A biochemical study of the antidiabetic and anticoagulant effects of *Tulbaghia violacea*

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Submitted in partial fulfilment of requirements for the degree of *Magister Scientiae* (MSc) in the Department of Biochemistry and Microbiology in the Faculty of Science at the Nelson Mandela Metropolitan University

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ABSTRACT

Secondary metabolites derived from plants, especially those used by traditional healers, are at the forefront of new drug development in combating diseases such as cancer and diabetes. Garlic is employed in indigenous medicine all over the world for the treatment of a variety of diseases. Dietary garlic has been recognized for its beneficial health effects. In particular, garlic consumption has been correlated with (i) reduction of risk factors for cardiovascular diseases and cancer, (ii) stimulation of immune function, (iii) enhanced detoxification of foreign compounds, (iv) hepatoprotection, (v) antimicrobial effects, (vi) antioxidant effects, and most importantly (vii) its hypoglycemic and anticoagulant properties. Due to these beneficial properties, garlic and its closely related genera which includes *Tulbaghia violacea*, may be useful as coadjuvant therapy in the treatment of type 2 diabetes and some of its physiological complications.

The aim of this study was to determine if *T. violacea* has antidiabetic and anticoagulant properties. This was performed *in vitro* using both aqueous and organic extracts of the roots, leaves and bulbs. An organic extract was able to improve glucose-stimulated insulin secretion (GSIS) in INS-1 pancreatic β-cells and glucose uptake in Chang liver cells. The BO extract had no effect on the glucose uptake in 3T3-L1 an adipose cell line and reduced glucose utilisation in C2C12, a skeletal muscle cell line. Some of the properties displayed by *T. violacea* in this study are consistent with those found in similar studies with garlic extracts. It was observed that the BO extract increased the membrane potential and Glut-2 expression in INS-1 cells cultured at hyperglycemic levels, however, at normoglycemic levels a reduction was observed. The oxygen consumption increased at both glycemic levels due to treatment with the BO extract.

Platelets were exposed to the extracts to determine their effects upon platelet aggregation, adhesion and protein secretion. Since the BO extract displayed the highest potential at inhibiting platelet aggregation and adhesion. A rat model was used in ex vivo studies to determine if the extract exhibited the same effect in a physiological model. It was noted that the BO extract exhibited a higher degree of inhibition on platelet aggregation and adhesion than the positive control, aspirin. The BO extract reduced clotting times in the prothrombin time (PT) test, but prolonged the clotting time in the actived partial thromboplastin time (APTT) assay in the *ex vivo* model; however, it had no affect on these clotting assays in the *in vitro* model using human blood. The BO extract increased the D-dimer and Fibrinogen-C levels in the *in vitro* model, but had no effect on the D-dimer concentrations and lowered the Fibrinogen-C levels in the *ex vivo* model. The active compounds in the extract remain to be elucidated.

Keywords: *Tulbaghia violacea, INS-1 cells, 3T3-L1 cells, Chang liver cells, C2C12, insulin secretion, glucose uptake, Glut-2 expression, oxygen consumption, membrane potential, antiplatelet, anticoagulant*

DECLARATION

In accordance with Rule G4.6.3, I hereby declare that the above-mentioned treatise/ dissertation/ thesis is my own work and that it has not previously been submitted for assessment to another university or for another qualification.

SIGNATURE: _____

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ACKNOWLEDGMENTS

I would like to thank my supervisors, Prof. Carminita Frost and Mrs. Ruby-Ann Levendal for their invaluable guidance, advice, patience and always “being there” for me at all times, making this research possible. I would also wish to thank my family for their continuous support during this time especially my mother. My grateful thanks also to my friends and colleagues in NMMU Biochemistry Department for their generous help and support. Thank you also to the National Research Foundation and NMMU for funding this project.

LIST OF ABBREVIATIONS

$\Delta\psi_m$:	Membrane potential
AAs:	Amino acids
ACE:	Angiotensin-converting enzyme
ADP:	Adenosine diphosphate
AGE:	Aged garlic extracts
Akt:	Protein kinase A
APTT:	Activated partial thromboplastin time
ArA:	Arachidonic acid
ATCC:	American tissue culture collection
ATP:	Adenosine triphosphate
ATPase:	ATP synthase
BA:	Bulb aqueous
BCA:	Bicinchroninic acid
BCIP/NBT:	5-Bromo-4-chloro-3indolyl phosphate/Nitroblue Tetrazolium
BO:	Bulb Organic
BSA:	Bovine serum albumin
BT:	Bleeding time
cAMP:	Cyclic adenosine monophosphate
COX:	Cyclooxygenase
DIC:	Disseminated intravascular coagulation
ddH_2O:	Double distilled water
DMEM:	Dulbecco's modified Eagle's medium
DMSO	Dimethyl-sulfoxide
DPPH:	2, 2'-diphenyl-1-picrylhydrazyl
EC:	Endothelial cells
ECM:	Extracellular matrix
EDTA:	Ethylenediaminetetraacetate
ER:	Endoplasmic reticulum
FBS:	Foetal bovine serum
Fc	Faraday's constant
FITC-PAC-1:	Fluorescent isothiocyanate-monoclonal antibody directed at glycoprotein IIb/IIIa platelet complex
FFA:	Free fatty Acids
FpA:	Fibrinopeptide A
FpB:	Fibrinopeptide B
FV:	Proaccelerin labile factor
FXa:	Activated stuart-power factor
FX:	Stuart-power factor

FVIII:	Antithemophilic factor A
FIX:	Antithemophilic factor B (Christmas factor)
FXI:	Plasma thromboplastin antecedent
FXII:	Hageman factor
FXIIa:	Activated Hageman factor
GLP-1	Glucagon-like peptide-1
G-6-P:	Glucose-6-phosphate
GLUTs:	Glucose transporters
Glut-2:	Glucose transporter 2
GP IIb/IIIa:	Glycoprotein IIb/IIIa
Grb:	Growth factor receptor bound protein
Grb2:	Growth factor receptor bound protein 2
GSIS:	Glucose-stimulated insulin secretion
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC:	High-performance liquid chromatography
IgG:	Immunoglobulin G
IP_3:	Inositol triphosphate
IRS:	Insulin receptor substrate
IRS-1:	Insulin receptor substrate one
JC-1:	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide / 1st J-aggregate-forming cationic dye
K_{ATP}:	ATP sensitive potassium channel
kDa:	Kilo Daltons
LA:	Leaves aqueous
LF:	Lachrymatory factor
LO:	Leaves organic
MTT:	3-(4, 5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide
NAD:	Nicotinamide adenine dinucleotide
NADH:	Reduced nicotinamide adenine dinucleotide
$NaHCO_3$:	Sodium bicarbonate
Nck:	Adapter protein
NMMU:	Nelson Mandela Metropolitan University
NO	nitric oxide
PAI-1:	Type-1 plasminogen activator inhibitor
PAF:	Platelet-activating factor
PAR:	Protease-activated receptor
PBS:	Phosphate buffered saline
PDK 1:	Phosphoinositide-dependent kinase 1

PEG	Polyethylene glycol
PGs:	Prostaglandins
PIP_2:	Phosphatidyl-inositol-3, 4-bisphosphate
PIP_3:	Phosphatidyl-inositol-3, 4, 5-triphosphate
PI3-K:	Phosphoinositide-3-kinase
PK:	Prekallikrein
PKA:	Protein kinase A
PKB:	Protein kinase B
PKC:	Protein kinase C
PLA_2:	Phospholipase A_2
PPP:	Platelet poor plasma
PRP:	Platelet rich plasma
PT:	Prothrombin time
PVDF:	Polyvinylidene difluoride
RA:	Rhizomes Aqueous
R_f:	Relative migration
RFI:	Relative fluorescent index
RIA:	Radioimmunoassay
RO:	Rhizomes organic
ROS:	Reactive oxygen species
RPMI:	Roswell Park memorial institute (medium)
RRP:	Readily releasable pool
RT:	Room temperature
RT-qPCR:	Real time quantitative polymerase chain reaction
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM:	Standard error of the mean
SIRP:	IRS-1 signal regulatory protein
SH_2:	Src homology 2
SHP_2:	Non-receptor protein tyrosine phosphatase
SNAP:	Synaptosomal-associated protein
SNAP-25:	Synaptosomal-associated protein 25
SNARES:	SNAP Receptors
STZ:	Streptozotocin
TAT:	Thrombin-antithrombin complex
TB:	Tuberculosis
TBS:	Tris-buffered saline
TF:	Tissue factor
Tris:	2-Amino-2-(hydroxymethyl)-1,3 propanediol

t-PA:	Tissue plasminogen activator
T1DM:	Type 1 diabetes mellitus
T2DM:	Type 2 diabetes mellitus
TT:	Thrombin time
TX:	Thromboxane
TXA_2:	Thromboxane A_2
TXB_2:	Thromboxane B_2
u-PA:	Urinary plasminogen activator
VAMP-2:	Vesicle-associated protein 2
VECs:	Vascular endothelial cells
VSMC:	Vascular smooth muscle cell
vWF:	von Willebrand Factor
WHO:	World Health Organisation

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CHAPTER ONE: LITERATURE REVIEW

1.1 INTRODUCTION

Both in modern and traditional medicine, medicinal plants continue to provide valuable therapeutic agents in the cure of disease and aliments. Doubts about the efficacy and safety of the oral hypoglycemic agents have prompted a search for safer and more effective drugs in the treatment of diabetes (Bannerman, 1993). In spite of the fact that insulin has become one of the most important therapeutic agents, researchers have been making efforts to find insulin substitutes from synthetic or plant sources for the treatment of diabetes. Many herbs have remained as an alternative to conventional therapy especially in poor areas where insulin is not readily available, due to the high cost and the lack of medical aid (El-Demerdash *et al*., 2005).

