinogen control in lane 2.
Degradation products of plasmin action could be observed around 45, 44, and 14.2 kDa. There was no difference between fibrinogen treated with M for 1.5 and 3 hours (lane 4 and 5; respectively), although there was visible degradation of the Bβ and the γ chain. The OL 3 extract completely degraded the Aα chain, while the Bβ and the γ chains were partially degraded after 1.5 and 3 hours (lane 6 and 7). After 3 hours, the AL 3 extract had degraded the Aα chain, (lane 9) but there was very little degradation of the Bβ and the γ chain, a degradation product of 48 kDa could be observed.

4.4 Platelet aggregation and adhesion
A microscopic image of the platelets attached to ECM plates is shown on figure 4.9A. There was no platelet aggregation observed for the untreated control platelets (C) compared to the PC (thrombin, 50 U/mL) tplatelets (figure 4.9A). No aggregation was observed for M (5 μg/mL) and the OL 3 extract (100 μg/mL) treated platelets with the strongest inhibition of platelet aggregation noted for M (5 μg/mL). To quantify and determine the mechanism of inhibition on platelet aggregation, the effect of the extracts and M on GP IIb/IIIa, a platelet surface glycoprotein was determined. This glycoprotein mediates platelet aggregation by binding fibrinogen when the platelets are activated; therefore, regulation of this glycoprotein is essential in platelet aggregation (Kim et al., 2008a).

Figure 4.9B displays typical histograms for thrombin-induced platelet aggregation using flow cytometry. The addition of OL and AL extract, or M on thrombin-activated platelets decreased the expression of the surface marker of platelet aggregation. These results confirm the microscopic investigation (figure 4.9A). More than 85% of the PC platelets were activated by thrombin, whereas the addition of the extracts significantly decreased the population of activated platelets. The percentage of inhibition of platelet aggregation was determined (figure 4.9C) and indicates that both the extracts and M inhibited the expression of GIib/IIIa in a concentration dependent manner. The OL 3 extract (100 μg/mL) and M 3 (5 μg/mL) were more potent than the AL (100 μg/mL) extract, showing 92.5, 91.6 and 69.5% platelet aggregation inhibition; respectively.
Figure 4.9: (A) Platelet aggregation on non-coated plates, where C = untreated control, PC = positive control, n = 5. PC: thrombin-induced platelet aggregation, forming ‘clumps’ or aggregates. C: no induction of platelet aggregation. M 3 (5 µg/mL) represented an equivalent concentration in the OL 3 extract (100 µg/mL). (B) Typical histograms obtained for the inhibitory effect of the extracts and M on thrombin-induced PAC-1 expression on platelets (n = 5), where OL = 25-100 µg/mL; AL = 25-100 µg/mL; M = 1.25-5 µg/mL; (C) Percentage inhibition of platelet aggregation by AL, OL and M.

When platelets were activated with thrombin (PC), they flattened and attached to the ECM plates, whereas inactive platelets remained unattached (C) (figure 4.10A).
Figure 4.10: (A) Platelet adhesion on ECM plates, n = 3. Activated platelets (PC) were flattened and adhered to the plates. The untreated platelets control (C) platelets did not attach to the plate, but floated. The various treatments displayed a similar trend; (B) The effect of the treatment, at different concentrations, on the adhesion of platelets. * p<0.05, # p<0.01, relative to the PC.

Although the OL 3 (100 µg/mL) and AL 3 (100 µg/mL) extracts also decreased platelet adhesion, it was not as significant as compared to M exposure (Figure 4.10A). Platelet adhesion was further quantified by using the cellular acid phosphatase assay (figure 4.10B). All concentrations tested in vitro with the exception of AL 1 extract (25 µg/mL), indicated that M and the extracts significantly decreased platelet adhesion. The percentage platelet adhesion in PC was 99.8%. Where there was no induction of platelet adhesion (C), percentage of adhered platelets was 24%. M 1 extract (1.25 µg/mL), significantly decreased platelet adhesion by 78.9% (p<0.05), relative to PC. When activated platelets were exposed to M 2 and 3 (2.5 and 5 µg/mL) adhesion to ECM plates
was significantly decreased to 50.6 (M 2) and 27.1% (M 3), p<0.01, respectively, relative to the PC. At the lowest concentration, OL 1 (25 µg/mL) significantly decreased platelet adhesion to 78.7% (p<0.05), relative to PC. The OL extract significantly decreased thrombin-induced platelet adhesion to 57.9 and 37.1% at 50 (OL 2) and 100 µg/mL (OL 3), p<0.01, respectively. The percentage of adhered platelets was significantly decreased to 52 and 37% by the AL 2 and 3 extract at 50 and 100 µg/mL, respectively, p<0.01.

4.5 Protein secretion
All concentrations tested significantly reduced protein secretion (figure 4.1). M decreased protein secretion by 31.8, 55.9 and 77.2% at 2.5, 5 and 10 µg/mL, respectively, relative to the control (PC).

![Figure 4.1](image.png)

Figure 4.11: The effect of marrubiin and the extracts on protein secretion relative to PC. All data represents mean ± SEM (n=3).

The OL extract had a similar trend to M, it inhibited protein secretion by 27.9, 50.7 and 77.4% at 50, 100 and 200 µg/mL, respectively, whereas the AL extract decreased protein secretion by 18.6, 55.4 and 60% at 50, 100 and 200 µg/mL, respectively, relative to PC.
The IC\textsubscript{50} values were determined to be 5.46, 110.6, and 138 µg/mL for M, OL, and AL extract, respectively. The OL extract was found to be more effective at inhibiting protein secretion than the AL extract, it had a lower IC\textsubscript{50} value.

4.6 Thromboxane B\textsubscript{2} assay

TXA\textsubscript{2} is formed from AA. It is a strong platelet agonist that causes irreversible platelet aggregation. It is unstable and is hydrolyzed non-enzymatically to form TXB\textsubscript{2} (Kim \textit{et al.}, 2008a). Therefore, the levels of TXB\textsubscript{2} are a measure of TXA\textsubscript{2} and TXA\textsubscript{2} synthase activity. The extracts and M significantly (p<0.05) inhibited the level of TXB\textsubscript{2} formation in thrombin-induced platelet aggregation in a dose-dependent manner (figure 4.12). The PC (thrombin, 0.1 U/mL) had a 4.3-fold increase in TXB\textsubscript{2} formation as compared to the C (p<0.01).

![Figure 4.12](image.png)

Figure 4.12: The effect of the extracts and M on TXB\textsubscript{2} formation in thrombin-stimulated platelets. All data represents mean ± SEM (n=3). * p< 0.05, # p<0.01, relative to the PC.

At 50 µg/mL, the AL and OL extracts inhibited TXB\textsubscript{2} formation by 1.8- (p<0.05) and 2.4-fold (p<0.01), respectively, relative to PC. At an equivalent concentration of M (2.5 µg/mL) a 2.4-fold inhibition was obtained (p<0.01), relative to PC. At 100 µg/mL, the AL and OL extracts inhibited TXB\textsubscript{2} formation 4.3- (p<0.01) and 6.8-fold (p<0.01),
respectively, while an equivalent M concentration in the OL extract, M inhibited TXB₂ formation by 4.3-fold (p<0.01), relative to PC. At 200 µg/mL, the AL and OL extracts showed a 4.3- (p<0.01) and 8.5-fold inhibition (p<0.01), respectively, while M inhibited TXB₂ formation 8.5-fold (p<0.01), relative to PC.

4.7 Calcium mobilization
The extracts and M reduced the intracellular levels of Ca²⁺ that was stimulated by both agonists in a dose-dependent manner (figure 4.13).

![Figure 4.13: The effects of the extracts and marrubiin on (A) thrombin- and (B) collagen-induced calcium mobilization. All data represents mean ± SEM (n=3).](image)

The extracts inhibited both thrombin- and collagen-induced calcium mobilization to the same degree. The AL extract inhibited thrombin-induced calcium mobilization by 15-
40% in the range tested (50-200 µg/mL, the IC$_{50}$ value was at 200 µg/mL. The OL extract and M inhibited calcium mobilization by 26-58% (50-200 µg/mL) and 27-60% (2.5-10 µg/mL), respectively, at the range tested (figure 4.13A).

The IC$_{50}$ value was at 150 µg/mL and 7 µg/mL for the OL extract and M, respectively. Collagen-induced calcium mobilization was inhibited by 37-61% by the AL extract at 50-200 µg/mL. The OL extract inhibited collagen-induced calcium mobilization by 47-71% at 50-200 µg/mL, where as M at equivalent concentrations (2.5-10 µg/mL) had a 48-72% inhibition (figure 4.13B). The IC$_{50}$ values were determined to be 124, 54 and 3 µg/mL for AL, OL and M, respectively. The OL extract was found to be more effective at inhibiting calcium mobilization than the AL extract based on the IC$_{50}$ values. Two agonists were used in this experiment to observe whether the extracts and M had any specificity, but based on the findings, they did not show specificity as the degree of inhibition was relatively similar for both thrombin- and collagen-induced calcium mobilization.

4.8 *Ex vivo* model
The effect of M (50 mg/kg) on platelet aggregation and on coagulation parameters was further studied in an *ex vivo* animal model as it takes into account the cell-based theory of coagulation. Similar to the results obtained *in vitro*, M had no effect on the extrinsic pathway (figure 4.14A); however, the clotting time was prolonged in the intrinsic pathway, figure 4.14B. M significantly prolonged the clotting time 1.9-fold (p< 0.05). Aspirin (50 mg/kg) displayed similar results to M. It increased the clotting time 1.5-fold (p<0.05). Aspirin is a known antiplatelet agent, which blocks thromboxane synthesis through the acetylation of platelet COX (Ashour *et al.*, 2009).
Aspirin inhibited platelet aggregation by 78%. However, M had a pronounced effect on thrombin-induced platelet aggregation. It inhibited platelet aggregation by 84.5%, figure 4.14C. M and aspirin had a similar effect on D-Dimer formation, they significantly reduced D-Dimer formation 1.8-fold (p<0.05). Although M reduced fibrin formation in vitro, this was not noted in the ex vivo model, figure 4.14E.
4.9 Discussion and conclusions

Two methods of quantifying M were used, TLC and RP-HPLC. TLC was easy, convenient and cost effective. An Rf value of 0.66 for M was obtained from TLC and this value was in agreement with literature for the identification of M (Scott et al., 2004; Rey et al., 1992). A similar value was obtained for the OL extract. It was determined that the OL extract has 5% M, however, the AL extract contained no M.

Previous literature on RP-HPLC completed on Leonotis leonurus extracts only obtained fingerprint profiles (Bienvenu et al., 2002; and Scott et al., 2004). Several attempts were made to quantify M using the same chromatographic conditions used for the HPLC fingerprints for Leonotis leonurus, this however did not allow for quantification of M in the plant extract (results not shown). The chromatographic column used was a VYDAC C18 column with 5 µm particle size (4.6 x 250 mm), (Luna). The mobile phase was 1% acetic acid and methanol at a flow rate of 1 mL/min. Different ratios of the mobile phase were applied, but the resolution and separation of the plant extract was poor and M could not be retained on the column based on its chemical characteristics.

Ion-pairing RP-HPLC was used as an alternative in an attempt to improve the separation of the plant extract which contained polar, ionic and non-polar components. Tetrabutylammonium phosphate (TBA), an ion-pairing reagent was utilised using a C18 column. TBA improved the binding of M to the column as it is a cation, which bound to the negatively charged M (due to hydroxyl group and carbonyl group) to enhance its retention on the column. The mobile phase was 10 mM ammonium dihydrogen phosphate/5 mM TBA (pH 3.5) and 1% acetonitrile at a flow rate of 1 mL/min. The absorbance was monitored at 225 nm. However, the disadvantages of this method were that TBA is expensive and it formed crystals that blocked the HPLC pumps, hence causing problems with the flow of the mobile phase. In addition the pH of the ammonium dihydrogen phosphate/5 mM TBA had to be at pH 3.5 to ensure binding of M to the column, this decreased the lifetime of the RP-HPLC column. This method was also time consuming, a complete program took 40 minutes (results not shown).
The results obtained in RP-HPLC formed a basis for further development of a RP-HPLC method for quantifying M. An ionic RP-HPLC method using a PFP chromatography was developed. This method was easy and cost effective as it required acetonitrile and water (mobile phase), TBA was not required to improve retention as the resin had pentafluorophenyl as a functional group. As can be seen the chromatographic profiles of the OL extract (figure 4.4) and M (figure 4.3) displayed a resolved M peak which could be easily quantified. The advantages of this method are that it is convenient, no additional equipment is required, no salt was required and it saves time. The program duration is only 10 minutes. It also provides a novel method for the quantification of M.