Medicinal plants are the oldest known health-care products, where renewed interest is growing based on the ethnological, medical and historical background of each country. Medicinal plants have always been important for pharmacological research and drug development, where the plant constituents are used directly as therapeutic agents, for the synthesis of drugs or as models for pharmacologically active compounds (Levetin and McMahon, 1999).

Researches into secondary metabolites derived from plants are being used in the development of new drugs to combat diseases such as cancer and diabetes. These compounds, named "nutraceutical" or "phytochemicals", are classified as non-essential micronutrients and are able to contribute to human homeostasis, playing a role in the maintenance of health and lessening the impact of chronic disease on the life quality of the patients. Such interest was due to the results of epidemiological studies that have correlated a semi-vegetarian diet with a decreased incidence of chronic- and acute-inflammatory diseases (Barnes and Prasain, 2005). The identification of organic compounds responsible for these activities has increased scientific studies of plants and the analysis of secondary metabolites from edible plants. Both garlic (*Allium sativum* L.), and onion, (*Allium cepa* L.), have been used since ancient times for the treatment of many diseases. Codex Ebers (1550 BC), an Egyptian medical papyrus, is one of the first reports of garlic and onion being used in

therapeutic formulas in the treatment of a variety of diseases. Therefore, garlic, onion and their family representatives are receiving renewed attention (Lanzotti, 2006).

Garlic is still being employed in indigenous medicine all over the world for the treatment of a variety of diseases (Benavides *et al*., 2007). Dietary garlic has been recognized for its beneficial health effects. In particular, garlic consumption has been correlated with (i) reduction of risk factors for cardiovascular diseases and cancer, (ii) stimulation of immune function, (iii) enhanced detoxification of foreign compounds, (iv) hepatoprotection, (v) antimicrobial effects and (vi) antioxidant effects. However, the active components and mechanisms of action of garlic to some degree still remain elusive (Banerjee *et al*., 2002; Benavides *et al*., 2007).

Evidence from several investigations suggests that the biological and medical functions of garlic and related genera are mainly due to their high organo-sulphur compound content. The primary sulphur-containing constituents are S-alk (en) yl-L-cysteine sulphoxides (ACSOs), such as alliin, and g-glutamyl cysteines, which serve as important storage peptides, are biosynthetic intermediates for corresponding ACSOs, such as allicin, and lipid-soluble sulphur compounds, such as diallyl sulphide (DAS) and diallyl disulphide (DADS). These compounds provide garlic and onion with their characteristic odour and flavour, as well as most of their biological properties (Lanzotti, 2006). Lectins, prostaglandins (PGs), fructan, pectin, adenosine, vitamins B_1, B_2, B_6, C and E, biotin, nicotinic acid, fatty acids, glycolipids (GLs), phospholipids (PLs) also contribute to garlic's biological properties (Lanzotti, 2006). Other characteristic chemical constituents of garlic include allixin and organo-selenium compounds. These chemical compounds are reported to exhibit several biological effects, which include cholesterol reduction, cancer prevention, and probably work synergistically with organo-sulphur compounds (Amagase, 2006).

Garlic and onion are both known to inhibit platelet aggregation *in vitro*. Several platelet inhibitors have been isolated and characterized from garlic and onion. Their effects on platelet aggregation and thromboxane (TX) formation have been studied (Allison *et al*., 2006). Organic solvents may cause denaturation of the enzyme allinase, which converts alliin to allicin, a potent inhibitor of platelet aggregation. Due to the variations in methods of preparation, the inhibitory effects of various garlic

products differ in their effectiveness. It has recently been reported that in plants of the genus *Allium*, such as garlic and onions, the anti-platelet activity is determined in part by the native concentration of organo-sulphur compounds. In the same study it was reported that the anti-platelet activity from four different genotypes of onion (*Allium cepa*) on human blood correlated with the sulphur content of the bulb (Allison *et al*., 2006).

Due to its hypoglycemic and antioxidant properties, garlic and its closely related genera may be useful as coadjuvant therapy in the treatment of type 2 diabetes mellitus (T2DM) and some of its physiological complications. Allicin, garlic's proposed active ingredient, is believed to compete with insulin for hepatic insulin-activating sites, providing a possible solution for the treatment of diabetes and its side effects (Benavides *et al*., 2007). It is estimated that 80% of diabetics die as a result of a thrombus and of these, 75% as a result of cardiovascular complications (Calles-Escandon *et al*., 1999 and Sacco, 1995). The atherothrombotic complications that are found in insulin resistance syndrome are caused by a dysregulation of hemostasis which induces a prothrombotic state. This state includes endothelial activation, enhanced platelet activity, enhanced coagulability, and reduction in fibrinolysis (Juhan-Vague *et al*., 2001). Aqueous and organic garlic extracts inhibit platelet aggregation induced by a number of physiologically important aggregating agents, thereby counteracting these thrombotic complications.

Traditional medicine plays an integral part of South African everyday cultural life, and is unlikely to change to a large degree in years to come. It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicines from as many as 700 indigenous plant species (Duncan *et al.,* 1999). Traditional and folk medicine came into being long before the development and spread of western medicine that originated in Europe with the advent of modern science and technology. The knowledge of traditional medicine is often passed on, verbally or otherwise, from generation to generation, and can be lost if cultural values are not sustained (Zhang, 2000 and Neuwinger, 2000).

Most people in non-industrialised countries receive traditional health care (THC) for their everyday health care needs. The World Health Organisation (WHO) has

consistently estimated that 60–80% of the population in these countries rely on THC for their basic health care needs, either on its own or in conjunction with modern medical care (Bannerman, 1993). The increased demand for traditional medicine in developing countries is likely due to their increased formalisation, low cost and safety standards, and their use as a complementary therapy (Bannerman, 1993 and Neuwinger, 2000).

1.2. THE FAMILY *ALLIACEAE*

The *Alliaceae* family has 600 species in 30 genera and the family is taxonomically intermediate between the *Liliaceae* and the *Amaryllidaceae. Alliaceae* is a widely distributed family and the major places of its distribution are the Mediterranean, Europe, Asia, North and South Americas and Southern African areas. The southern African genera are *Agapanthus, Tulbaghia* and *Allium* (Dahlgren and Thorne, 1984).

1.3. THE GENUS *ALLIUM*

Allium is the largest and most important representative genus of the *Alliaceae* family and comprises 450 species. Besides the well known garlic and onion, several other species are widely grown for culinary use, such as leek (*Allium porrum* L.), scallion (*Allium fistulosum* L.), shallot (*Allium ascalonicum* Hort.), wild garlic (*Allium ursinum* L.), elephant garlic (*Allium ampeloprasum* L. var. *ampeloprasum*), chive (*Allium schoenoprasum* L.) and Chinese chive (*Allium tuberosum* L.) (Lanzotti, 2006).

1.3.1. *ALLIUM SATIVUM*

Garlic (*Allium sativum*) has long been used both for flavoring and for the potential benefits of preventing and curing ailments in many cultures. Garlic exhibits hypolipidemic, antiplatelet, and procirculatory effects (Amagase, 2006). The systematic classification of garlic is as follows (Rahman, 2003):

Family:	*Alliaceae*
Order:	*Asparagales*
Superior order:	Lilane
Subclass:	Liliidae
Class:	Monocotyledones

1.3.2 CHEMISTRY OF ALLIUM

1.3.2.1. VOLATILE COMPOUNDS

The major sulphur-containing compounds within intact garlic are g-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulphoxides (alliin). Both are abundant sulfur compounds. Alliin is the primary odorless, sulphur-containing amino acid (AA) and is a precursor of allicin, methiin, (1)-S-(trans-1-propenyl)-L-cysteine sulphoxide, and cycloalliin. These sulphoxides, except cycloalliin, are converted into thiosulphinates (such as allicin) through enzymatic reactions when raw garlic is cut or crushed. Thus, no thiosulphinates are found in intact garlic (Amagase, 2006). Thiosulphinates are the best-studied compounds arising from the *Allium* species. The differences in the thiosulfinates among the species arises due to the structure and relative amounts of their precursors (Fig.1.1: 1a–d). For example, in garlic the compound with a propyl residue (Fig.1.1: 1d) is absent, while an allyl residue, i.e. alliin (Fig.1.1: 1a), is a major compound. This last compound is the precursor of allicin (Fig.1.1: 3a). On the other hand, in onion (1a) it is totally absent while, isoalliin (1b) which contains al-propenyl residue, is a major metabolite. Compounds possessing a 1-propenyl residue at the thiolic site exist as a mixture of the *E*, *Z* isomers (Fig. 1.1: 3e–3f, 3g–3h, 3l–3m) because of a sigmatropic rearrangement (Lanzotti, 2006).

The sulphenic 1-propenyl acid intermediate (Fig.1.1: 2b) gives rise also to other compounds (Fig. 2), such as (*Z*, *E*)-propanethiol *S* oxide, or lachrymatory factor (LF) (4, *Z*-isomer) cepaenes and 2, 3-dimethyl-5, 6-dithiabicyclo [2.1.1] hexane 5-oxides, or *cis* and *trans*-zwiebelane (Fig. 1.2: 6a and 6b, respectively) (Lanzotti, 2006).

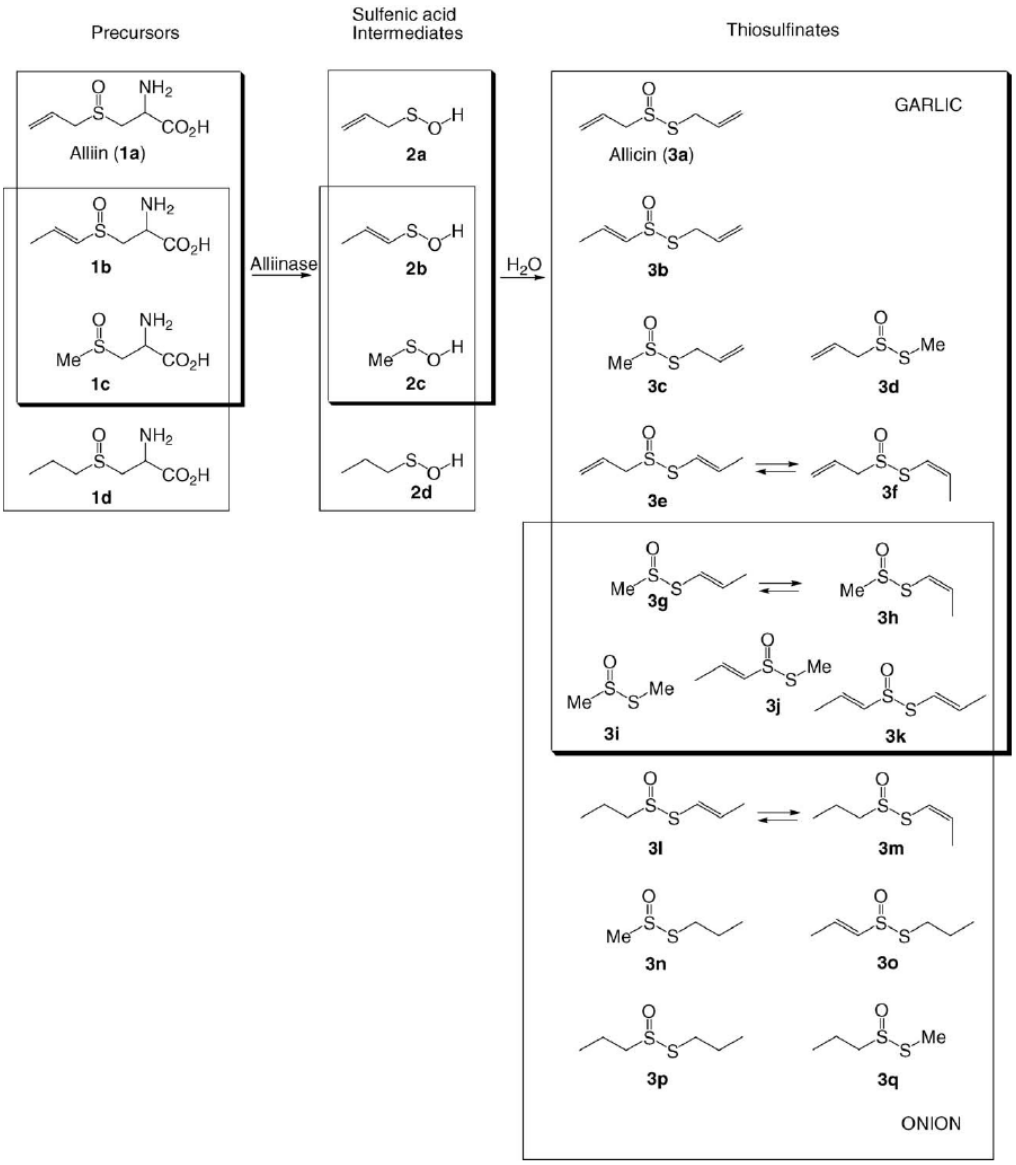

Figure 1. 1: Biosynthetic pathway of thiosulphinates, present in onion and garlic extracts showing similarity in the compounds formed. The heavy set boxes demonstrating the compounds found exclusively in garlic, the light set boxes demonstrating the compounds exclusively found in onion and the overlay of the boxes demonstrating compounds common to both (Lanzotti 2006).

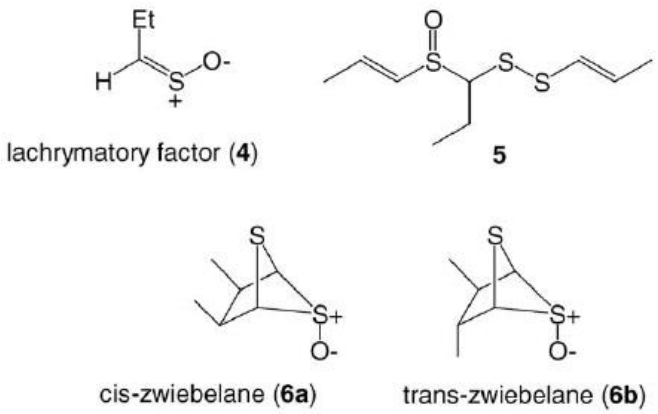

Figure 1. 2: Volatile organosulphur compounds in onion (Lanzotti, 2006).

Thiosulphinates are very unstable compounds and give rise to further rearrangements leading to a wide variety of derived sulphur compounds (Fig. 1.3), which take part in further transformations and still exhibit biological activity. Among these, thiosulphonates (Fig. 1.3: 7a and 7b), di- and tri-sulphur compounds (Fig.1.3: 8a–8f), 2-vinyl-2, 4-dihydro-1, 3-dithiin (Fig.1.3: 9), 3-vinyl-3, 4- dihydro-1, 2-dithiin (Fig 1.3: 10), and ajoene. At temperatures of 100 °C poly-sulphur compounds are formed (Fig 1.3: 12a, 12b and 13) and may contain up to five sulphur atoms (Lanzotti, 2006).

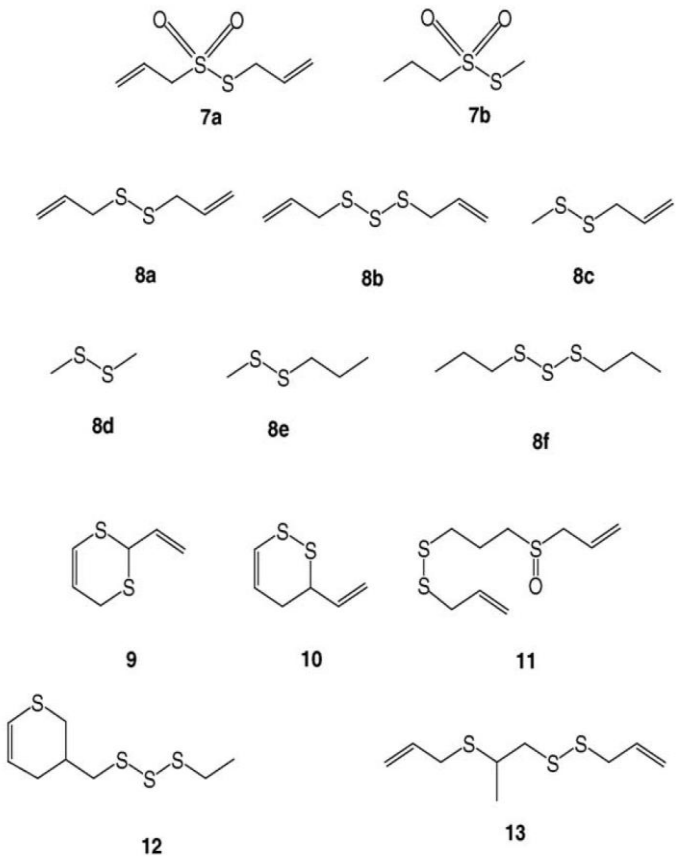

Figure 1. 3: Organosulphur compounds formed by thiosulphonates degradation (Lanzotti, 2006).

1.3.2.1.1. THIOSULFINATE FORMATION.

The disruption of garlic bulbs causes the formation of thiosulfinates, such as allicin. These are formed through the enzymatic reaction of cytoplasmic sulphur-substituted cysteine sulphoxides(CSs) with alliinase situated in the vacuole, with sulphur-substituted sulphenic acids forming as a highly reactive intermediate (Fig. 1.4) (Amagase, 2006).

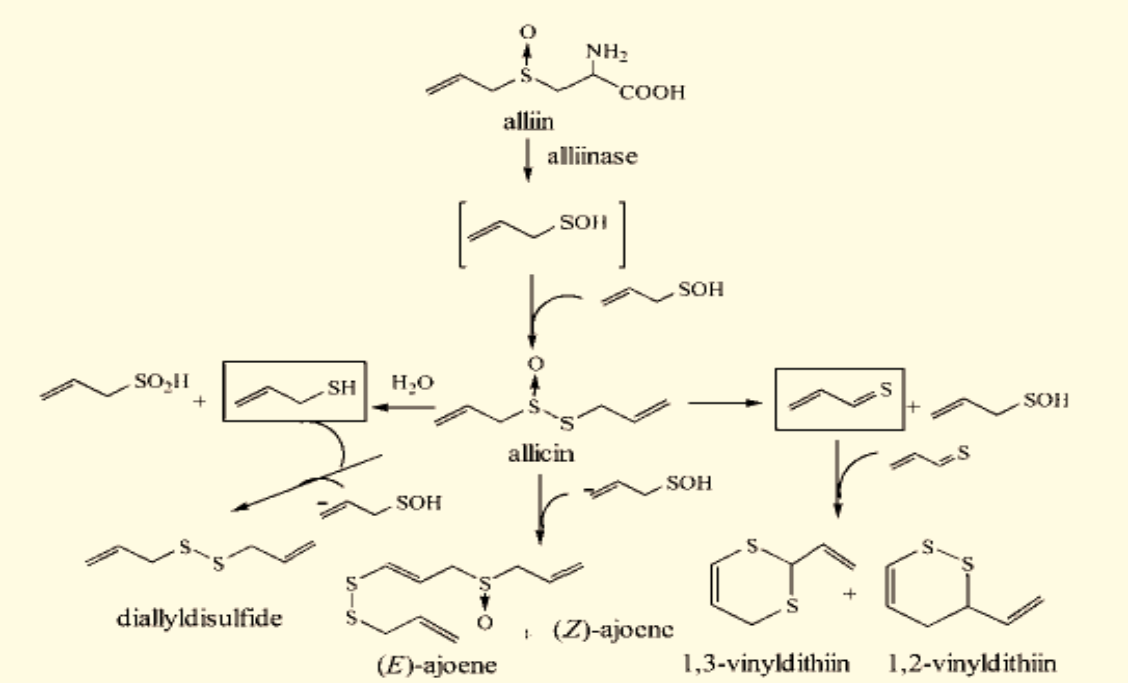

Figure 1. 4: Formation of thiosulphinates (Amagase, 2006).