The PT and APTT tests are used to distinguish between the effects of test agents on the extrinsic and intrinsic pathways (Brown, 1988). Experiments completed in vitro for the OL extract significantly prolonged PT clotting time, however, did not significantly affect the PT in the ex vivo model. M (1.25 µg/mL) and the AL extract (50 µg/mL) prolonged PT clotting time, however the M and AL range of concentrations tested had no effect on PT in the in vitro assays. These results were compared to heparin, a commonly used anticoagulant. Heparin belongs to a family of sulphated glycosaminoglycans and acts as an anticoagulant by activating antithrombin III which inhibits thrombin (Rang et al., 1999). When heparin binds to antithrombin III, a complex is formed. This complex enhances the antiprotease activity of antithrombin III by promoting the binding of the complex to its substrate (Guglielmone et al., 2002; Fu et al., 1995). In a study conducted by Kim et al. 2008a, heparin (10 µM) was found to prolong the PT clotting by 19.53±0.86 sec (p<0.05) which is comparable to our findings, where heparin (0.1 U/mL) prolonged the clotting time by 21.3±0.02 sec.

Alterations in the levels of factors VIII, IX, X, XI, and XII have impacted on the APTT which measures the integrity of the intrinsic pathway (Azevedo et al., 2007). Other studies have shown that heparin prolonged APTT clotting time by 46.77±1.79 sec at 1 µM (p<0.05) (Kim et al., 2008a). Our findings displayed a significant prolongation of the APTT clotting time for heparin, the OL extract, AL extract and M. The degree of prolongation of clotting time (in vitro) by the OL extract and M was similar. The OL
extract, AL extract and M prolonged the APTT clotting time 2.5-, 2- and 2.5-fold, respectively, relative to C. Heparin prolonged the clotting time 5.3-fold. Similar findings of plant extracts have been obtained for *Jatropha curcas*, a tropical Africa plant, which had little or no effect on PT, however, it displayed an anticoagulant effect by prolonging the APTT clotting time (Osoniyi and Onajobi, 2003). These *in vitro* results provided some evidence for an anticoagulant effect of the AL extract, OL extract and M which could possibly be mediated by the inhibition of intrinsic pathway factors. Additional investigation is required to determine the specificity of the plant extracts and M to FVIII, IX, X, XI and XII.

It has been found that prolongation of the intrinsic and the extrinsic pathways are associated with a slow rate of fibrin formation (Azevedo *et al.*, 2007). Fibrinogen levels in plasma can be reflected by PT and APTT clotting curves. The absorbance change in PT and APTT reflect plasma fibrinogen levels, and fibrinogen levels can be estimated from either PT or APTT clotting curves (Zhang and Bai, 2008). Heparin has been found to decrease thrombin generation and fibrin formation in patients with CAD (Linder *et al.*, 1999). Fibrinogen plays a key role in haemostasis. It prevents blood loss by forming a haemostatic plug. It is a precursor of fibrin and its plasma concentration directly influence the rate of fibrin formation and deposition. Elevated fibrinogen levels can cause CAD due to the hypercoagulable state it creates. The binding of fibrin to GPIIb/IIIa results in the activation of platelets and the release of platelet granules. Therefore fibrinogen plays a key role in the balance of thrombotic and thrombolytic events. The fibrin(ogen) lowering drugs would help in the management of chronic disease such as DM, hypertension and CAD (Handley and Hughes, 1997). The *in vitro* experimentation noted a decrease in fibrin formation in treated plasma compared to the untreated plasma (C) in the fibrinogen-C test by M, AL and OL extracts. The degree of inhibition of fibrin formation by the OL extract and M were similar. Heparin decreased fibrin formation by 8-fold.

D-Dimers are a product of fibrin degradation. The plasma levels of D-Dimer are used to predict the DVT and PE, but can also be used as a marker of thrombus formation. Ziai *et
*al.* (2005) found a correlation between coagulation and D-Dimer formation. Patients with thrombotic events had significantly high plasma levels of D-Dimer. The reduction in clot formation by heparin (40 IU/kg) treated patients was found to reduce D-Dimer plasma levels. Heparin treatment (5000 IU) in CAD patients reduced D-Dimer formation by 20% (Linder *et al*., 1999). Based on the above studies, it can be stated that the inhibition of clot formation results in reduced D-Dimer formation. In this study, heparin reduced D-Dimer formation by 40%. D-Dimer formation was reduced by 31%, 38% and 38% by the AL (100 µg/mL), OL (100 µg/mL) and M (5 µg/mL) at the highest concentration tested *in vitro*.

Fibrinogen consists of two sets of non-identical polypeptide chains: Aα (66 062 kDa), Bβ (54 358 kDa) and γ (48 529 kDa), crosslinked by 29 disulfide bonds (Ajjan and Ariëns, 2009; Wolberg, 2007; Vadseth *et al*., 2004). Plasmin specifically cleaves the Aα, Bβ, and γ chains of fibrinogen at regions binding the central E fragment with the two C-terminal D fragments (Odriljin *et al*., 1996). In this study, plasmin did degrade fibrinogen on the Aα, Bβ, and γ chains. M had no effect on fibrinogen degradation but the extract showed a partial degradation of the Aα. Although the extracts displayed a proteolytic-like activity, further investigation is required.

Platelets have various receptors for different proteins such as collagen, fibronectin, fibrinogen, vWF, laminin, thrombin and vitronectin (Bellavite *et al*., 1994). The interaction of the receptors with their respective adhesive proteins, results in platelet adhesion, intracellular signalling and activation. Platelet activation results in TXA₂ release (Olas *et al*., 2005). Microscopic analysis provided evidence of the inhibition of platelet aggregation for the extracts as no platelet aggregates were observed. However, the microscopic method used was subjective, but allowed for the screening of a large volume of sample concentrations and was cost effective. Therefore flow cytometry was used to quantify these results both *in vitro* and *ex vivo*.

Flow cytometry analysis not only quantified, but provided a mechanism of action for the inhibition of platelet aggregation at the GPIIb-IIIa complex. The OL extract and M
inhibited platelet aggregation by 90% at 100 µg/mL and 5 µg/mL, respectively, indicating that M could be attributed as the inhibitory compound in the OL extract. The AL extract also inhibited platelet aggregation by 69.5% at 100 µg/mL. *Andrographis paniculata* which is a plant found in India, contains diterpenoids. The 3 diterpenoids isolated from this plant were tested for inhibitory effect on platelet aggregation *in vitro*. Two of the diterpenoids inhibited thrombin-induced platelet aggregation in a dose-dependent manner (1-100 µM). The IC₅₀ values ranged from 10-50 µM. The highest concentration (100 µM) of one of the diterpenoids inhibited platelet aggregation by 95%. The mechanism of anti-platelet effect of these diterpenoids is attributed to the inhibition of the ERK1/2 signalling pathway. ERKs are activated by thrombin and collagen in platelets. ERK 2 signalling pathway stimulates calcium mobilization (Thisoda *et al*., 2006). Another plant extract, *Actinostemma lobatum* was found to inhibit platelet aggregation in a dose-dependent manner. To elucidate the mechanism of the antiplatelet effect of this plant, expression of GPIIb/IIIa was evaluated using flow cytometry. *Actinostemma lobatum* significantly inhibited the binding of fibrinogen to GPIIb/IIIa complex. Based on these results, it was concluded that the antiplatelet effect of *Actinostemma lobatum* is through the GPIIb/IIIa pathway (Kim *et al*., 2008a). These findings are similar to those found for *Leonotis leonurus* extracts, where the diterpenoid M displays inhibition of platelet aggregation both *in vitro* and *ex vivo*.

The adhesion of platelets to the site of injury is one of the important mechanisms by which platelets function (Bellavite *et al*., 1994). Various agonists bind different GPs. GPIa/IIa and GPIIb/IIIa are not only involved in platelet adhesion but also in signalling transducing elements (figure 1.15) (Bellavite *et al*., 1994). Activation of platelets results in their morphological change, adhesion to one another and to the site of injury, granule secretion and aggregation (Olas *et al*., 2005). Therefore platelet adhesion plays a key role in the functioning of platelets. A microscopic method was used to evaluate the effect of the extracts and M on platelet adhesion. Adhesion to ECM plates was inhibited by the extracts and M, however, the OL extract and M appeared to be more effective at inhibiting platelet adhesion than the AL extract. In a study conducted by Shahriyary and Yazdanparast (2007), *Artenisia dracunculus*, a plant whose leaves have been traditionally
used to alleviate CAD in humans, was found to inhibit platelet adhesion. The microscopic examination of platelet adhesion was completed in the study by Shahriyary and Yazdanparast (2007). This method could not be used to quantify platelet adhesion. It was used to observe if the extract would show an effect on platelet adhesion and for the optimization of the OL and AL extract concentrations to be used for quantification, in flow cytometry experiments.

The activity of acid phosphatase was used to evaluate and quantify the effect of the extracts and M on platelet adhesion. Adhesion to ECM coated plates was inhibited by 63% for the OL extract (100 µg/mL) and the AL extract (100 µg/mL) and 70% by M (5 µg/mL). Linked to this, protein secretion was inhibited by 77% (OL extract and M at 100 and 5 µg/mL, respectively) while the AL extract inhibited protein secretion by 60%. In a study conducted by Shahriyary and Yazdanparast (2007), *Artemisia dracunculus* leaves extract was found to inhibit platelet aggregation, adhesion and protein secretion but the mechanism of the inhibition of platelet aggregation and adhesion was unknown. To elucidate the mechanism of the inhibition of platelet adhesion, evaluation of adhesion receptors such as GPIB/IX/V complex, vWF, and GPIa/IIa would provide answers. vWF (mediates the attachment of platelets to the subendothelium) binds collagen in the vessel wall. vWF can also bind GP Ib receptors, while subendothelial collagen binds to GP Ia/IIa (Jennings, 2009). Another plant extract, *Tripterygium wilfordii* was found to inhibit ICAM-1, VCAM-1 and E-selectin which are surface adhesion molecules involved in inflammation (Chang et al., 1999). The major diterpenoid in this plant is believed to be the major active component (Duan et al., 2000). The AL extract, OL extract and M inhibited platelet adhesion and protein secretion. However, the OL extract was more effective at inhibiting platelet adhesion and protein secretion and the degree of its inhibition was similar to that of M.

Calcium mobilization is induced by the activation of a membrane receptor with thrombin, collagen, or ADP (Kim et al., 2008a and b). The extracts and M inhibited both collagen and thrombin-induced calcium mobilization. Platelet activation and aggregation is suppressed by compounds that inhibit calcium mobilization (Liu et al., 2005). Thrombin
and collagen show different platelet aggregation mechanisms. Thrombin interacts with protease activated receptors (PARs) which are coupled to G proteins and phospholipase Cβ. The production of diacylglycerol by phospholipase Cβ stimulates protein kinase C which is linked to protein secretion. Inositol triphosphate, which is also produced as a result of phospholipase Cβ activation, leads to calcium mobilization. However, collagen induces platelet activation via a tyrosine kinase-based signalling pathway. This pathway involves the kinase Syk and phospholipase Cγ2. Calcium mobilization, shape change, and granule release (Jin et al., 2005). TXA₂ amplifies platelet activation, aggregation, and secretion (Liu et al., 2005). Our findings show that the extracts and M could potentially have a direct inhibitory effect on cyclooxygenase or thromboxane synthase activity based on the suppression of TXB₂ production. Several studies have shown that the AL and OL extract of Leonotis leonurus reduce inflammation (Fennell et al., 2004a). Therefore, the anticoagulant activity of Leonotis leonurus could be mediated primarily through this pathway. The cyclooxygenase-TXA₂ pathway is responsible for the inhibition of COX and COX-1 activity (Jäger et al., 1996; Steenkamp et al., 2004). Kim et al. (2008a) found that Artemisia dracunculus leaves extract did not affect the cyclooxygenase-TXA₂ pathway, therefore it was concluded that the antithrombotic effect of this plant are not mediated by this pathway. Jeng et al. (2007) reported that sanguinarine, an alkaloid present in roots of plants suppressed calcium mobilization in platelets. These results indicated that the alkaloid blocked calcium mobilization related to platelet aggregation. This alkaloid, further inhibited platelet COX-1, hence it was concluded that the anticoagulant and antiplatelet effect of sanguinarine occur via the cyclooxygenase-TXA₂ pathway. As it has been stated earlier, diterpenoids isolated from Andrographis paniculata suppressed calcium mobilization through the ERK1/2 signalling pathway (Thisoda et al., 2006). Based on our findings, M not only suppressed calcium mobilization but also inhibited TXB₂ production and could potentially mediate its action, similar to other findings, via the cyclooxygenase-TXA₂ pathway.

The in vitro coagulation studies treated the intrinsic and the extrinsic pathways as separate entities. The interaction of the extrinsic and intrinsic pathway factors is not reflected by the in vitro coagulation tests (PT and APTT). These tests do not allow for
complete elucidation of the mechanisms that result in haemostasis in vivo. In addition, PT and APTT do not provide adequate information with regards to the pathophysiology of the haemostatic system (Hoffman, 2003). Recent articles indicate the key role various cells play in coagulation, i.e. the cell-based model. TF is required for the initiation of blood coagulation in vivo. The exposure of cells expressing TF to flowing blood initiates blood coagulation process. Therefore, based on the cell-based model of coagulation the intrinsic pathway does not have a true role in haemostasis (Hoffbrand et al., 2005). As a result of these limitations an ex vivo model was utilised to mimic this physiological haemostatic state. Similar to the in vitro studies, APTT clotting time was prolonged by M, however, M had no effect on PT. Although in vitro M inhibited the formation of fibrin, this was not observed ex vivo. However, M inhibited platelet aggregation and D-Dimer formation relative to the untreated control (C). Based on the results, M was found to be more effective than aspirin (platelet aggregation studies). In a study conducted by Janssen et al. (1995) and Huang et al. (2002), aspirin significantly decreased ex vivo thromboxane synthesis in rats by 39% and ≥50%, respectively. Petroselinum crispum (parsley) a medicinal herb used to treat CAD was evaluated for its antiplatelet activity in vitro, ex vivo and in vivo (Gadi et al., 2009). This herb was found to inhibit collagen-, ADP-, epinephrine- and thrombin-induced platelet aggregation in vitro. The ex vivo findings showed that the herb prolonged bleeding time and inhibited platelet aggregation without changing the amount of platelets. It was concluded that the inhibition of platelet aggregation was the contributing factor in the prolongation of the bleeding time.

In this study, the observed findings suggest that the extracts and M possess antithrombotic/anticoagulant activity. This study has shown that the AL and OL extracts mediate their anticoagulant activities through the extrinsic pathway by significantly prolonging APTT in vitro and in the ex vivo model. The mechanism of antithrombotic/anticoagulant activity of the extracts and M was through the cyclooxygenase-TXA2 synthase pathway. Since coagulation was prolonged, less fibrin was formed. Therefore, the anticoagulant effect of the extracts and M was further enhanced by their inhibition of fibrin formation. As a result of reduction of
fibrinogen/fibrin formation, D-Dimer which is the degradation product of fibrinogen/fibrin was inhibited by the extracts and M.

Platelet adhesion receptors found on the platelet surface serve as ligands for specific cell receptors. These receptors include vWF, GPIb-IX-V, GPIb, GPIa/IIa, fibrinogen, fibronectin and vitronectin (Lee et al., 2002; Liu et al., 2005, Bellavite et al., 1994). *Leonotis leonurus* extracts and M significantly decrease platelet adhesion in thrombin activated platelets. However, the mechanism of inhibition of platelet adhesion was not established as the expression of the adhesion ligands was not studied. The extracts and M inhibited platelet adhesion and protein secretion, hence, decreasing the amplification of platelet aggregation. Flow cytometry could be used to evaluate the effect of *Leonotis leonurus* extracts and M on the expression and activity of the adhesion receptors.

Platelet aggregation is the integral part of platelet activation. GPIIb/IIIa is the most abundant cell surface receptor on platelets and it accounts for 15% of the entire platelet membrane receptor proteins (Lee et al., 2002). Therefore, the blockage of this receptor results in substantial inhibition of platelet aggregation and subsequent thrombus formation. Fibrinogen is the ligand that binds to this receptor. The extracts and M suppressed the binding of fibrinogen to the surface receptor GPIIb/IIIa, thereby providing the potential mechanism by which platelet aggregation was inhibited. Earlier it was reported (section 1.13) that there are 4 strategies for platelet function and antiplatelet targets, namely: (1) inhibition of agonist generation, (2) receptors inhibition, (3) G-protein inhibition and (4) inhibition of the enzymatic cascade (Xiang et al., 2008). Our current findings have shown that the *Leonotis leonurus* extracts and M inhibited platelet aggregation by targeting firstly, the inhibition of agonist and secondly the enzymatic cascade. These two targets were inhibited through the prolongation of the APTT clotting time, inhibiting the generation of thrombin which is the potent agonist of platelet aggregation. This dampens the amplification effect of the blood coagulation cascade, where thrombin is the hallmark enzyme in blood coagulation (refer to the coagulation cascade, figure 1.12). Our findings have also shown that target (3) was inhibited by the blockage of GPIIb/IIIa, hence inhibiting platelet aggregation. The AL extract showed
anticoagulant and antiplatelet properties, but, the OL extract was more effective than the AL at an equivalent concentration. For the OL extract, where an equivalent concentration of M was used, it is postulated that the biological effects noted could be attributed to M. Table 4.1 lists the properties of an ideal anticoagulant. M fulfilled some of the properties listed in table 4.1. M administered in rat models have reported that doses ≥1600 mg/kg caused negative reaction on the rats, i.e. significantly decreased red blood cells, packed cell volume, haemoglobin concentration, platelets, white blood cells and differential counts (Maphosa et al., 2008). The concentration of M used in the ex vivo studies was below ≥1600 mg/kg, equivalent to 1000 mg/kg of the OL extract.

Table 4.1: Properties of an ideal anticoagulant (Weitz et al., 2008).

<table>
<thead>
<tr>
<th>Properties</th>
<th>Benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally active</td>
<td>Ease of administration</td>
</tr>
<tr>
<td>Rapid onset of action</td>
<td>Obviates need for overlap with a parenteral anticoagulant</td>
</tr>
<tr>
<td>No food or drug interactions</td>
<td>Simplified dosing</td>
</tr>
<tr>
<td>Predictable anticoagulant effect</td>
<td>No routine coagulation monitoring</td>
</tr>
<tr>
<td>Extra-renal clearance</td>
<td>Safe in patients with renal insufficiency</td>
</tr>
<tr>
<td>Rapid offset of action</td>
<td>Simplifies management in case of bleed or need for intervention</td>
</tr>
<tr>
<td>Safe antidote</td>
<td>Useful in case of major bleed</td>
</tr>
<tr>
<td>Favourable net clinical benefit</td>
<td>Treatment benefit outweighs risk</td>
</tr>
</tbody>
</table>

Most importantly, treatment with M and its efficacy outweighed risks as no side effects at the concentration administered were noted. This is supported by the toxicity studies completed by Maphosa et al., 2008. M can be used as a lead compound for future studies.
4.10 Diabetic studies in vitro

4.10.1 GSIS

The effect of the OL extract and M on GSIS was completed on INS-1 cells (figure 4.15).

Figure 4.15: GSIS conducted on INS-1 cells maintained under normo- and hyperglycaemic conditions. All conditions were evaluated relative to the untreated normoglycaemic and hyperglycaemic control cells (A) Chronic insulin secretion, (B) basal insulin secretion response, (C) Stimulatory response, (D) insulin content and (E) stimulatory index of INS-1 cells in the presence and absence of OL and M, n= 4. *p<0.05 relative to normoglycaemic C, †p<0.01: relative to normoglycaemic C, ‡p<0.05 relative to hyperglycaemic C, ‡‡p<0.01: relative to hyperglycaemic C.
The hyperglycaemic C increased insulin secretion 1.5-fold relative to the normoglycaemic C p<0.05. For chronic samples, exposure of INS-1 cells to the extract for 48 hours in 11.1mM glucose increased insulin secretion only at 10 µg/mL (OL3) by 1.1-fold, however, INS-1 cells, by exposure to M, improved insulin secretion in a dose-dependent manner compared to the untreated control cells (C). M3 (500 ng/mL) significantly increased insulin secretion 2.0-fold (p<0.05), while M2 (250 ng/mL) increased insulin secretion 1.3-fold relative to normoglycaemic C (figure 4.15A). INS-1 cells that were exposed to OL and M under hyperglycaemic conditions increased insulin secretion in a dose-dependent manner. M3 (500 ng/mL) and OL3 (10 µg/mL) significantly increased insulin secretion 1.5- and 1.2-fold (p<0.05), respectively relative to the hyperglycaemic C (figure 4.15A). Under hyperglycaemic conditions, the OL2 and OL3 extract increased insulin secretion 1.3- and 1.8-fold (p<0.05), respectively, relative to the normoglycaemic C, while M2 and M3 increased insulin secretion 1.3- and 2.1-fold (p<0.05), respectively relative to the normoglycaemic C.

For basal treatment, insulin secretion in hyperglycaemic C was relatively similar to that of the normoglycaemic C. The OL extract and M increased insulin secretion in a dose-dependent manner. The OL1 (2.5 µg/mL) extract and M1 (125 ng/mL) both decreased insulin secretion 1.4-fold, relative to normoglycaemic C (p<0.05). The OL2 (5 µg/mL) extract decreased insulin secretion 1.0-fold, while OL3 (10 µg/mL) extract insulin secretion was relatively similar to that of the normoglycaemic C. M showed a similar trend, where it decreased insulin secretion 1.2-fold at M2 (250 ng/mL), while in M3 (500 ng/mL) insulin secretion was relatively similar to the normoglycaemic C (figure 4.15B). A similar trend was observed in the hyperglycaemic conditions, the OL1 (2.5 µg/mL) decreased insulin secretion 1.4-fold relative to the hyperglycaemic C (p<0.05), while OL2 (5 µg/mL) and OL3 (10 µg/mL) extract were relatively similar to the normoglycaemic and hyperglycaemic C. M1 (125 ng/mL) and M2 (250 ng/mL) decreased insulin levels 0.9- and 0.9-fold, respectively relative to hyperglycaemic and normoglycaemic C. However, M3 (500 ng/mL) insulin secretion was relatively similar to both controls.
Glucose-stimulated insulin secretion was 2.4-fold higher in normoglycaemic C as compared to the hyperglycaemic C (p<0.05), figure 4.15C. The extract and M decreased insulin secretion in a dose-dependent manner, relative to C under normoglycaemic conditions. The OL1, OL2 and OL3 significantly decreased stimulatory effect 3.5- (p<0.01), 3.0- (p<0.01) and 1.5 (0.05), relative to normoglycaemic C, respectively. M showed a similar trend, where it significantly decreased the stimulatory effect 2.6- (p<0.01), 2.1- (p<0.01) and 1.8-fold (p<0.05) at M1, M2 and M3, respectively relative to normoglycaemic C. Insulin secretion was significantly increased in a dose-dependent manner by both OL and M under hyperglycaemic conditions. The OL extract increased secretion 1.4 and 2.5-fold (p<0.01) at 5 (OL2) and 10 µg/mL (OL3), respectively, while M increased secretion 1.4 and 2.0-fold (p<0.01) at respective equivalent OL concentrations, relative to hyperglycaemic C. Under hyperglycaemic conditions, the OL1 and OL2 extract decreased insulin secretion 3.0 (p<0.01) and 1.7-fold (p<0.05), respectively relative to the normoglycaemic C, while the OL1 extract increased insulin secretion 1.2-fold relative to normoglycaemic C. Under the same conditions, M1, M2 and M3 decreased insulin secretion 3.0- (p<0.01), 1.7- (0.05) and 1.2-fold, respectively relative to the normoglycaemic C.

Insulin content (figure 4.15D) of INS-1 cells showed that the extract and M had no concentration dependent effect on the INS-1 insulin content at normoglycaemic levels, although an increase in insulin secretion was observed in OL3 and M3 treated cells. OL3 and M3 both increased insulin secretion 1.4-fold, relative to normoglycaemic C. However, at hyperglycaemic levels insulin levels were increased by the extract and M in a dose-dependent manner. Insulin levels for the content samples in hyperglycaemic conditions were increased 1.4- and 2.4-fold by the OL extract at 5 (OL2) and 10 µg/mL (OL3), respectively, while M increased insulin content levels 1.3- and 2.0-fold at 250 (M2) and 500 ng/mL (M3), respectively, relative to hyperglycaemic C (p<0.05). Under hyperglycaemic conditions, insulin content of both OL2 and M2 was increased 1.4-fold relative to the normoglycaemic C, while OL3 and M3 both increased the insulin content 1.5-fold, relative to the normoglycaemic C (p<0.05).
The stimulatory index (figure 4.15E) shows that insulin secretion was stimulated in a dose-dependent manner by the extract under hyperglycaemic conditions. The hyperglycaemic C decreased the stimulatory index 2.4-fold relative to normoglycaemic C, p<0.01. The OL extract and M decreased insulin secretion under normoglycaemic conditions. The OL1, OL2 and OL3 extract decreased the stimulatory index 2.4- (p<0.01), 2.7- (p<0.01) and 1.6-fold (p<0.05), respectively relative to normoglycaemic C. Similar to the OL extract, M decreased the stimulatory index 1.8- (p<0.05), 1.7- (p<0.05) and 1.8-fold (p<0.05), relative to normoglycaemic C. Insulin secretion under hyperglycaemic conditions was increased 1.6-fold (p<0.05) and 1.9-fold (p<0.01) by the OL extract at 5 (OL2) and 10 µg/mL (OL3), while M significantly increased insulin secretion 1.5-fold (p<0.05) and 1.9-fold (p<0.01) at 250 (M2) and 500 ng/mL (M3) respectively, relative to hyperglycaemic C.