Other thiosulfinates, including allylmethyl-, methylallyl-, and trans-1-propenyl-thiosulfinate, found in garlic homogenates, and, like allicin, are all unstable.

Allicin easily reacts with AAs and proteins, creating a SH group. Allicin binds to protein and fatty acids in the plasma membrane and is therefore trapped before absorption and cannot circulate in the blood. Alliinase is the key enzyme that facilitates the transformation of cysteine sulphoxides to thiosulfinates. The purified enzyme possesses a pH optimum of 6.5 with S-methyl-L-cysteine as substrate. In addition, pyridoxal phosphate stimulates alliinase activity as a co-factor. Allicin is thought to be a transient compound that is rapidly decomposed into other sulphur-containing compounds and is therefore not be the active compound of garlic (Amagase, 2006 and Bocchini *et al.*, 2001*)*.

1.3.2.1.2. ORGANOSULPHUR VOLATILES

Processed garlic contains a wider variety of organosulphur volatiles than the intact garlic clove (Fig. 1.5). Typical volatiles that have been identified in crushed garlic and garlic essential oil include diallyl disulphide, diallyl sulphides, diallyl trisulphide, methylallyl disulphide, methylallyl trisulphide, 2-vinyl-4H-1, 3-dithiin, 3-vinyl-4H-1, 2-dithiin, and (E,Z)-ajoenes (Corzo-Martınez *et al*., 2007).

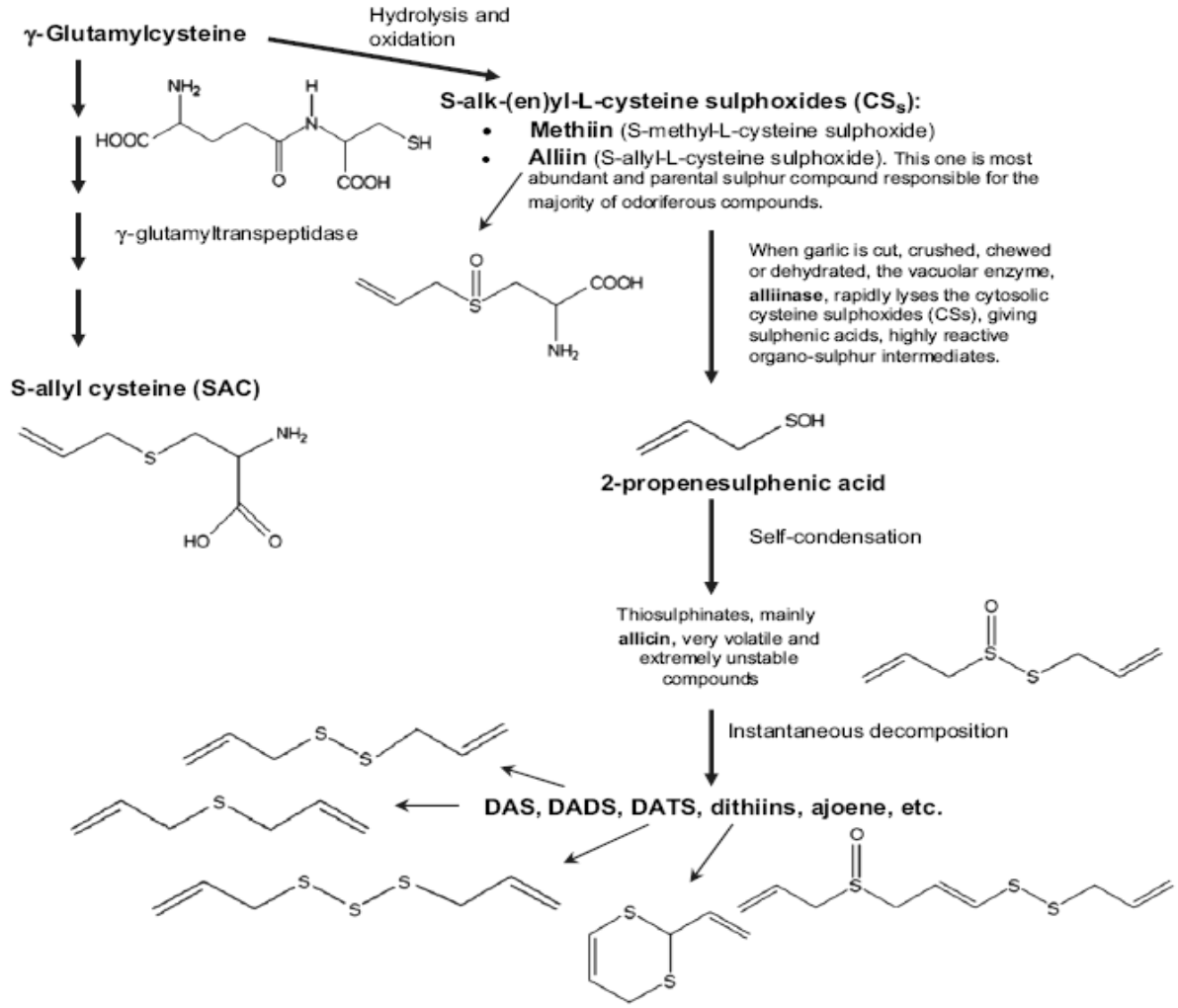

Figure 1. 5: Formation of organosulphur compounds during metabolic pathways in processed garlic (Corzo-Martınez *et al*., 2007).

Over 20 sulphides have been identified in steam-distilled garlic oil and oil-soluble extracts of garlic. The major sulfides in garlic oil include diallyl sulphides (57%), allylmethyl (37%), and dimethyl (6%) mono- to hexasulfides, in some cases, together with a small amount of allyl 1-propenyl and methyl 1-propenyl di-, tri-, and tetrasulphides. Diallyl trisulphide is the most abundant in fresh garlic oil. Vinyldithiins were first demonstrated to be thermal-degradation products derived from allicin during gas chromatographic analysis of allicin. These structures were elucidated to be 2-vinyl-4H-1, 3-dithiin and 3-vinyl-4H-1, 2-dithiin on the basis of spectroscopic analysis (Amagase, 2006). The mechanism of vinyldithiins synthesis has been confirmed to be a type of Diels-Alder dimerization of thioacrolein derived from the β-elimination of allicin. A remarkable production of vinyldithiins from allicin is observed when less-polar solvents such as hexane are used. Vinyldithiins, especially 2-vinyl-4H-1, 3-dithiin, are rich in the oil macerate of raw garlic (Amagase

2006). The first isolated organo-sulphur compound was ajoenes. It was isolated from an ether fraction of garlic, and was found to be a potent antithrombotic agent. Ajoene has both an E and Z isomers of 4, 5, 9-trithiadodeca-1, 6, 11-triene-9-oxide. Another ajoene-type organo-sulphur compound, E-4, 5, 9-tritriadeca-1, 7-diene-9-oxide, was isolated from oil macerated garlic extracts; however the no known biological activity with this compound has been identified (Amagase, 2006).

1.3.2.1.3. WATER-SOLUBLE ORGANOSULPHUR COMPOUNDS.

Previous studies indicate that alcohol and aqueous garlic extracts contain primarily S-allyl-L-cysteines derived from g-glutamyl-S-allyl-L-cysteines. S-Allyl-L-cysteine and trans-S-1-propenyl-L-cysteine, together with a small amount of S-methyl-L-cysteine (SAC), are found in garlic extracts such as aged garlic extracts (AGE). These cysteine derivatives are colorless crystals, which are odorless and stable in the solid state or aqueous solution under neutral or slight acidic conditions. SAC provides protection against oxidation, free radicals, cancer, and cardiovascular diseases. S-allylmercapto-L-cysteine demonstrates an *in vivo* hepato-protective effect, an *in vitro* cancer preventive effect in human prostate carcinoma cells, as well as antioxidant activity *in vitro* (Amagase 2006).

1.3.2.2 NON-VOLATILE COMPOUNDS

1.3.2.2.1 NONSULPHUR COMPOUNDS: SAPOGENINS AND SAPONINS

Many steroid saponins have been reported in plants and animals, especially in the *Liliaceae* family, which includes garlic. Saponins are generally classified into two groups, triterpenoid and steroid saponins, based on the molecular structure of aglycone. Steroid saponins are further divided into furostanols and spirostanols. Furostanol saponins have a β-glucosyl unit at the 26^{th} position of the aglycone moiety and are easily transformed into spirostanols by an enzymatic reaction to close a ring with β-glucosidase. Furostanols are reported to be present in fresh plants and are gradually converted into spirostanols during drying. (Amagase, 2006).

The presence of steroid saponins has been previously detected in garlic extracts by thin-layer chromatography (TLC). Proto-eruboside-B was the first furostanol saponin isolated from the bulbs (Amagase, 2006). Further studies on steroid saponins from

garlic led to the isolation and the structural determination of the furostanol saponin named sativoside-B1, and to the discovery of a known furostanol saponin, proto-desgalactotigonin. Further studies on the distribution of steroid saponins have yielded the isolation of two new steroid saponins, sativoside-R1 and sativoside-R2, from the roots. Their structures have been established to be gluco-proto-desgalactotigonin and its corresponding spirostanol saponin (Lanzotti, 2006).