4.10.2: Oxygen consumption studies

Figure 4.16 shows a typical respiration trace which was obtained before and after the addition of oligomycin in all experimental conditions. The change in slope was used to calculate oxygen consumption rate. Proton leak was observed after the addition of oligomycin (100 µM) at 500 sec.

![A typical respiration trace obtained from INS-1 cells (10⁶ cells/mL).](image)

Figure 4.16: A typical respiration trace obtained from INS-1 cells (10⁶ cells/mL).

Figure 4.17 displays the effect of the OL extract and M on oxygen consumption of INS-1 cells.
Figure 4.17: The oxygen consumption per minute per 1 million cells for the INS-1 cells. Oligomycin decreased oxygen consumption under both normoglycaemic and hyperglycaemic conditions in the presence and absence of OL and M. * p<0.05, # p<0.01, relative to relevant C and between – oligomycin and + oligomycin treatment (n= 4).

Oxygen consumption was enhanced within the INS-1 cells with OL and M treatment under both the normo- and hyperglycaemic conditions. OL and M showed similar results by increasing the oxygen consumption rates 1.6-fold (p<0.05) at normoglycaemic conditions, while at hyperglycaemic conditions oxygen consumption rate was increased 1.7-fold (p<0.05), relative to hyperglycaemic C for both OL and M. After the addition of oligomycin, oxygen consumption in M under normo- and hyperglycaemic conditions, continued to increase 1.8- and 1.7-fold (p<0.05), respectively relative to the respective control cells. After the addition of oligomycin under normoglycaemic conditions, the oxygen consumption rate of the OL-treated cells was relatively similar to that of the control cells, however, under hyperglycaemic conditions, the OL extract increased oxygen consumption rate 1.7-fold (p<0.05). These results support the results observed in the GSIS studies. The oxygen consumption rate of the hyperglycaemic C was increased 1.5-fold compared to the normoglycaemic C (p<0.05). Both the normoglycaemic and hyperglycaemic controls had a decrease in oxygen consumption rates after the addition of
oligomycin. Under normoglycaemic conditions, the oxygen consumption rate decreased 1.4-fold after the addition of oligomycin, while under hyperglycaemic conditions a 1.5-fold decrease was observed (p<0.05).

4.10.3 Mitochondrial membrane potential
Treatment with M significantly increased MMP of INS-1 cells in relation to the untreated control cells (figure 4.18).

![Figure 4.18: Membrane potential in INS-1 cells exposed to OL (10 µg/mL), M (500 ng/mL), and Camptothecin (NC) (100 µM). * p<0.05, # p<0.01, n= 4, relative to the relevant control cells.]

The extract and M significantly increased MMP 1.8 (p<0.05) and 2.9-fold (p<0.01), respectively under normoglycaemic conditions. Stimulation of MMP was observed under hyperglycaemic conditions, with the extract and M significantly increasing MMP 2.0-fold (p<0.01). Camptothecin significantly decreased MMP of INS-1 cells under hyperglycaemic conditions 2.0-fold (p<0.01) compared to the C, however, this effect was not observed under normoglycaemic conditions.

4.10.4 RT-qPCR studies
Glut-2 was up-regulated by both OL and M in 33.3mM glucose media. The gene expression was significantly increased 11- (p<0.01) and 12-fold (p<0.01) by the OL and M; respectively (figure 4.19A). This data correlated with GSIS findings, oxygen consumption and MMP studies.
Figure 4.19: The effect of the organic extract and marrubiin on the expression of (A) Glut-2 and (B) insulin genes in INS-1 cells relative to the relevant control cells. *p<0.05, # p<0.01, n = 4.

Glut-2 under hyperglycaemic conditions for the control cells was down-regulated possibly due to the constant exposure to a high glucose concentration. Under normoglycaemic conditions, M significantly decreased Glut-2 expression 2.0-fold (p<0.01), while there was a slight increase in the OL extract exposure.

Insulin expression was up-regulated in INS-1 cells exposed to OL or M under hyperglycaemic conditions, which correlates to the findings in GSIS studies. Insulin expression was increased 3.7- (p<0.01) and 4.5-fold (p<0.01) by the OL and M; respectively (figure 4.19B). Both the extract and M decreased insulin expression under normoglycaemic conditions 1.5- and 1.3-fold (p<0.05), relative to control cells, which supports the findings of the GSIS studies. Insulin expression was relatively similar for the controls.
4.11 *In vivo* studies

4.11.1 Body weight and fasting blood glucose levels

Table 4.2 shows the effect of treatment on body weight and blood glucose levels in rats fed a cafeteria diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-experiment weight (g) (week 8)</th>
<th>Post-experiment weight (g)</th>
<th>Degree of weight gain</th>
<th>Pre-experiment glucose (mmol/L) (week 8)</th>
<th>Post-experiment glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>346.75±5.0</td>
<td>367.5±2.90</td>
<td>21±3.05</td>
<td>4.6±0.11</td>
<td>5.6±0.12</td>
</tr>
<tr>
<td>OC</td>
<td>375.71±5.6a</td>
<td>405.28±9.94a</td>
<td>30±7.17a</td>
<td>8.4±0.26b</td>
<td>8.9±0.11a</td>
</tr>
<tr>
<td>PC MET</td>
<td>364.83±5.9a</td>
<td>388.71±7.19ac</td>
<td>24±6.11c</td>
<td>8.2±0.03b</td>
<td>5.4±0.20c</td>
</tr>
<tr>
<td>PC SU</td>
<td>374.71±5.3a</td>
<td>376.00±7.19c</td>
<td>1±0.23bd</td>
<td>8.2±0.18b</td>
<td>5.4±0.20c</td>
</tr>
<tr>
<td>PC ASP</td>
<td>380.00±3.1ac</td>
<td>398.80±7.10ac</td>
<td>18±3.11ac</td>
<td>8.1±0.18b</td>
<td>7.4±0.20ac</td>
</tr>
<tr>
<td>OL</td>
<td>367.50±8.1a</td>
<td>395.83±8.05a</td>
<td>28±7.67d</td>
<td>8.4±0.12b</td>
<td>6.5±0.60a</td>
</tr>
<tr>
<td>M</td>
<td>383.00±3.2ac</td>
<td>395.00±5.45a</td>
<td>12±0.1bd</td>
<td>8.3±0.17b</td>
<td>4.7±0.21ad</td>
</tr>
</tbody>
</table>

*a*p<0.05 relative to LC, *b*p<0.01: relative to LC, *c*p<0.05 relative to OC, *d*p<0.01: relative to OC.

It is important to note that the average weight of the collective obese rats was 370±3.3 g while the average weight of LC was 346.75±5.0 g (*p*<0.01). Therefore, the cafeteria diet did induce weight gain in the obese rats. The LC, OC, metformin-, sulfonylurea-, aspirin-, M- and the OL-treated groups gained 21±3.05, 30±7.17, 24±6.11, 1±0.07, 18±3.11 and 12±0.1, 28±7.67 g post-treatment. The OC group gained 9 g relative to LC, *p*<0.05. Metformin group gained 3 g relative to LC group. The OL extract group gained 7 g relative to the LC group, while the aspirin group gained 3 g relative to LC group, *p*<0.05. M-treated group lost 9 and 18 g, respectively, relative to the LC and OC groups, *p*<0.01. Although no record of food consumption was completed, it was observed that M-treated group ate less as compared to the other groups. Despite the reduction in food
consumption, no abnormalities or toxicity was found in M-treated rats when the rats were sacrificed and their organs harvested. The sulfonylurea-treated group significantly lost 20 and 29 g, respectively, relative to the LC and OC groups, p<0.01. The metformin- and aspirin-treated groups significantly reduced weight by 6 and 14 g, relative to OC group, p<0.05. The OL extract caused a 2 g weight loss, relative to the OC group.

The fasting blood glucose levels of all rats that were fed a cafeteria diet was significantly higher than those of the LC group (p<0.01), table 4.2. The average fasting blood glucose levels were 4.6±0.11 mmol/L for the LC, while the average fasting blood glucose levels were 8.3±1.14 x 10^{-5} mmol/L for rats fed on the cafeteria diet (pre-treatment). The pre-treatment OC (8.4±0.26 mmol/L) had elevated blood glucose levels which were 1.8-fold higher relative to the LC group, p<0.01. Post treatment, the OC group (8.9±0.11 mmol/L) increased fasting blood glucose levels 1.6-fold relative to LC, p<0.05. Supplementation with metformin (5.4±0.20 mmol/L) and sulfonylurea (5.4±0.20 mmol/L) normalised the fasting blood glucose levels relative to the LC (5.6±0.12 mmol/L). Similarly, M significantly reduced the fasting blood glucose levels (4.7±.21 mmol/L) 1.2-fold relative to the LC, p<0.05. The supplementation of aspirin (7.4±0.20 mmol/L) significantly increased fasting blood glucose levels 1.3-fold relative to the LC group (p<0.05). The OL extract (6.5±0.60 mmol/L) increased fasting blood glucose levels 1.1-fold relative to the LC group, p<0.05. Metformin and sulfonylurea significantly lowered the blood glucose levels 1.6-fold compared to the OC group (p<0.05). However, M was more effective than all the drugs tested in reducing fasting glucose levels relative to the OC group. It reduced the fasting blood glucose levels 1.9-fold, relative to OC group (p<0.01). The OL extract reduced fasting blood glucose levels 1.4-fold, while aspirin was the least effective drug reducing the blood glucose levels 1.2-fold relative to the OC group, p<0.05.

4.11.2: IPGTT and IPITT
IPGTT was performed after treatment with metformin, sulfonylurea, aspirin, the OL extract and M to determine their effect on glucose tolerance (figure 4.20). There was a significant difference in the blood glucose levels of the LC and the OC group from time 15 to 120 minutes after glucose load (p<0.05). The OC increased blood glucose levels 1.1-, 1.2-, 1.6 (p<0.05)-, 1.8- (p<0.05) and 1.4-fold, relative to the LC group. Treatment
with metformin significantly improved glucose clearance when compared to the OC (p<0.05) and this reduction of blood glucose levels was similar to the LC group. After 2 hours of glucose load, metformin decreased blood glucose levels 1.8-fold, relative to the OC group, p<0.01. From time 15 to 60 minutes, metformin reduced blood glucose levels 1.3-, 1.6- (p<0.05) and 1.4-fold, relative to OC group. At time 0, 30 and 60 minutes, metformin increased blood glucose levels 1.0-, 1.0- and 1.3-fold, however, at time 15 and 120 minutes blood glucose levels were reduced by 1.1- and 1.2-fold relative to LC. Sulfonylurea significantly increased glucose clearance after a glucose load (p<0.01) when compared to the OC group. This difference was observed from time 15 to 120 minutes after glucose load. From time 15 to 60 minutes, blood glucose levels were reduced by 1.4-, 1.6- (p<0.05) and 1.4-fold, relative to OC group. After 2 hours of glucose load, blood glucose levels were decreased 1.7-fold (p<0.05), relative to OC group. At time 0 and 60 minute, sulfonylurea increased blood glucose levels 1.2- and 1.3-fold relative to LC group. However, at 15 and 120 minutes, blood glucose levels were both reduced by 1.2-fold.

The overall glucose tolerance curve shows that aspirin did not have an effect on glucose tolerance, although there was a marked difference between the OC group and the aspirin group after 30 minutes of glucose load. The OL extract decreased blood glucose levels by 1.1-, 1.2-, 2 (p<0.01), 1.3- and 1.3-fold, relative to OC group. Between 0 and 30 minutes, the OL extract decreased blood glucose levels 1.0-, 1.0- and 1.3-fold, relative to the LC group. However, at time 60 to120 minutes blood glucose levels were increased 1.4- and 1.1-fold, relative to LC group. M also decreased blood glucose levels when compared to the OC group (p<0.05), this reduction was comparable with the LC group. After 2 hours of glucose load, M decreased blood glucose levels by 1 point lower than the LC group. M decreased blood glucose levels 1.1-, 1.4-, 1.6- (p<0.05), 1.3- and 1.7-fold (p<0.05) relative to the OC group. At time 15, 30 and 120 minutes, M reduced blood glucose levels by 1.3-, 1.0- and 1.2-fold relative to the LC group and increased the levels 1.0- and 1.4-fold at 0 and 60 minutes, respectively. From the IPGTT data, the sum of AUC was calculated (table 4.3).
Figure 4.20: The effect of (A) metformin, (B) sulfonylurea, (C) aspirin, (D) the OL extract and (E) M on IPGTT (post-treatment). *p<0.05 relative to LC, **p<0.01: relative to LC, †p<0.05 relative to OC, ‡p<0.01: relative to OC, n = 4.
Table 4.3: The total AUC for IPGTT and IPITT (n = 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>AUC_g (IPGTT) (mmol/minute/L)</th>
<th>AUC_i (IPITT) (mmol/minute/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>912±2.38</td>
<td>428±8.54</td>
</tr>
<tr>
<td>OC</td>
<td>1380±5.62^a</td>
<td>499±8.71</td>
</tr>
<tr>
<td>PC MET</td>
<td>931±5.90^c</td>
<td>273±2.31^ac</td>
</tr>
<tr>
<td>PC SU</td>
<td>947±8.42^c</td>
<td>489±6.71</td>
</tr>
<tr>
<td>PC ASP</td>
<td>1286±9.87</td>
<td>411±6.43</td>
</tr>
<tr>
<td>OL</td>
<td>994±11.54</td>
<td>549±12.34</td>
</tr>
<tr>
<td>M</td>
<td>932±8.43^c</td>
<td>496±6.17</td>
</tr>
</tbody>
</table>

*p<0.05 relative to LC, ^p<0.01: relative to LC, ^p<0.05 relative to OC, ^p<0.01: relative to OC.

The AUC_g associated with IPGTT for the OC group was 1.5-fold higher than that of the LC group, p<0.05. Metformin and M had a similar effect on IPGTT based on the AUC_g, they both decreased the AUC_g 1.5-fold relative to the OC group, and they both reflected an AUC_g relatively similar to that of the LC group. Sulfonylurea decreased the AUC_g 1.5-fold relative to the OC group, while also reflecting an AUC_g relatively similar to the LC group. Aspirin reflected an AUC_g similar to the OC group, and increased it 1.4-fold relatively similar AUC_g to the LC group. The OL extract decreased the AUC_g 1.4-fold relative to the OC group and relatively similar AUC_g to the LC group.

IPITT was performed after treatment with metformin, sulfonylurea, aspirin, the OL extract and M to determine their effect on insulin sensitivity (figure 4.21). IPITT curve showed that the LC group’s blood glucose levels were significantly lower to that of the OC group (p<0.05). Metformin significantly decreased blood glucose levels compared to other groups (p<0.05). This reduction in rate of blood glucose levels was evident from 0 to 120 minutes after insulin administration. Aspirin significantly decreased blood glucose levels between 15 and 60 minutes (p<0.05) after insulin administration but this reduction was overturned after 120 minutes. M and sulfonylurea showed a similar trend in the decrease in blood glucose levels (p<0.05). The OL extract lowered insulin sensitivity as did M; as a result, the glucose curve for this treatment was very similar to that of the OC group. The sum of the AUC_i was calculated for each group (table 4.3).
Figure 4.21: The effect of (A) metformin, (B) sulfonylurea, (C) aspirin, (D) the OL extract and (E) M on IPITT (post-treatment). $^a$p<0.05 relative to LC, $^b$p<0.01 relative to LC, $^c$p<0.05 relative to OC, $^d$p<0.01: relative to OC, n = 4.

The AUC$_i$ of the OC group was increased 1.2-fold relative to the LC group. Metformin decreased the AUC$_i$ 1.8-fold relative to the OC group, while it decreased AUC by 1.6-fold relative to the LC group (p<0.05). Sulfonyurea decreased AUC$_i$ 1.1-fold relative to
OC group, and increased it 1.1-fold relative to the LC group. Aspirin decreased the AUCi 1.2-fold and relatively similar to the OC and LC groups, respectively. The AUCi of the OC group and M group were similar, and increased the AUCi 1.2-fold relative to the LC group. The OL extract increased the AUCi 1.1- and 1.3-fold relative to the OC and LC groups, respectively.

4.11.3 Fasting plasma insulin levels
Table 4.4 displays the effect of the compounds on insulin secretion.

Table 4.4: The effect of various treatments on insulin secretion in cafeteria diet-induced obesity (n = 5).

<table>
<thead>
<tr>
<th>Group</th>
<th>[Insulin] ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>0.96±0.01</td>
</tr>
<tr>
<td>OC</td>
<td>1.24±0.02</td>
</tr>
<tr>
<td>PC MET</td>
<td>1.4±0.01</td>
</tr>
<tr>
<td>PC SU</td>
<td>1.6±0.01*</td>
</tr>
<tr>
<td>PC ASP</td>
<td>1.4±0.02</td>
</tr>
<tr>
<td>OL</td>
<td>4.75±0.01**</td>
</tr>
<tr>
<td>M</td>
<td>2.75±0.03**</td>
</tr>
</tbody>
</table>

*p<0.05: relative to LC, ^p<0.01 relative to LC, *p<0.05: relative to OC, ^p<0.01: relative to OC, n = 5.

Table 4.4 shows that the OC group had chronic insulin levels which were 1.3-fold higher to the LC group. The insulin levels of metformin-treated, aspirin-treated and the OC groups were relatively similar, while metformin and aspirin increased insulin levels by 1.4-fold relative to the LC group. Sulfonylurea insulin levels were relatively similar to that of the OC group, however, it increased insulin 1.7-fold (p<0.05) relative to the LC groups. The OL extract significantly increased insulin secretion 4.0- (p<0.01) and 5.0-fold (p<0.01) relative to the OC and LC groups, respectively. M significantly increased insulin secretion 2.2- (p<0.01) and 2.8-fold (p<0.01) relative to the OC and LC groups, respectively. M did not only improve insulin secretion relative to LC and OC, it also enhanced secretion more effectively than the insulin-sensitizing drug sulfonylurea. M increased insulin secretion 2.1-fold relative to sulfonylurea.
4.11.4 Coagulation and platelet aggregation studies in vivo

Treatment with metformin, sulfonylurea, aspirin, the OL extract and M had no effect on PT (figure 4.22A) relative to LC and OC controls, although there was a slight increase in clotting time in rats treated with metformin and aspirin. There was no significant difference noted in PT clotting time of the OC group relative to LC group.

APTT (figure 4.22B) showed that the clotting time of the OC group was significantly shortened 1.6-fold when compared to their LC counterparts (p<0.05). Metformin, aspirin, sulfonylurea, M and OL prolonged the clotting time 1.6-, 1.94-, 1.8-, 2.1- and 1.4-fold, respectively compared to the OC group (p<0.05). There was no significant difference between treated groups and the LC group.

Fibrin formation was 1.9-fold higher in OC when compared to LC (p<0.01), figure 4.22C. Metformin, sulfonylurea and OL significantly decreased fibrin formation 1.3-, 1.3- and 1.2-fold, respectively (p<0.05). Aspirin and M were the two compounds which showed the most significant reduction in fibrin formation. They decreased fibrin formation by 1.9-fold relative to the OC group (p<0.01). The aspirin and M normalised the fibrin levels, as fibrin formation of these two compounds was similar to that of the LC group. The metformin, sulfonylurea and the OL extract increased fibrin formation 1.5-, 1.5- and 1.3-fold, respectively relative to LC, but these findings were not significant.

D-Dimer formation was significantly increased 1.5-fold (p<0.05) in OC group relative to the LC group (figure 4.22D). Metformin decreased D-Dimer formation 0.9-fold (p<0.05) relative to the OC group. Sulfonylurea increased D-Dimer formation 1.4-fold to OC group. Similar to fibrin formation, aspirin and M were the compounds that had the most effect on D-Dimer formation. These compounds reduced D-Dimer formation 1.6- and 2-fold, respectively (p<0.05) relative to the OC group, while OL decreased D-Dimer formation by 1.7-fold (p<0.05). The OL extract normalised the D-Dimer levels, while M decreased the levels by 1.5-fold relative to the LC group (p<0.05). Metformin, sulfonylurea and aspirin treatment had elevated D-Dimer levels, 1.4-, 2- and 1.2-fold relative to the LC group (p<0.05).
Figure 4.22: The effect of metformin, sulfonylurea, aspirin, organic extract and marrubiin on coagulation markers on rats that were fed a cafeteria diet. Data is presented as mean±SEM, n=7. 

\[ a \] p<0.05: relative to LC, 
\[ b \] p<0.01 relative to LC; 
\[ c \] p<0.05: relative to OC, 
\[ d \] p<0.01: relative to OC.

Evaluation of platelet activity showed that the tested compounds inhibited platelet aggregation relative to the LC group (figure 4.23). The degree of inhibition was similar for metformin and sulfonylurea (93.7% and 94.4%, respectively). Aspirin, the OL extract and M displayed a similar level of inhibition of platelet aggregation, where the inhibition was ±104% (figure 4.23). The results indicate that all treatments improved the hyperplatelet state observed in the OC group.
4.11.5: Plasma triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol and atherogenic index
Table 4.5 indicates that, the triglyceride levels were very high in the OC group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Triglycerides (mmol/L)</th>
<th>Total cholesterol (mmol/L)</th>
<th>HDL-cholesterol</th>
<th>LDL-cholesterol</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC</td>
<td>2.1 ±0.03</td>
<td>145.6±0.02</td>
<td>120.2±0.12</td>
<td>25.4±0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>OC</td>
<td>3.8±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>245.0±0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>78.0±.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.1±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC MET</td>
<td>2.6±0.50&lt;sup&gt;c&lt;/sup&gt;</td>
<td>164.0±0.13&lt;sup&gt;c&lt;/sup&gt;</td>
<td>131.0±.007&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.3±.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC SU</td>
<td>2.3±0.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>163.0±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>135.0±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.2±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PC ASP</td>
<td>2.9±0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>176.0±0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>120.0±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.2±0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.5&lt;sup&gt;ad&lt;/sup&gt;</td>
</tr>
<tr>
<td>OL</td>
<td>2.9±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>200.0±0.07</td>
<td>150.0±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50.3±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>M</td>
<td>2.0±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>145.0±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>123.0±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.1±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>p<0.05: relative to LC, <sup>b</sup>p<0.01 relative to LC  <sup>c</sup>p<0.05: relative to OC,  <sup>d</sup>p<0.01: relative to OC
The OC group had triglyceride levels significantly higher (3.8±0.02 mmol/L, p<0.05) than the LC group, 2.1±0.03 mmol/L, which was a 1.8-fold increase. Metformin, sulfonylurea and M had a 1.5-, 1.7- and 1.9-fold (p<0.05) reduction in plasma triglycerides relative to the OC group. M triglyceride levels were reduced to levels lower than the LC triglyceride levels. Aspirin and the OL extract decreased the plasma triglyceride levels 1.3-fold (p<0.05), relative to the OC group. Metformin, sulfonylurea, aspirin and the OL extract increased triglyceride levels 1.2-, 1-, 1.3- and 1.3-fold, respectively relative to the LC group.

The plasma total cholesterol levels of the OC control rats were significantly higher (1.7-fold) than that of the LC group (p<0.01). Both metformin and sulfonylurea significantly decreased the total cholesterol levels 1.5-fold, p<0.05, while aspirin decreased the levels 1.4-fold (p<0.05) relative to the OC group. M was the compound which showed the most pronounced effect on total cholesterol, decreasing the levels 1.7-fold (p<0.05), to levels similar to the LC group. The OL extract showed the least effect by decreasing the total cholesterol levels 1.2-fold relative to the OC group. Metformin, sulfonylurea and aspirin increased total cholesterol levels 1.1, 1.1 and 1.2-fold relative to the LC group.

HDL levels were significantly lower in the OC group (1.5-fold, p<0.05) when compared with the LC group. All the drugs or compounds increased the HDL levels when compared with the OC group, with the OL extract showing the most pronounced effect. The OL extract significantly increased HDL levels 1.9-fold relative to the OC group, p<0.05. M and aspirin showed levels similar to the LC group. These two compounds significantly increased HDL levels 1.6- and 1.5-fold, respectively relative to the OC group (p<0.05). Metformin and sulfonylurea both significantly increased HDL levels 1.7-fold relative to the OC group (p<0.05). Aspirin and M normalised the HDL levels relative to the LC group. Metformin and sulfonylurea both increased HDL levels 1.1-fold relative to the LC group. The OL extract improved HDL levels 1.3-fold relative to the LC group.