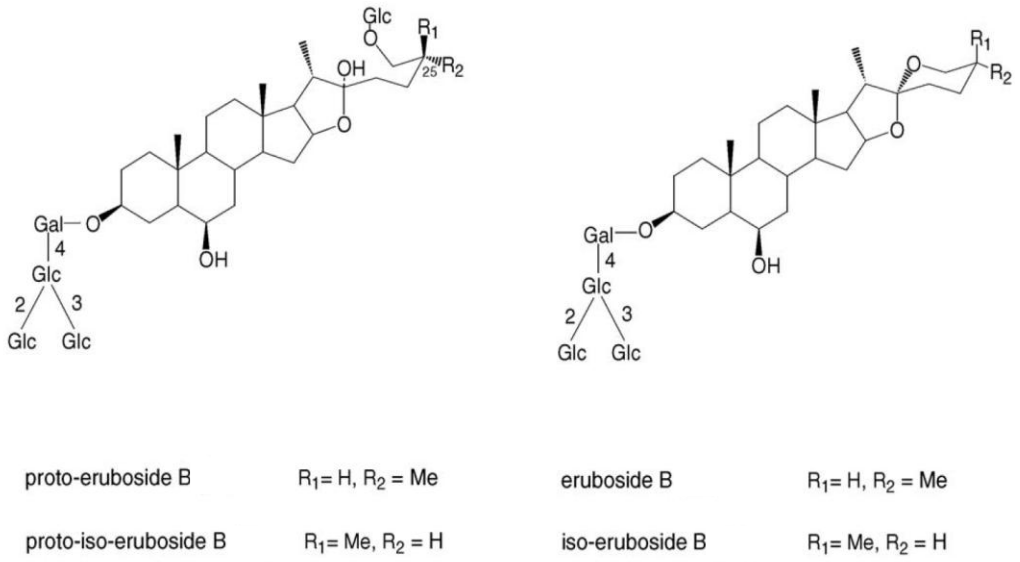

Figure 1. 6: Saponins isolated from garlic bulbs (Lanzotti, 2006).

Among the biological activities of steroid saponins isolated from the garlic bulb, eruboside-B exhibited antifungal activity for *Candida albicans*, antitumor, and cytotoxic activities *in vitro* (Amagase 2006). Generally saponins have a positive effect on the prevention of platelet aggregation, blood coagulation and fibrinolysis (Lanzotti, 2006).

1.3.2.2.2. FLAVONOIDS AND PHENOLICS: CHEMICAL STRUCTURE AND BIOACTIVITY

Garlic has a high flavonoid content, which is considered one of the important factors in the overall antioxidant activity of dietary plants. Analysis of garlic revealed the presence of two phenolics, *N*-feruloyltyrosine and *N*-feruloyltyramine (Fig. 1.7). Because garlic roots are known in traditional medicine used in infusions for eliminating worms, *N*-feruloyltyrosine and *N*-feruloyltyramine have been subjected to antifungal assays. Activity against *Fusarium culmorum* for both compounds was observed. Antifungal activity was described for the flavonoid quercetin and for isoflavones such as genistein (4, 5, 7-trihydroxyisoflavone) (Lanzotti, 2006).

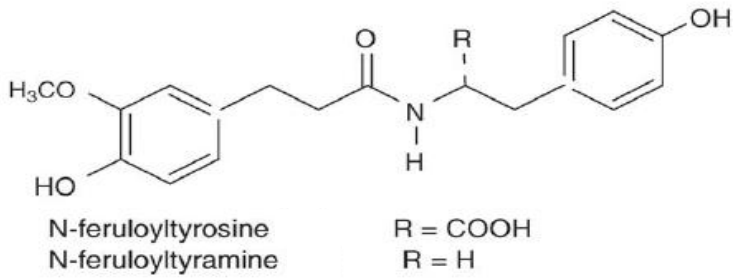

Figure 1.7: Phenolics found in *Allium sativum* (Lanzotti 2006).

1.4. THE GENUS: *TULBAGHIA*

Tulbaghia is a small genus of approximately 20 species and entirely African in distribution. The distribution extends from Namaqualand in the Western Cape to the southern parts of Tanzania in southern tropical Africa (Kubeca *et al*., 2002).

1.4.1. *TULBAGHIA VIOLACEA*

T. violacea Harv. (*Alliaceae*) (Fig. 1.8) is a small bulbous herb indigenous to Natal, Gauteng and the Eastern Cape region in South Africa where it grows in rocky grasslands to a height of 50 cm. The evergreen leaves of *T. violacea* exhibit a garlic-like smell when bruised and has been used in some cultures as a substitute for garlic and chives (Maoela, 2005). The leaves are narrow, hairless and strap-shaped, and grow to 30 cm in length and are 1.5 cm wide, arising from several white bases. The leaves are dark green, leathery in texture and smell strongly of garlic. It has umbels of up to 20 violet flowers.

Figure 1.8: Typical examples of *T. violacea* plants (SANBSA, 2009) Mountain Valley Growers Inc. (2009) (Altervista 2009).

The plant is known by several common English names including society garlic, sweet garlic, wild garlic and by a number of indigenous names, Wildeknoffel (Afrikaans), Icinsini (Zulu), Itswele lomlambo (Xhosa) and Mothebe (Sotho) (van Wyk and Gericke, 2000). *T. violacea* has traditionally been used for the treatment of fever and colds, asthma, tuberculosis, and gastrointestinal ailments. However, extensive consumption of this plant has been associated with a variety of undesirable symptoms, such as abdominal pain, inflammation, and gastroenteritis, due to the high sulphur (2, 4, 5, 7-tetrathiaoctane-2, 2-dioxide and 2, 4, 5, 7-tetrathiaoctane) and steroidal saponin content (Burton, 1990). The characteristic to inhibit normal muscle contraction was found by Burton (1990) to be the result of a β-adrenergic agonist. Its antibacterial activity against a number of bacterial strains was confirmed by Burton (1990). It has been reported that *Tulbaghia violacea* deters moles, due to its odour. The Zulu nation of South Africa grows this plant around their homes, as it is believed to repel snakes (Kubeca *et al.,* 2002).

1.5. ACTIVE COMPOUNDS FOUND IN *T. VIOLACEA* AS COMPARED TO *ALLIUM SATIVUM*

To date research on *T. violacea* has been conducted (Table 1). Jacobsen e*t al*., (1968) reported the presence of a C–S lyase and three unidentified S-substituted cysteine sulfoxide derivatives and an enzyme similar in action to that found in garlic, i.e. alliinase, has been reported to be present. The enzyme also catalyses the same reaction in other plants belonging to the *Allium* genus. However, in different plants allinase has been found to differ in physical and kinetic properties although they catalyze the same reaction i.e. the hydrolysis of S-1-propenyl, S-propyl, and S-methyl. The sulphur containing AA, (R(S) R(C))-S (methylthiomethyl) cysteine-4-oxide was identified by Kubec *et al*., (2002). Burton and Kaye (1992) isolated 2, 4, 5, 7-tetrathiaoctane- 2, 2-dioxide and 2, 4, 5, 7-tetrathiaoctane from the leaves of *T. violacea* and the presence of two flavonols: kaempferol and quercitin, was confirmed (Burton 1990). Burton (1990) confirmed the presence of sulphur compounds, although they were different to those found in other *Allium* species, indicating their uniqueness to *T. violacea.* Recently, papers published by Bungu *et al*., (2006), found that *T.a violacea* had anti-cancer properties and had effectively inhibited the growth of various cancer cell lines. Bungu *et al.,* (2008) demonstrated that the antithrombotic activity depends on the extraction method and is affected by seasonal variations.

Other studies have found the age of the plant also affects the amount of secondary metabolites present and therefore the biological activity (Burton, 1990). Further studies have shown that it reduces systemic arterial blood pressure in the Dahl rat due to a decrease in renal AT1 receptor gene expression (Mackraj *et al.*, 2008).

Table 1. 1: Literature summary of compounds isolated or identified, and known biological functions of *T. violacea*

Author	*Method of extraction*	*Isolated compound (IC) / results obtained (RO)/ traditional use (TU)*
Jacobsen *et al*., (1968)	**methanol**	**IC: C–S lyase and three unidentified S-substituted cysteine sulphoxide derivatives.**
Burton (1990)	**methanol**	**IC: flavonols: kaempferol and quercetin, identified**
Burton and Kaye (1992)	**methanol**	**IC: 2, 4, 5, 7-tetrathiaoctane- 2, 2-dioxide 2, 4, 5, 7-tetrathiaoctane.** **RO: Anti-bacterial activity**
Duncan *et al*., (1999)	**Methanol and aqueous extracts of leaves and roots**	**RO: ACE inhibitor *in vitro*** **72% inhibitor in leaves** **49% inhibitor in roots** **TU: gastrointestinal, febrile-colds**
Zheng and Wang (2001)	**Aqueous (phosphate buffer)**	**IC: +/- 1 mg /g (Gallic acid equivalents) phenolics compounds per fresh weight material** **RO: oxygen radical absorbance capacity, was 7.5 umol /g of fresh weight**
Kubeca *et al*., (2002).	**Methanol**	**IC: Sulphur containing amino acid, (R(S) R(C))-S (methylthiomethyl) cysteine 4-oxide** **TU: Treatment of fever, colds, asthma, tuberculosis, gastrointestinal ailments. Deters moles and repels snakes (Zulu)** **Undesirable symptoms: abdominal pain, inflammation, gastroenteritis**
Motsei *et al*., (2003)	**Aqueous bulb and leaf extracts***	**IC: allicin suspected active compound** **RO: Treatment of oral *Candida albicans***