The OC group increased LDL levels 2.7-fold relative to the LC group (p<0.01). The OL extract and aspirin decreased LDL levels relative to the OC group but these results were not significant. Metformin, sulfonylurea and M significantly decreased LDL levels 2-,
2.4- and 3-fold relative to the OC group (p<0.01). M showed levels which were lower than the LC group, thus effectively enhancing the lipid profile of the obese rat model. Metformin and sulfonylurea increased LDL levels 1.3- and 1.1-fold, respectively relative to the LC group. The OL extract and aspirin significantly increased LDL levels 2- and 2.2-fold, respectively relative to the LC group (p<0.05).

The AI of the OC group was 10-fold greater than in the LC control rats (p<0.01). M and sulfonylurea enhanced the AI profile, similar to the levels observed in the LC group, and reduced the AI profile 10.5-fold relative to the OC group (p<0.01). Metformin and OL both decreased AI profile 7-fold (p<0.01) relative to the OC group. Aspirin increased AI profile 4.2-fold (p<0.01) when compared to the OC group. Metformin and the OL extract both increased the AI profile 1.5-fold relative to the LC group, while aspirin significantly increased the AI profile by 2.5-fold (p<0.05) relative to the LC group.

4.12 Discussion and conclusions
Control of insulin secretion by pancreatic β-cells is critical for glucose homeostasis. Glucose signals control GSIS in pancreatic β-cells (Špacek et al., 2008). Hypoglycaemia 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 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 the 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. Metformin treatment causes a minimum risk of hypoglycaemia. The side effects that are caused by metformin treatment include lactic acidosis and gastrointestinal symptoms. Sulfonylurea can cause hypoglycaemia and it acts in low blood glucose levels. In addition, it causes weight gain (Nathason and Nyström, 2009; Jellinger and Mace, 2007; Stunvoll et al., 2005). Therefore, there is a need for a more effective and safer agent that can be used to manage this disease (Kaleem et al., 2006). In a previous study, the OL extract and M were found to induce insulin secretion in rat pancreatic islet cells (Mnonopi, 2007). One of the aims of this study was to investigate the mechanism by which these effects of the OL extract and M were induced.

INS-1 cells were used to evaluate if the results obtained in rat pancreatic islet cells would be reproducible because the utilization of primary rat pancreatic cells could not be sustained economically. Prior to the GSIS studies, cytotoxic studies were completed for a range of OL concentrations 2.5-40 µg/mL (equivalent M concentrations were also tested). Concentrations below 10 µg/mL and 500 ng/mL for the OL extract and M, respectively were found not to be toxic in INS-1 cells (results not shown). The chronic insulin levels of INS-1 cells exposed to the OL extract and M under normoglycaemic conditions, were relatively similar. M3 was the only concentration which induced insulin secretion when compared to the untreated control cells. However, insulin secretion was significantly enhanced in cells exposed to the OL extract and M under hyperglycaemic conditions (figure 4.15). The stimulatory index shows that insulin secretion was enhanced and was more pronounced under hyperglycaemic conditions than under normoglycaemic conditions. Insulin content was not significantly increased under hyperglycaemic conditions. Glut-2 gene expression is known to be modulated by glucose in vitro and in
vivo (Chen et al., 1992; Yasuda et al., 1992). As a result of the increase in insulin secretion in the OL- and M-treated cells under hyperglycaemic conditions, there was an increased expression of Glut-2 and insulin genes. The increase in expression of Glut-2 correlated with that of the increased insulin gene expression. The INS-1 cells upregulated the expression Glut-2 transporters, thus increasing insulin secretion as a mechanism to maintain minimal levels of glucose, hence, protecting them against glucotoxicity, thus a lower insulin content. These results support the results that were obtained in the in vitro studies using isolated rat islet β-cells (Mnonopi, 2007). In the diabetic studies conducted by Mnonopi (2007), under hyperglycaemic condition, the OL (10 μg/mL) extract and M (500 ng/mL) significantly increased insulin secretion by 2.0-fold and 4.0-fold, respectively, relative to the control. The OL extract and M stimulated the release of insulin; the stimulatory index was significantly increased by 4.5- and 5.0-fold, respectively, relative to the control. In the apoptotic studies, under the normoglycaemic and hyperglycaemic 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. M did not have an effect on apoptosis under hyperglycaemic condition, but it decreased the occurrence of apoptosis by 2.0-fold under normoglycaemic conditions. The OL extract increased proliferation by 1.5-fold and 1.6-fold in normoglycaemic and hyperglycaemic conditions, respectively. The same effect was observed for M, where, proliferation was increased by 1.8-fold and 2.0-fold in normoglycaemic and hyperglycaemic conditions, respectively (Mnonopi, 2007).

GSIS relies heavily on mitochondrial energy transduction since ATP is closely associated with glucose oxidation (Affourtit and Brand, 2008). Affourtit and Brand (2008) reported that UCP-2 contributes to the proton leak in INS-1 cells, resulting in the decrease in ATP/ADP ratio. UCP-2 contributes to about 20% of INS-1 respiration and it is expressed in a number of tissues including pancreatic islets. It is a negative regulator of insulin secretion (Affourtit and Brand, 2008; Zhang et al., 2006). β-cell dysfunction, T2DM and obesity is improved in a UCP-2 gene-knockout mice model (Affourtit and Brand, 2008; Zhang et al., 2006). An extract of Gardenia jasminoides was found to increase MMP in isolated pancreatic islets (Zhang et al., 2006). Genipin, which is the active compound
from this plant, closed $\text{K}_{\text{ATP}}$-dependant channels, resulting in the stimulation of insulin secretion. The mechanism of insulin secretion by genipin was through the inhibition of UCP-2. In this study, MMP was significantly enhanced under both normo- and hyperglycaemic conditions by the OL extract and M, but these effects were more pronounced under hyperglycaemic conditions (figure 4.18). As it has been stated, oxidative phosphorylation (respiration) is closely linked with mitochondrial energy transduction. The increase in MMP was reflected in the respiration studies, and M seemed to increase respiration under both normo- and hyperglycaemic conditions (figure 4.17). Similar to GSIS, the increase in MMP and oxygen consumption rate was more pronounced under the hyperglycaemic conditions. After the addition of oligomycin (an ATPase inhibitor) oxygen consumption of cells exposed to the OL extract and M was significantly higher than the control cells. Therefore, M could be uncoupling mitochondrial thermogenesis. Whether the stimulated insulin secretion is mediated by inhibition of UCP2 by the OL extract and M requires further investigation. Similar to MMP and respiration studies, the increase was more pronounced under hyperglycaemic conditions. Camptothecin is an anticancer drug that induces the apoptosis of cancerous cells by decreasing the MMP. Depolarisation of MMP is induced by the activation of caspase-8 and 3 (Wang et al., 2008). Camptothecin-treated cells significantly reduced MMP under hyperglycaemic conditions although there was a slight increase in MMP under normoglycaemic conditions.

DM is a metabolic disease which is characterized by the development of IR. For research purposes, this metabolic disease can be induced in vivo in several ways i.e. (a) pharmacological induction, (b) surgical models of diabetes, (c) genetic models of diabetes and (d) models which allow the study of the reduction of pancreatic $\beta$-cell mass. Most of these procedures are carried out in rodents (Fröde and Medeiros, 2008).

STZ and alloxan are the most used drugs that induce DM. These drugs lead to the formation of ROS; however, their mode of action is different. STZ is transported into the pancreatic $\beta$-cells by GLUT-2. DNA is alkalyated by STZ. STZ also causes the release of NO and the activation of polyadenosine ribosylation leading to necrosis of these cells.
Although STZ has been successfully used to induce DM, it has toxic effects. STZ does not cause cytotoxic effects only in pancreatic β-cells. Renal injury, oxidative stress, inflammation and endothelial dysfunction are possible toxic effects caused by its use. Alloxan and dialuric acid (alloxan reduction product) lead to the formation of superoxide radicals. Hydrogen peroxide which formed from the superoxide radicals causes the destruction of pancreatic β-cells. Alloxan is toxic, with a small overdose being lethal to animals due to kidney tubular cell necrotic toxicity. The administration of both drugs leads to hypoglycaemia which is lethal to animals. Hence it is required that after administration during the fasting period a glucose solution should be given to the animals (Fröde and Medeiros, 2008).

Surgical methods that induce DM include the complete/partial removal of the pancreas. The problem with this method is that (a) the technique requires a skilled person with the expertise and an adequate surgical room, (b) the animals are exposed to high risk surgery, (c) the administration of antibiotics and analgesia after surgery requires technical skills, (d) the animals lose their ability to respond to hypoglycaemic conditions and (e) supplements that contain pancreatic enzyme to avoid mal-absorption are required (Fröde and Medeiros, 2008).

Genetic models of DM include animals that spontaneously develop diabetes and genetic engineering to acquire diabetes. The big limitation with these models is the high cost involved (Fröde and Medeiros, 2008).

Animal models are living organisms with an inherited, naturally acquired or an induced pathological disease that resembles one or more aspects in the same disease in man. An ideal animal model should respond predictably and have relevance to a particular problem (Gitel et al., 1997). Due to the various limitations of these procedures, an animal model of obesity was used in this study. The onset of obesity depends on the duration of the feeding and types and levels of dietary fats (Kim and Park, 2008). Obesity is closely associated with IR which is a pre-diabetic state that often results in T2DM. An imbalance between food intake and energy expenditure result in obesity. The excess energy is stored
as fat primarily in the adipose tissue, secondarily in the skeletal muscle, liver, pancreas and brain tissues (Gómez-Ruiz et al., 2009). Rats fed with a cafeteria diet have been a very useful model to study human obesity. A cafeteria diet mimics the common practises of a Western style diet which is rich in carbohydrates and fats. It leads to hyperphagia and weight gain. It is widely used to induce experimental obesity (Sclafani et al., 1976). Numerous studies have reported weight gain in Wistar rats fed a cafeteria diet (Gómez-Ruiz et al., 2009; Beltowski et al., 2000; Thomas-Moya et al., 2008). The findings observed in this study are in agreement with literature, where the OC rats, which were fed a cafeteria diet, significantly gained weight relative to the LC group (pre- and post-experimental), and presented with an elevated lipid profile (table 4.5) and hypercoagulable state (figure 4.22).

Weight loss improves IR, hyperglycaemia and hyperinsulinemia in T2DM. The metformin-treated obese rats gained weight at a lower rate when compared with the OC group, however it significantly increased weight relative to the LC group. Metformin has been reported to cause a body weight loss in T2DM patients (Kadoglou et al., 2009; Erdem et al., 2008; DeFronzo and Goodman, 1995). Metformin reduces body weight by reducing appetite thereby reducing calorie intake (Nathason and Nyström, 2009; Jellinger and Mace, 2007; Stunvoll et al., 2005).

Sulfonylurea significantly decreased body weight relative to the OC group, only gaining 1 g in relation to the 30 g gained in the OC group. Our findings display a marginal weight gain as compared to literature, sulfonylurea has been reported to induce weight gain due to water retention and improvement of osmotic diuretics. Gliclazide has been shown to increase mean body weight in T2DM patients and Zucker rats (Belcher et al., 2005; Shi et al., 1999). The increase in body weight has been attributed to its effect on circulating insulin rather than any direct effect on adipocytes. SUR modulates energy storage, thereby contributing to body weight gain (Shi et al., 1999). It is possible that the treatment period was too short to induce a significant weight gain in the sulfonylurea-treated group. In a study conducted by Biederman, rats were treated for 18 to 26 weeks to induce a significant weight gain (Biederman et al., 2005).
Aspirin which is an anticoagulant drug caused a decrease in weight gain relative to the OC and LC groups (18 g relative to 30 g and 21 g, respectively). Aspirin is often used by T2DM patients to treat CAD events since it has antiplatelet and anti-inflammatory properties (Sirois *et al*., 2008). Aspirin has been found to cause water retention in rats (Feldman and Couropmitree, 1976).

The OL extract caused a weight gain relative to the LC group, while it caused a weight loss relative to the OC group. However M caused a weight loss. Two terpenoids isolated from *Pycnathus angolensis* caused a weight gain and improved insulin secretion in a T2DM mouse model (T2DM induced by obesity, *ob/ob* mouse) (Luo *et al*., 1998). The hypoglycaemic effect of these terpenoids contributed to their ability to improve insulin-mediated glucose disposal. However, in a study conducted by Kamgang *et al*. (2007), a water-ethanol extract of *Kalanchoe crenata*, improved glucose uptake, insulin sensitivity and resistance to weight gain in diet-induced diabetic rats. A phytochemical analysis of this extract revealed that this plant contains terpenoids amongst other chemical compounds.