Fennell *et al.*, (2004)	**Aqueous extracts***	**RO: Showed antifungal activity with minimum lethal concentration values of between 6.25 and 12.5 mg/ml and antibacterial activity against gram negative bacteria and demonstrated anthelmintic activity**
Serra *et al.*, (2005)	**Aerial part in methanol–HEPES (20:80) extracts**	**RO: ACE inhibitor 30.8% in HPLC assay ACE inhibitor 35.7% in colorimetric assay**
Reid *et al.*, (2006)	**Dichloromethane (DCM): methanol (10:90) ratio**	**TU: Oesophageal cancer, fever, colds, asthma and tuberculosis RO: No anti-mutagenic and mutagenic properties found**
Bungu *et al.*, (2006)	**Ethanol and aqueous extracts i.e. bulbs and leaf**	**RO: Anti-cancer properties against the following cell lines: HT-29, HeLa, MCF-7 and WHCO3 RO: Induction of apoptosis was noted**
Koduru *et al.*, (2007)	**Bulbs boiled in water**	**TU: Treatment of cancer**
Braga *et al.*, (2007)	**Ethanol aerial parts**	**RO: 35% ACE inhibitor properties**
Mackraj *et al.*, (2007)	**Aqueous extracts***	**RO: (50 mg/kg b.w.), of the extract to rats intraperitoneally, reduced AT1a mRNA expressions by 5.03-fold reductions in systolic blood pressure (SBP) of 9.12±0.31%, it reduced systemic arterial blood pressure in the Dahl rat by decreasing renal AT1 receptor gene expression and hence modulating sodium and water homeostasis.**
Bungu *et al.*, (2008)	**Methanol extracts**	**RO: Seasonal studies on its antithrombotic activities (thrombin) and anticoagulant effect**
Mackraj *et al.*, (2008)	**Aqueous extracts ***	**RO: Reduced systemic arterial blood pressure by decreasing renal AT1 receptor gene expression thus modulating sodium and water homeostasis TU: fever, asthma, constipation, oesophageal cancer, hypertension**
Verschaeve and van Staden (2008)	**Dichloromethane followed by methanol: water (9:1) leaves, bulbs, and roots**	**TU: asthma and TB RO: No mutagenic and non-mutagenic activities confirmed**

*Details of solvent or extraction method not provided

In literature, it is postulated that *Tulbaghia violacea* may have similar biological activities to garlic as they belong to the same family and have a characteristic sulphur smell. Since there are shared secondary metabolites between these species it is possible that the biological activity observed in *Tulbaghia violacea* may be due to compounds homologous to those found in garlic.

1.6. DIABETES

Diabetes mellitus (DM) has reached epidemic proportions and affects more than 170 million individuals worldwide. T2DM is a complex metabolic disorder that affects between 6 and 20% of the population in Western industrialized societies. Around the globe, the prevalence of T2DM is expected to increase exponentially, especially among the young. By definition, DM is categorized as a metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. The first category, T1DM, is caused by an absolute deficiency of insulin secretion. The second, much more prevalent category, T2DM, results from a combination of resistance to insulin action and an inadequate compensatory insulin-secretory response and progressive loss of β-cell function throughout the course of the disease. It is associated with dyslipidemia, hypertension, and obesity (components of the metabolic syndrome) (American Diabetes Association, 2005).

Changes in metabolic factors, such as increased oxidative stress, endothelial dysfunction, inflammation, and the prothrombotic state, observed in diabetics play an important role in cardiovascular complications of diabetes (WHO, 1978). Diabetes is generally grouped into two types: insulin dependent diabetes mellitus and non-insulin dependent diabetes mellitus. Both types are associated with excessive morbidity and mortality (Mandrup-Poulsen, 1998). Poorly controlled blood glucose is believed to be the most important factor in the development of diabetic complications in both T1DM and T2DM (Liu *et al.*, 2006).

1.6.1. INSULIN

Insulin is the most important hormone in the regulation of blood glucose concentration and is essential in the post-prandial state. As blood sugar concentrations

rise, insulin is secreted into the bloodstream by the pancreatic β-cells of the pancreas (Islets of Langerhans). Insulin stimulates glucose uptake into fat and muscle cells to promote the storage of glucose as intracellular triglycerides and glycogen. In addition, insulin inhibits the production and release of glucose from the liver (gluconeogenesis and glycogenolysis). In healthy individuals, this prevents the rise of blood glucose concentrations that would occur after meal ingestion. However, in the early stages of T2DM, the blood glucose concentration remains increased, despite the presence of normal to high insulin concentration in the bloodstream. The combined inability of muscle and adipose tissue to facilitate glucose uptake and the liver to suppress glucose output in response to increasing amounts of insulin is referred to as insulin resistance. This is regarded as the hallmark characteristic of T2DM. The later stages of T2DM are characterized by low plasma insulin concentrations due to a decrease insulin production, leading to the need for exogenous insulin (Liu *et al.*, 2006). The actions of insulin can be divided into (i) short-term (glucose uptake and metabolism), (ii) intermediate-term (protein and lipid turnover) and (iii) long-term (cell growth and mitogenesis) effects (Duckworth *et al*., 1998). Insulin has anabolic functions which include the promotion of glycogen, triglyceride and protein synthesis.

1.6.1.1. INSULIN STRUCTURE

Insulin is a polypeptide hormone composed of two polypeptide chains, A and B, joined by two disulphide bonds (Fig.1.9). This small protein with 51 amino acid residues and molecular mass of 5800 Da, is the most important hormone in the regulation of metabolic control in the maintenance of normoglycaemia and normolipidaemia (Pirola *et al*., 2006). Its short plasma half life of approximately 4-6 min is necessary to ensure rapid changes in blood glucose levels (Duckworth *et al*., 1998).

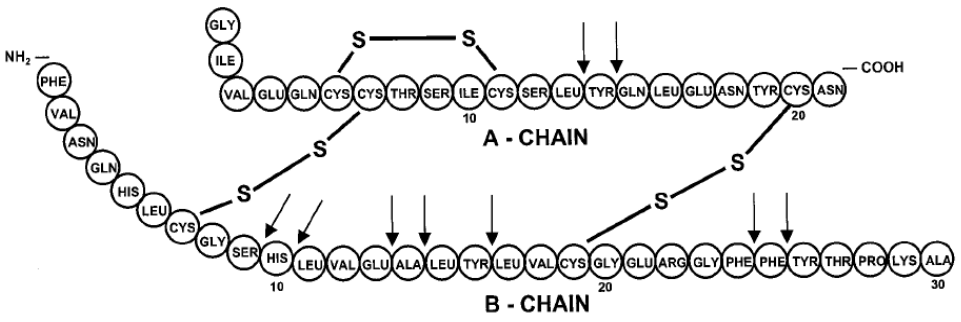

Figure 1. 9: Amino acid composition of insulin. Arrows indicate cleavage sites of insulin during degradation (Duckworth *et al.*, 1998).

1.6.1.2. INSULIN SYNTHESIS

The production of insulin and its release from islets is complex and tightly regulated. Glucose affects insulin at all levels, including transcription, translation and release. Insulin is synthesized in significant quantities solely by β-cells in the pancreas. Insulin mRNA is translated as a single chain precursor called preproinsulin (Fig. 1.10). The synthesis of preproinsulin ensures that the final product, insulin is adequately or correctly folded. Preproinsulin is an inactive single-chain precursor with an amino-terminal "signal sequence" that directs its passage into secretory vesicles. The signal sequence is needed to direct the preproinsulin into the endoplasmic reticulum (ER) for post translational processing. Removal of its signal peptide during insertion into the ER generates proinsulin.

Proinsulin consists of three domains: N-terminal B-chain, a C-terminal A-chain and a connecting peptide in the middle known as the C peptide. Proinsulin is then transported along the microtubule network system in transport vesicles to the ER, where it is exposed to several specific endopeptidases which excise the C peptide via cleavage of 2 peptide bonds, thereby generating the mature form of insulin. Insulin (crystal form) and free C peptide are packaged in the Golgi apparatus into secretory granules which accumulate in the cytoplasm until its released or degraded (Ren *et al*., 2007).

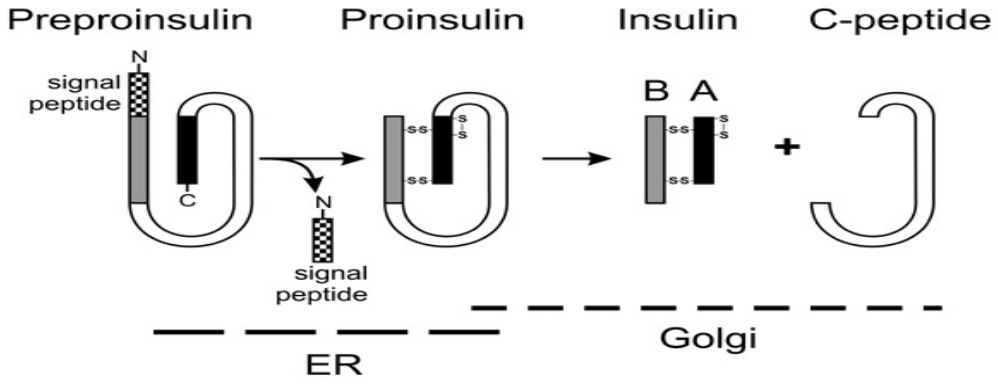

Figure 1. 10: Formation of mature insulin. Upon translation of insulin mRNA, preproinsulin (inactive) is produced in the pancreas. The signal peptide is then cleaved off from the preproinsulin forming proinsulin in the ER, and proinsulin is further processed by removal of C-peptide in the ER into A and B chains of insulin linked by disulphide bonds to form active insulin (Morrissey, 2006).