The interaction of insulin with its receptors leads to the autophosphorylation of certain tyrosine residues, resulting in the activation of the receptor kinase (Meshkani and Adeli, 2009; LeRoith and Gavrilova, 2005; Schinner *et al*., 2005). However, when the insulin receptor is dephosphorylated by protein tyrosine phosphatase-1B (PTP1B), the metabolic action of insulin is inhibited. The negative regulation of the insulin gene by PTP1B results in hyperglycaemia (Taha *et al*., 2007). The rats that were on the cafeteria diet (OC group) were hyperglycaemic relative to the LC group. The OC group failed to clear glucose levels after 2 hours (IPGTT). Although the insulin levels of OC and LC were relatively the same, the OC group failed to improve glucose uptake. These rats were rendered glucose intolerant (IPGTT) and their insulin sensitivity decreased (IPITT) compared to the LC group.

Metformin acts mainly on increasing glucose utilization and decreases hepatic glucose production (Yoshida *et al*., 2009; Erdem *et al*., 2008; Bailey and Mynett, 1994). In this
study, two weeks treatment with metformin decreased fasting blood glucose levels. The fasting blood glucose levels after treatment with metformin were similar to that of the LC group. These results correlate with Radziuk et al. (1997) who showed that chronic treatment with metformin decreased plasma glucose levels in rodents. Metformin was the drug which showed the most improvement in IPGTT and IPITT. The reduction in fasting glucose levels was even more pronounced in the presence of insulin (IPITT). Bailey and Mynett (1994) showed that metformin requires insulin for its acute hypoglycaemic effect. The hypoglycaemic effect of metformin was lost in diabetic rats which had low or no insulin concentrations, but the effects were re-gained when insulin was administered. In this study, metformin significantly induced chronic insulin secretion in the OC group. Jang et al. (2010) reported that metformin increased chronic insulin secretion in diabetic (db/db) mice. Metformin has been reported to inhibit PTP1B activity. It indirectly activates the insulin receptor by activating its phosphorylation through the inhibition of PTP1B (Holland et al., 2004). The activation of the insulin receptor results in an increase in glucose uptake.

Sulfonylurea which was used in this study was gliclazide, a drug which is used to stimulate insulin secretion in DM patients (Tahrani et al., 2009). Similar to metformin, treatment with sulfonylurea decreased fasting blood glucose levels and improved IPGTT and IPITT. Guerra et al. (2009) showed that gliclazide improved insulin secretion in human islet β-cells that were exposed to hyperglycaemic conditions. It increased the gene and protein expression of PDX-1 and Ki67, molecules that are involved in β-cell differentiation and regeneration, respectively. Gliclazide normalised glucose and insulin levels, improved lipid profiles in obese rats (Marquié et al., 2004). It was found to increase the expression of PDX-1 in Wistar rats and INS-1 cells. The increase in PDX-1 expression was correlated to GSIS (Sun et al., 2007). Therefore, it is possible that gliclazide improved glucose clearance and insulin sensitivity in the Wistar rats on a cafeteria diet by increasing the gene and protein expression of PDX-1 and Ki67. Our findings are in agreement with literature, as sulfonylurea increased insulin secretion relative to LC and OC groups. As it was mentioned earlier, one of the side effects of sulfonylurea is hypoglycaemia as insulin secretion can be stimulated under both normo-
and hyperglycaemic conditions (Nathason and Nyström, 2009; Jellinger and Mace, 2007; Stunvoll et al., 2005).

In this study, aspirin showed increased insulin sensitivity (IPITT) rather than evidence of insulin secretion. In a study conducted by Devarakonda et al. (2009), aspirin prevented the development of T2DM in rats by reducing IR, improving insulin sensitivity and fasting insulin levels. A human clinical trial showed that aspirin improves insulin sensitivity and fasting insulin levels by inhibiting IKKβ activity (Hundal et al., 2002). In a study conducted by Abdin et al. (2009) administration of aspirin to T2DM rats improved insulin levels, and it was concluded that inhibition of IKKβ by aspirin could provide a valuable tool in discovering new drugs for the treatment of T2DM. The dosage of aspirin which was used in the studies conducted by Abdin et al. (2009) was 120 mg/kg, which is 2.4-fold higher than the aspirin dose which was used in this study (50 mg/kg), while Devarakonda et al. (2009) used 10 mg/kg/day for 1 to 2 months.

This study shows that M significantly lowered the fasting blood glucose levels in a cafeteria diet-induced obesity model. Due to reduced M levels in OL condition, OL did not improve glucose clearance to the same degree as M. The effect of the OL extract on IPGTT was not as significant as that exhibited by M. A methanol extract of *Ichnocarpus frutescense* increased plasma insulin secretion by 69% in STZ-induced DM in rats (Subash-Babu et al., 2008). It was concluded that the stimulation of chronic insulin release could be attributed to the presence of terpenoids and steroids in the plant extract. Li et al., (2004) reported that the mechanism of action of terpenoids and steroids was to stimulate insulin secretion and increase the sensitivity of islets to promote glucose uptake in pancreatic β-cells. *Tectona grandis* has been traditionally used to treat DM (Ghaisas et al., 2009). Ghaisas et al. (2009) scientifically evaluated the antidiabetic effects of this plant. A phytochemical study revealed that this plant contained terpenoids amongst other compounds. The ethanolic extract of this plant reduced plasma glucose levels and triglycerides in dexamethasone-induced T2DM, and these effects were contributed to the presence of terpenoids. It also improved glucose uptake and decreased IR. Based on these findings in literature, it is proposed that M, a diterpenoid present in the *Leonotis leonurus*
extract is responsible for the stimulation of insulin secretion in INS-1 cells and in the obese rat model. M enhanced insulin secretion 2.2-fold relative to the OC group. These results correlated with GSIS where the stimulatory index was increased by 2.0-fold relative to the hyperglycaemic C. However, M also enhanced insulin secretion 3.0-fold, relative to LC groups. This could possibly be due to the cells being directly exposed to M, while the *in vivo* studies possibly resulted in metabolites of M stimulating the release of insulin under normoglycaemic conditions (LC group). The M did not significantly improve IPITT relative to both controls (LC and OC group), therefore it could be eliciting its effects directly on islet cells, causing secretion of insulin. This however needs to be further investigated using primary islet cells. The OL extract had the most prominent effect on insulin secretion. The enhanced effect of the OL extract could be caused by other phytochemical compounds present in the extract or the synergy of the compounds, as it was displayed on the TLC and HPLC chromatogram, the OL extract has multiple compounds.

DM is known as a hypercoagulable disease due to hypercoagulation and hyperplatelet activity observed in DM patients (Albert *et al*., 2005). As a result of this state, T2DM patients are predisposed to the development of DVT (Bouzegharane *et al*., 2008). CAD is known to be the cause of morbidity and mortality in T2DM patients (Plutzky *et al*., 2002). Atherosclerosis is the cause of the increase in risk of CAD. Endothelial destruction by the formation of ROS through chronic inflammation, dyslipidemia, hypertension and hypercoagulation result in the development of atherosclerotic lesions. The formation of foam cells is caused by the accumulation of cholesterol esters. Mature atherosclerotic plaques are formed by the continuous recruitment of leukocytes and the migration of smooth muscle cells into the intima (Plutzky *et al*., 2002). Endothelial damage increases the expression of adhesion molecules such as VCAM-1, selectins and ICAM-1. Pro-inflammatory cytokines (IL-6 and TNF-α) are released and promote the expression and activation of adhesion molecules (Bakker *et al*., 2009; Ferroni *et al*., 2004; Plutzky *et al*., 2002).
As a result of an increased rate in coagulation, fibrin levels in the OC group were significantly higher when compared to the LC group. DVT and PE were associated with increased levels of D-Dimer (Ghys et al., 2008). D-Dimer is used as a diagnostic tool in DVT and PE. D-Dimer levels are also used as a marker of thrombin generation (Chaireti et al., 2009). Obesity results in a prothrombotic state caused by hypercoagulation. Hyperplatelet activity is observed in obesity. Obesity and IR patients have been shown to have high levels of P-selectin, which is a marker of platelet activity (De Pergola et al., 2008). This study confirmed the theory that hyperglycaemia affects coagulation because plasma from the OC group significantly increase coagulability, this was evident by the short time of clot formation (APTT) and the increase in the levels of fibrin formation when compared to the LC group. Hypercoagulation was accompanied by hyperplatelet activity (figure 4.23) and D-Dimer formation was significantly elevated in the OC group relative to the LC group (figure 4.22).

Hyperglycaemia results in the glycation of coagulation factors, altering the haemostatic balance. Metformin promoted glucose uptake in the obese model, thus possibly reducing the glycation of the coagulation factors which would result in the prolongation of the clotting time. Metformin significantly prolonged the APTT clotting time, reduced fibrin formation and platelet aggregation relative to the OC group. Although D-Dimer formation was reduced it was not significant relative to the OC group. The prolongation of the APTT clotting time and the inhibition of platelet aggregation were normalised as their levels were similar when compared to the LC group. Metformin has been reported to have anti-thrombotic properties by reducing the in vivo levels of FVII (Grant 2003). This was evident by the decrease of fibrin formation from fibrinogen in rats that were treated with metformin. It has been reported to reduce PAI-1 in obese, non-diabetic and T2DM patients (Charles et al., 1998; Testa et al., 1996). In vitro and in vivo investigations have shown that metformin interferes with fibrin polymerisation and reduces FXIII crosslinking, thus resulting in clots which are easier to dissolve (Charles et al., 1998; Standeven et al., 2002). Inhibition of coagulation would result in less platelet activity, fibrin and D-Dimer formation, as observed in this study.
Sulfonylurea significantly prolonged the clotting time (APTT) relative to the OC group and normalised it relative to the LC group. It significantly reduced fibrin formation and platelet aggregation although D-Dimer formation was significantly increased relative to the OC group. Gliclazide has been reported to reduce permeability of a clot structure, thus forming a prothrombotic clot that is resistant to fibrinolysis (Standeven et al., 2002). Based on the results, gliclazide does not have cardiovascular protective effects. However, in a study conducted by Freed et al. (2000) and Cefalu et al., (2002), sulfonylurea (glibeclamide and gliplizide, respectively) was found to reduce PAI-1 levels in T2DM patients. The inhibition of PAI would result in the activation of plasmin through tPA. Plasmin activation leads to an increase in fibrinolytic activity, hence elevated D-Dimer (fibrin degradation product) levels were observed in sulfonylurea-treated obese rats.

Aspirin is a known cyclooxygenase inhibitor; therefore, it inhibits platelet aggregation. It acetylates COX-1, thereby inhibiting the formation of TXA2 resulting in the reduction of platelet aggregation. In addition to its antiplatelet activity, aspirin has been reported to possess antithrombotic properties. Aspirin acetylates fibrinogen, resulting in clots which are easier to lyse (Henschen-Edman, 2001; Bjornsson et al., 1989). Aspirin has also been shown to modulate FXIII activity in vivo (Bjornsson et al., 1989). The results obtained in this study are in agreement with those found in literature. The APTT clotting time was prolonged relative to the OC group, showing the anti-thrombotic property of aspirin. The prolongation of the clotting time resulted in less fibrin and D-Dimer formation (figure 4.22). Platelet aggregation was inhibited through the inhibition of COX-1 activity. Aspirin was able to normalise the clotting time, fibrin and D-Dimer formation, as the degree of clot prolongation, fibrin and D-Dimer formation was similar to that of the LC control (figure 4.22).

The OL extract and M prolonged the clotting time of the APTT time, indicating that OL and M are specific for the intrinsic pathway. These results, correlate with the results that were obtained from the ex vivo model whereby only the intrinsic pathway was affected by M. The degree of the prolongation of the clotting time by M was similar to that of aspirin. M showed a more pronounced effect on fibrin formation (figure 4.22). It reduced fibrin
formation to the same level as the LC group. Similar to the results obtained for the APTT, the effect of M on fibrin formation was similar to that of aspirin. The OL extract did decrease fibrin formation but not to the same level as M. M was the only compound which reduced D-Dimer levels to the same or comparable levels as that of the LC group. The OL extract and M significantly inhibited platelet aggregation (figure 4.23). These results correlate with the results obtained in the in vitro and ex vivo models. They indicate that the OL extract and M mediate the inhibition of thrombin-induced platelet aggregation via the blockage of GP IIb/IIIa. The anticoagulant and antiplatelet activity of the OL extract can be attributed to M.

Dyslipidemia which can be caused by either hypercholesterolemia or hyperlipoproteinemia is one of the risk factors in CAD and atherosclerosis (West, 2000; Tatsuki et al., 1997). Lipid abnormalities which are found in diabetic dyslipidemia can cause an altered balance in metabolic states of DM, i.e. hyperglycaemia and IR (Bhandari et al., 2002). Therefore, drugs that control glycaemic states improve dyslipidemia in DM patients (West, 2000; Tatsuki et al., 1997). The development of IR in obesity impairs the normal release of FFAs from adipose tissue. This allows the development of dyslipidemia associated with elevated levels of triglyceride, low HDL cholesterol and high levels of circulating FFAs. The elevated levels of FFAs leads to the overproduction of LDL and VLDL cholesterol in IR observed in T2DM patients (Meshkani and Adeli, 2009; Stunvoll et al., 2005). The atherogenic lipoprotein phenotype enhances cardiovascular risk in T2DM (Holmes et al., 2008).

In this study, the OC group had significantly high levels of plasma cholesterol, triglycerides and LDL with a significant decrease in HDL levels. High levels of serum triglycerides are used as a diagnostic tool for vascular disease, however, the increase in serum cholesterol levels serves as a predictor of atherosclerosis (O’ Keefe et al., 1999). Reduced levels of HDL in DM patients could be caused by an elevated triglycerides load in HDL particles. Triglycerides are hydrolyzed by lipase. This hydrolysis results in a small HDL particle which is filtered by the kidneys, which result in decreased levels of apolipoprotein A and HDL levels (Groenendijk et al., 1999). The AI of the OC group was
2.1 which classified this group to be in a high risk category for AI, as its AI profile was >0.21 (Holmes et al., 2008).

Metformin, M and sulfonylurea significantly decreased triglyceride, total cholesterol and LDL cholesterol levels, while HDL cholesterol levels were significantly improved relative to the OC group. The AI of metformin-treated rats was significantly lower than that of the OC group but higher than that of the LC group. The AI profile of metformin-treated rats was 0.3 which classified this group into the high risk category for AI. Metformin has been reported to reduce circulating levels of triglycerides, FFAs, and LDL cholesterol, while it increases the levels of HDL cholesterol (Nathason and Nyström, 2009; Jellinger and Mace 2007, Stunvoll et al., 2005).

Sulfonylurea significantly decreased triglycerides, total cholesterol, LDL cholesterol and AI, while it increased HDL cholesterol. The AI of sulfonylurea was similar to that of metformin, placing this group into the same risk category for the AI as that of the metformin group. Tessier et al. (1999) reported elevated levels of HDL and reduced levels of total cholesterol, LDL cholesterol and triglycerides in T2DM rats. The high levels of HDL were thought to protect LDL from lipid oxidation.

In this study, aspirin improved the lipid profile by decreasing triglyceride, total cholesterol, and LDL cholesterol levels, while it increased HDL levels. Aspirin is commonly recommended for CVD patients. However, aspirin showed the least effect on AI profile (0.5), placing this group into the high risk category for the AI. In a study conducted by Abidin et al. (2009), aspirin improved dyslipidemia. Yuan et al. (2001) demonstrated that salicylates (including aspirin) can reverse hyperglycaemia, hyperinsulinemia, and dyslipidemia by sensitizing insulin signalling and improvement of IR.

M was more effective in decreasing triglyceride, cholesterol and LDL levels, while it significantly increased HDL levels. OL also improved lipid profiles of the OC rats but not to the same degree as M. M normalised the HDL, LDL, total cholesterol and the AI
levels relative to the LC group. The AI profile of M-treated group was 0.2, placing this group in the intermediate risk category for the AI profile. The M-treated group is the only group besides LC group which is in this AI category. In a study conducted by Herrera-Arellano (2004), *Marrubium vulgare* significantly decreased cholesterol and triglyceride levels in human T2DM patients by 4.16 and 5.78%, respectively. *Ichnocarpus frutescens*ce and *Tectona grandis* were observed to decrease triglyceride, LDL cholesterol levels, and increased HDL levels (Subash-Babu *et al*., 2008; Ghaisas *et al*., 2009). The presence of terpenoids in these two plants is proposed to be responsible for insulin secretion and the anti-atherogenic properties, providing evidence that M is the agent responsible for improving lipid profiles in the obese model tested.

T2DM is a progressive disease which is characterised by IR in peripheral tissues. The elevated blood glucose levels result in additional complications, including changes in the coagulation and fibrinolytic system. Atherosclerosis develops in DM patients because of hypercoagulability which is caused by the inhibition of natural anticoagulants (protein C, TF, heparin-antithrombin pathway and thrombomodulin). The glycation of these natural anticoagulants results in their inactivity causing hypercoagulation. Elevated fibrinogen levels lead to its binding to GPIIb/IIIa complex, where it only binds the activated GPIIb/IIIa complex. This complex is activated by various platelet agonists which are released during coagulation and platelet activity. Binding of GPIIb/IIIa to fibrinogen causes hyperplatelet activity and adhesion. Hyperglycaemia causes an imbalance between the blood coagulation and fibrinolytic system. The impairment of the fibrinolytic system caused by elevated levels of PAI, results in clots which are not dissolved. These clots prevent normal blood flow, leading to the development of CAD. The pathogenesis of DVT is associated with DM. The elevated incidences of DVT with an increase in morbidity and mortality are associated with obesity and DM (Bouzeghrane *et al*., 2008). IR is the common link between obesity and T2DM (Taha *et al*., 2007). Diabetes is defined as a state whereby haemostasis of lipid and carbohydrate metabolism is improperly regulated by insulin. Therefore, T2DM often co-exists with visceral obesity, dyslipidemia and hypertension. This cluster is referred to as MetS (Plutzky *et al*., 2002). These cardiovascular risk factors promote the development of atherosclerosis in T2DM.
There is a need for the development of new antidiabetic drugs because the prevalence of this pandemic has escalated. A multi-model therapeutic approach is required for the treatment of DM since it is a multifactorial pathogenetic disease. Medicinal plants offer a large scope for combating the threat of the diabetic pandemic (Tiwari and Rao, 2002). In this study, M was found to stimulate insulin secretion \textit{in vitro} and \textit{in vivo}. The increase in MMP and respiration correlated with the increase in GSIS. The \textit{in vivo} animal model was utilised to evaluate the effect of M in a physiological environment. An obese model is associated with IR, hypercoagulation and hyperplatelet activity. These conditions are associated with T2DM. The stimulation of insulin release by M, resulted in an improved lipid profile. M exhibited anti-atherogenic properties by decreasing triglyceride, total cholesterol and LDL cholesterol, while it improved HDL cholesterol levels. Insulin prevents the release of free fatty acids and is an anti-atherogenic hormone (Jellinger and Mace, 2007). Several studies have reported that terpenoids have antidiabetic activities because they stimulate insulin secretion and improve the AI (Subash-Babu \textit{et al.}, 2008; Ghaisa \textit{et al.}, 2009). M is the active agent in the OL extract due to similarities in findings associated with equivalent M concentrations. M therefore presents as a lead compound that could be used in the treatment of T2DM patients who are at risk of CVD, because of its antidiabetic, anti-atherogenic, anticoagulant and antiplatelet properties.
CHAPTER 5
5.1 Future perspectives
The aim of this study was to evaluate the mechanism of action of *Leonotis leonurus*’s anticoagulant, antifibrinolytic, antiplatelet and antidiabetic effects.

Based on the results that were obtained in this study, it can be concluded that M is the primary diterpenoid in the *Leonotis leonurus* extract responsible for the anticoagulant, antiplatelet and antidiabetic activities associated with this extract.

For the coagulation and platelet studies, coagulation factors that are inactivated in APTT can be determined. The effect of M on ERK1/2 signalling pathway can be studied, to see if calcium mobilization suppression is through this pathway. Protein expression and gene expression of ERK1/2 could be evaluated using Western blot analysis and RT-qPCR. The mechanism of inhibition of platelet adhesion can be evaluated by determining the effect of M on the expression and activity of adhesion surface ligands such as vWF, GPIb-IX-V, GPIb, GPIa/IIa using flow-cytometry.

Hyperglycaemia results in glycation of coagulation Fs and the suppression of fibrinolysis, therefore, the effect of M on plasma advanced glycation products could be evaluated. RANTES and MCP-1 are pro-atherosclerotic chemokines. The levels of these two chemokines were found to be increased in obese Zucker rats (Schäfer et al., 2008). RANTES and MCP-1, increase platelet adhesion resulting in hyperplatelet activity. Immunoassays could be used to determine serum levels of RANTES and MCP-1. Therefore, the effect of M on these two pro-inflammatory chemokines could be evaluated.

Elevated circulating levels of IL-6 are used to predict the development of DM (Pickup et al., 1997). The levels of IL-6 and TNF-α are elevated in T2DM. Gene and protein expression studies could be conducted to see the effect of M on these two pro-inflammatory markers. Hyperglycaemia induces oxidative stress. The production of ROS species results in elevated levels of thiobarbituric acid-reactive species (TBARS) (Ferroni
et al., 2004. The liver and kidney tissues have been isolated from the in vivo rat obese model, they can be used to evaluate hepatic glucose -6-phosphatase activity, cytochrome P450, and TBARS.

Calcium mobilization studies on β-cells can be conducted to evaluate how M induces insulin secretion. Western blots and RT-qPCR can be completed to evaluate the effect of M on the expression of UCP-2 as it is well established that the blockage of this protein results in an increase in insulin secretion. At this point in time, there is little known in literature about M receptors in cells, particularly β-cells. Therefore one can look at M receptors to determine how M elicits its effect on target tissues. RT-qPCR can be completed to evaluate the effect of M on the expression of IRS-1 and IRS-2 as the downstream regulation of these two receptors is important in the secretion of insulin. The skeletal muscle, heart muscle and adipose tissue from all groups can be used to investigate the role of fatty acid uptake and fatty acid β-oxidation in mediating IR in heart and skeletal muscle.

The product standardization, efficacy, safety and therapeutic risk/benefit associated with the extract needs to be investigated. The absorption rate of M in Wistar rats could not be completed in the present study based on time constraints. The pharmacokinetics of M therefore requires investigation, where the metabolic effects of M needs to be investigated i.e. its rate of absorption and elimination and its half-life. The therapeutic index of M has to be evaluated, where the maximum and the minimum safe dose is obtained. If M is to be commercialised, the solubility of M has to be investigated to ensure capsulation which can be orally administered to ensure increased patient compliance.
CHAPTER 6
REFERENCES


Ramanadham S., Zhang S., Ma Z., Wohltmann M., Bohrer A., Hsu F.F. and Donald M.J. (2002). Delta 6, stearoyl CoA-, and delta 5-desaturase enzymes are expressed in beta-cells and are altered by increases in exogenous PUFA concentrations. Biochimica et Biophysica Acta. 1580, 40-56.


**Internet references**

APPENDIX

ANNEXURE A

Ref: [A09-SCI-BCM-003/Approval]
Contact person: Mrs U Spies

25 November 2009

Prof C Frost
NMMU
Faculty of Science
Department of Biochemistry and Microbiology
South Campus

Dear Prof Frost,

THE MECHANISM OF ANTICOAGULANT ACTIVITIES OF TULBAGHIA VIOLACEAE AND MARRUBIUM

Your above-entitled application for ethics approval served at the October 2009 ordinary meeting of the Research Ethics Committee (Animal).

We take pleasure in informing you that the application was APPROVED by the Committee.

The Ethics clearance reference number is A09-SCI-BCM-003, and is valid for three years.

Please note that each year you will have to affirm that the approved protocol is still in place, or apply for ethics approval for any modification. At the end of the third year you will have to affirm that the project is complete, or reapply for ethics approval. You will receive the appropriate reminder and documentation each year well in time for any applicable deadline.

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

Yours sincerely

Dr G Dealtry
Chairperson: Research Ethics Committee (Animal)

cc: Faculty Officer. Faculty of Science
Department of Research Capacity Development

/us
ANNEXURE B

Ref: [A09-SCI-BCM-001/Approval]
Contact person: Mrs U Spies
23 November 2009
Prof C Frost
NMMU
Faculty of Science
Department of Biochemistry and Microbiology
South Campus

Dear Prof Frost,

THE MECHANISM OF ANTICOAGULANT AND ANTIDIABETIC ACTIVITIES OF LEONOTIS LEONURUS

Your above-entitled application for ethics approval served at the September 2009 ordinary meeting of the Research Ethics Committee (Animal).

We take pleasure in informing you that the application was APPROVED by the Committee.

The Ethics clearance reference number is A09-SCI-BCM-001, and is valid for three years.

Please note that each year you will have to affirm that the approved protocol is still in place, or apply for ethics approval for any modification. At the end of the third year you will have to affirm that the project is complete, or reapply for ethics approval. You will receive the appropriate reminder and documentation each year well in time for any applicable deadline.

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

Yours sincerely,

[Signature]

Dr G Dealty
Chairperson: Research Ethics Committee (Animal)

cc: Faculty Officer, Faculty of Science
    Department of Research Capacity Development

Nelson Mandela Metropolitan University
For tomorrow

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Ref: N 01/1/33/07 [09688-003/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