1.6.1.3. C-PEPTIDE

The C-peptide, also known as the connecting peptide is composed of a single chain of 31 amino acids and has a molecular mass of 3020 Da. The C-peptide links the A and B chain of insulin, that allows correct folding and inter-chain disulphide bond formation and as a result the correct synthesis of insulin. When the C-peptide is removed from proinsulin by proteolytic processing, the COOH-terminal part of the B chain becomes exposed and free to assume an appropriate conformation for effective interaction with the insulin receptor. Figure 1.11 shows the linear representation of human proinsulin indicating the amino acid sequence of the C-peptide and the position of the COOH-terminal pentapeptide (Wahren *et al*., 2000).

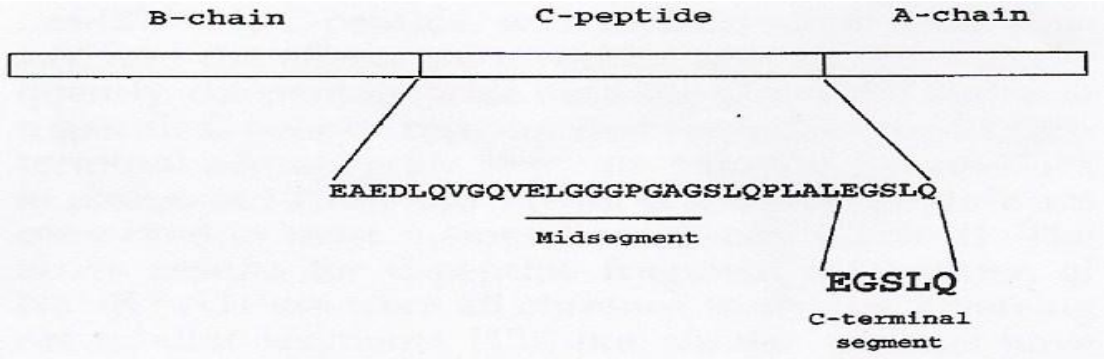

Figure 1. 11: **Amino acid sequence of C-peptide in human proinsulin with COOH-terminal indicated (Wahren *et al*., 2000).**

1.6.1.4. INSULIN SECRETION

The secretion of insulin is the direct or primary response to elevated blood glucose levels and the presence of AAs and gastrointestinal hormones, such as glucagon-like peptide-1 (GLP-1). Insulin secretion is affected by metabolic signals and transcription factors that are required for suitable differentiation and growth of various types of pancreatic islet cells (Fig.1.12). Insulin secretion is the result of changes in the β-cell's membrane and calcium-dependent action potentials (Ren *et al*., 2007).

Nearly all insulin released by β-cells is from insulin secretory granules. To release insulin, the granules must be recruited from the cytoplasm, translocated to the plasma membrane where they are docked, fuse with the plasma membrane, and release their contents into the extracellular space. A group of proteins known as the synaptosomal associated protein (SNAP) Receptors (SNARES) are important for directing the insulin vesicles to the plasma membrane. The actual docking of the vesicle with the plasma membrane involves the linking of plasma membrane proteins syntaxin and

synaptosomal-associated protein 25 (SNAP-25) with the vesicle protein vesicle-associated protein 2 (VAMP-2) or synaptobrevin-2. This process is initiated when there is an increase in blood glucose levels and is characterised by two phases. This biphasic pattern consists of a rapidly initiated, but transient first phase of insulin release and a sustained second phase (Ren *et al*., 2007) (Fig 1.12).

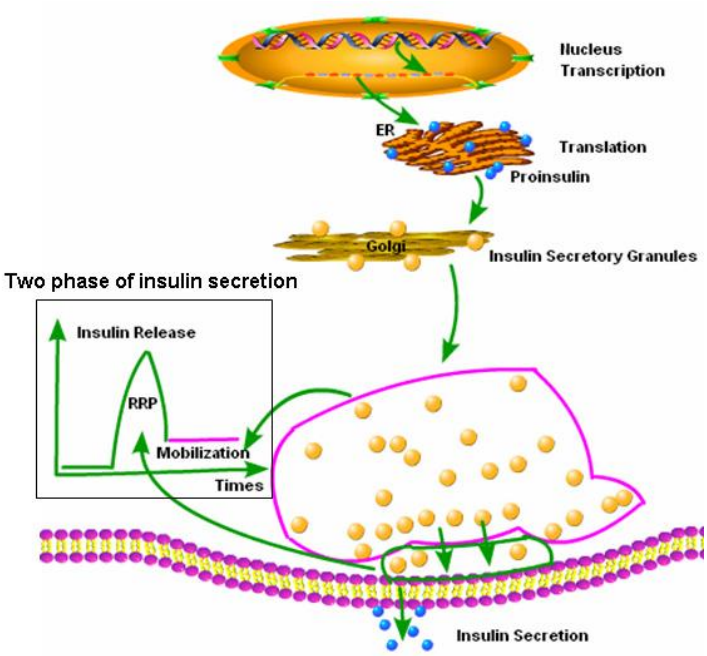

Figure 1. 12: Insulin synthesis and secretion process readily releasable pool (RRP) (Ren *et al.*, 2007).

1.6.1.5. GLUCOSE CONTROL OF INSULIN SECRETION.

Glucose-stimulated insulin secretion occurs via two mechanisms: the triggering and the amplifying pathway. In the triggering pathway (Fig 1.13) glucose enters the β-cells through the GLUT-2 transporter were it undergoes glycolysis, and it is converted to glucose-6-phosphate (G-6-P) by glucokinase (GK). ATP is subsequently generated resulting in an increase in the intracellular ATP/ADP ratio. The increase in ATP/ADP ratio leads to the inhibition of ATP-sensitive K_{ATP}-channels due to the high concentration of G-6-P in the cell. The increased ATP/ADP ratio causes the opening of the K^+ channel, allowing K^+ to flow out. The resulting change in cell membrane potential or depolarization triggers the opening of voltage-dependent calcium channels (VDCC) in the cell membrane and Ca^{2+} enters the cell. The linking of the plasma membrane proteins syntaxin and SNAP-25 to vesicle protein VAMP-2/synaptobrevin-2 causes the docking of the vesicle, bringing the readily releasable pool (RRP) of insulin granules in close contact with the plasma membrane and calcium channels. This close proximity of the RRP of insulin granules to the high

Ca^{2+} concentration, leads to the degranulation of the RRP of insulin granules and secretion (Halvorsen and Levine, 2001, Ren *et al.*, 2007). The second process of insulin secretion is the result of acidification of the insulin granular pool. The simultaneous mobilization or priming process of a V-type H^+-ATPase and Cl^- channels is critical for this mechanism to occur. The uptake of Cl^- provides the counter-ion to allow continuous H^+ pumping, which in turn determines the extent of granular acidification. ADP is an inhibitor of the Cl^- channel activity, but as glucose metabolism reduces the ADP level, the ability of ADP to inhibit the Cl^- channels is reduced, leading to the acidification of insulin secretory granules and the subsequent release of insulin (Ren *et al.*, 2007 and Eliasson *et al.*, 2008*)*.

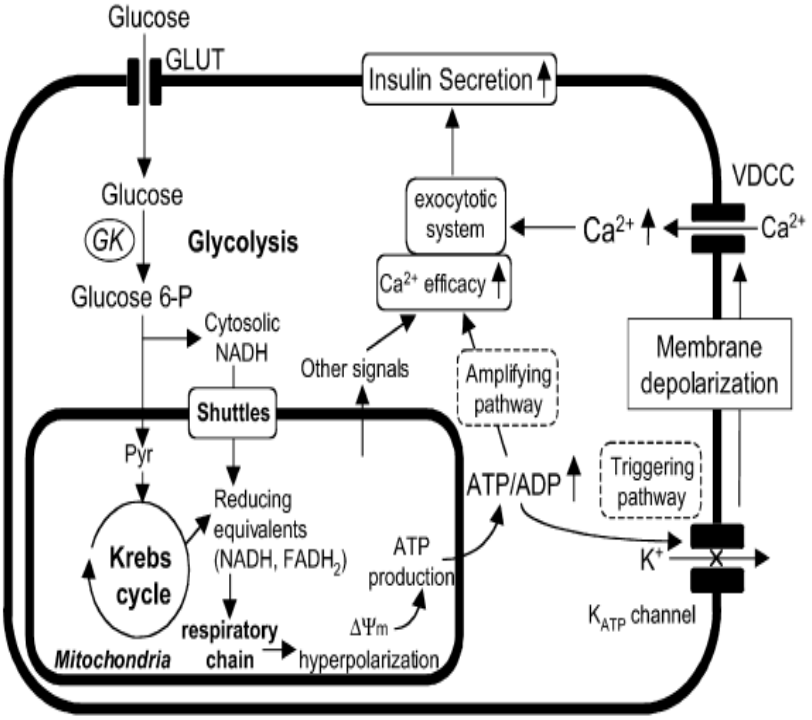

Figure 1. 13: A schematic diagram of the coupling of glucose metabolism to insulin secretion in pancreatic β-cells. GLUT, glucose transporter; GK, glucokinase; glucose-6-P, glucose- 6-phosphate; Pyr, pyruvate; VDCC, voltage-dependent Ca^{2+} channel (Fujimoto *et al*., 2007).

1.6.1.6. INSULIN SIGNALING

The insulin signalling pathway consists of a complex array of protein kinases and protein phosphatases that regulate insulin action. Insulin receptor substrates (IRS)-1 and IRS-2 are docking proteins that are essential in initiating the pleotropic effects of insulin through phosphatidylinositol 3–kinase (PI3-K) (Del Auigulia *et al.*, 1999) (Fig.1.14).

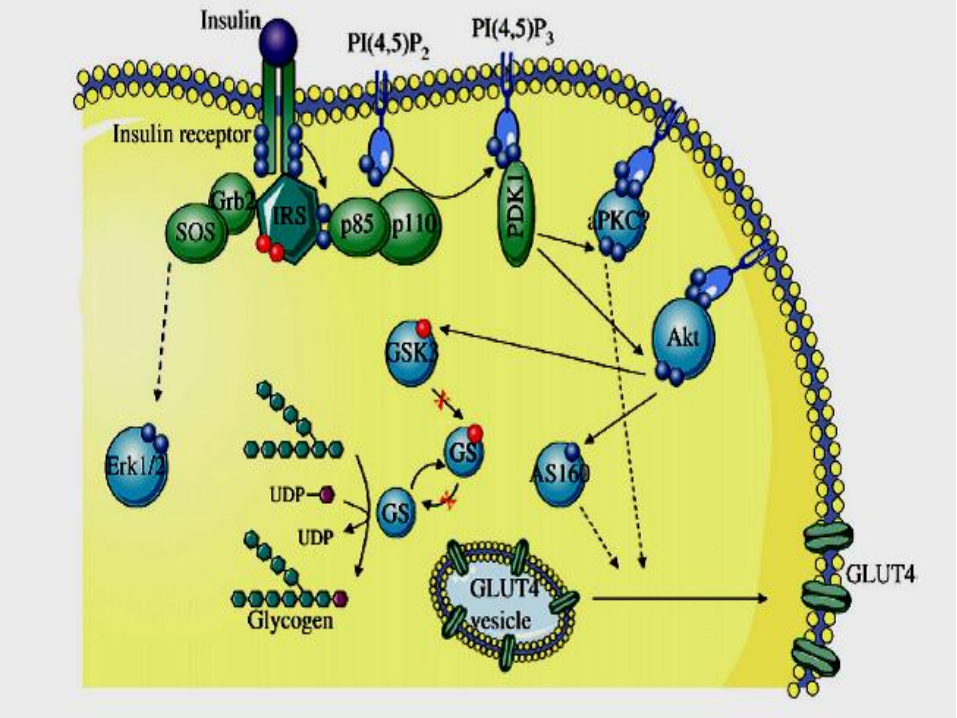

Figure 1. 14: Schematic depiction of the insulin regulated intracellular signal transduction cascade, reporting the signalling steps whose alterations (in red) have been studied in insulin resistance and T2DM subjects, depicted in adipocytes (Fröjdö *et al.*, 2009).

Insulin action is initiated through the binding to and activation of its cell-surface receptor, which consists of two α subunits and two β subunits that are disulfide-linked into a α2β2 heterotetrameric complex. Insulin binds to the extracellular α subunits, transmitting a signal across the plasma membrane that activates the intracellular tyrosine kinase domain of the β subunit. The receptor then undergoes a series of intramolecular phosphorylation reactions in which one β subunit phosphorylates tyrosine residues on it's adjacent partner.

Once activated, the insulin receptor phosphorylates tyrosine residues of a number of important proximal substrates, including members of the IRS family (IRS1/2/3/4), the Shc adapter protein isoforms, adenosine diphosphate (ADP), thrombin-antithrombin complex (TAT), protein kinase C (PKC) cyclooxygenase (COX-1), amino acid, Insulin receptor substrates (IRS-1) and signal regulatory protein (SIRP) family members. Tyrosine phosphorylation of the IRS proteins creates recognition sites for additional effector molecules containing Src homology 2 (SH2) domains. These include the small adapter proteins Grb2 and adapter protein Nck, the non-receptor SHP2 protein tyrosine phosphatase and, most importantly, the regulatory subunit of the type 1A PI3-K (Watson and Pessin, 2006).

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 then bind to phosphoinositide-dependent kinase 1 (PDK 1). Known substrates of the PDKs are protein kinase B (PKB), also known as Akt and also typical forms of protein kinase C (PKC). Akt is a serine/threonine (Ser/Thr) kinase. The Akt pathway mediates the effects of insulin on glycogen synthesis, protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis. The Akt pathway regulates both glucose uptake, via facilitated GLUTs and intracellular glucose metabolism in insulin sensitive tissues (eg. 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 1.14 (LeRoith and Gavrilova, 2005 and Schinner *et al.,* 2005).

1.7. THE BLOOD COAGULATION PATHWAY

Hemostasis is a fundamental defence mechanism of all vertebrates and involves two complementary processes: the formation of a blood clot, or thrombus, to stem blood loss from a damaged vessel and the process of thrombus dissolution, or fibrinolysis, once endothelial repair has occurred. These are complex processes involving multiple interdependent interactions between platelets, endothelial cells, white cells and plasma proteins (Gentry, 2004). Classically, the coagulation pathway may be divided into three interlinking pathways, namely the intrinsic, extrinsic and the common pathway.

1.7.1. THE WATERFALL/CASCADE COAGULATION MODEL

The intrinsic pathway is ultimately a cascade of proteases in action, with these reactions initiated by factors that are present within the blood. When blood comes into contact with a foreign or negatively charged surface such as glass or the membrane of an activated platelet, the intrinsic pathway it activated. FXII, (Hageman factor) is activated to FXIIa with kininogen, a high molecular weight kininogen, anchoring FXIIa to the charged surface. As FXIIa accumulates, prekallikrein is converted by FXIIa to kallikrein, anchored by HMWK. The kallikrein in turn accelerates the conversion of FXII to FXIIa, in addition to amplifying its own generation. FXIIa (together with HMWK) proteolytically cleaves FXI, to form FXIa. The newly formed

FXIa then proteolytically cleaves FIX to form more FIXa. With FIXa, FXa and thrombin, FVIII is proteolytically cleaved to form FVIIIa, which acts as a co-factor in the next reaction (Riddel *et al*., 2007). Lastly, FIXa and FVIIIa together with Ca^{2+} (which is generated from activated platelets) and negatively charged phospholipids (the major constituents of cell membranes) form a trimolecular complex, *tenase*. Tenase in turn converts FX to FXa (Boron and Boulpaep, 2005). In a parallel series of interactions, FXa binds to the co-factor FVa, to generate a complex with enzymatic activity known as prothrombinase. This complex converts the proenzyme prothrombin to its active form, thrombin. Thrombin reacts with fibrinogen to generate the fibrin monomer. The fibrin monomers rapidly polymerize to form the fibrin clot. In clinical laboratory analysis of blood clotting, the intrinsic pathway of blood coagulation is evaluated using the activated partial prothrombin time (APTT) (Hoffman and Monroe *et al*., 2005 and Riddel *et al*., 2007).

The integral membrane protein tissue factor (TF) (variably known as FIII or tissue thromboplastin), which is expressed by non-vascular cells, is a receptor for the plasma protein FVII (Kumar *et al*., 2005).The formation of a complex between TF (located on cell surfaces) and FVIIa, (located outside the vascular system) initiates the extrinsic pathway (Gentry, 2004). When FVII comes into contact with TF as a result of a break or tear of the endothelium, the TF non-proteolytically activates FVII to FVIIa. FVIIa then binds to TF to form an enzyme complex, which activates FX to FXa. FXa binds to the co-factor FV, on membrane surfaces in the presence of calcium ions to generate the prothrombinase complex. The prothrombinase complex converts prothrombin to thrombin, which converts fibrinogen to fibrin to generate the fibrin clot. During laboratory analysis of blood clotting, the extrinsic pathway of blood coagulation is evaluated using the prothrombin time (PT) (Hoffman and Monroe *et al*., 2005, Riddel *et al*., 2007).

The common pathway begins with the activation of FX within the intrinsic pathway, the extrinsic pathway, or both. FXa from either the intrinsic or extrinsic pathway is the first protease of the common pathway. FXa, in the presence of FV, $Ca^{2+,}$ and phospholipids, converts prothrombin to its active form, thrombin (Riddel *et al.*, 2007). Thrombin catalyzes the proteolysis of the soluble plasma protein fibrinogen to form fibrin monomers that remain soluble. Fibrin monomers then polymerize to form a gel

of fibrin polymers that trap blood cells. Thrombin also activates FXIII, which is converted to FXIIIa and mediates the covalent cross-linking of the fibrin polymers to form a mesh termed stable fibrin, which is less soluble than fibrin polymers (Boron and Boulpaep, 2005). Thrombin can catalyze the formation of new thrombin from prothrombin and can catalyze the formation of the co-factors FVa and FVIIIa, resulting in efficient amplification of coagulation. Because the common pathway contains the factors FX, FV, and FII, these factors may be monitored by both the PT and APTT assays (Harmening, 2002).

The cascade/waterfall hypothesis does not fully and completely reflect the events of hemostasis in the *in vivo* cascade. Despite this shortfall, it has served for many years as a useful model (Hoffman and Monroe, 2001). Recently clinical and experimental observations have shown fundamental deficiencies in the model, which include:

a) The absence of a clinical bleeding tendency if there are deficiencies of FXII, prekallikrein, or HMWK, even though deficiencies in any one of these factors for hemostasis *in vitro* showed prolong surface-activated coagulation assays or
b) A valid explanation as to why deficiencies in either FVIII or FIX, can cause severe bleeding, even though the extrinsic pathway would theoretically be able to bypass the need for FVIII and FIX (Riddel *et al*., 2007).

These two key observations led to revisions of earlier models of coagulation. Firstly, the complex of FVIIa and TF activates not only FX, but also FIX. Secondly, the activity of the FVIIa/TF complex is the major initiating event in hemostasis *in vivo* (Hoffman and Monroe, 2005 and Riddel *et al*., 2007). With these observations in mind a new model for coagulation was introduced, i.e. the cell-based model of coagulation.

1.7.2. THE CELL-BASED MODEL OF COAGULATION

In the cell-based model, hemostasis requires the formation of an impermeable platelet and fibrin plug at the site of vessel injury and the procoagulant substances activated in this process remain localized at the site of injury. The blood coagulation process is initiated by the exposure of the blood to cells that express TF (Fig.1.15).

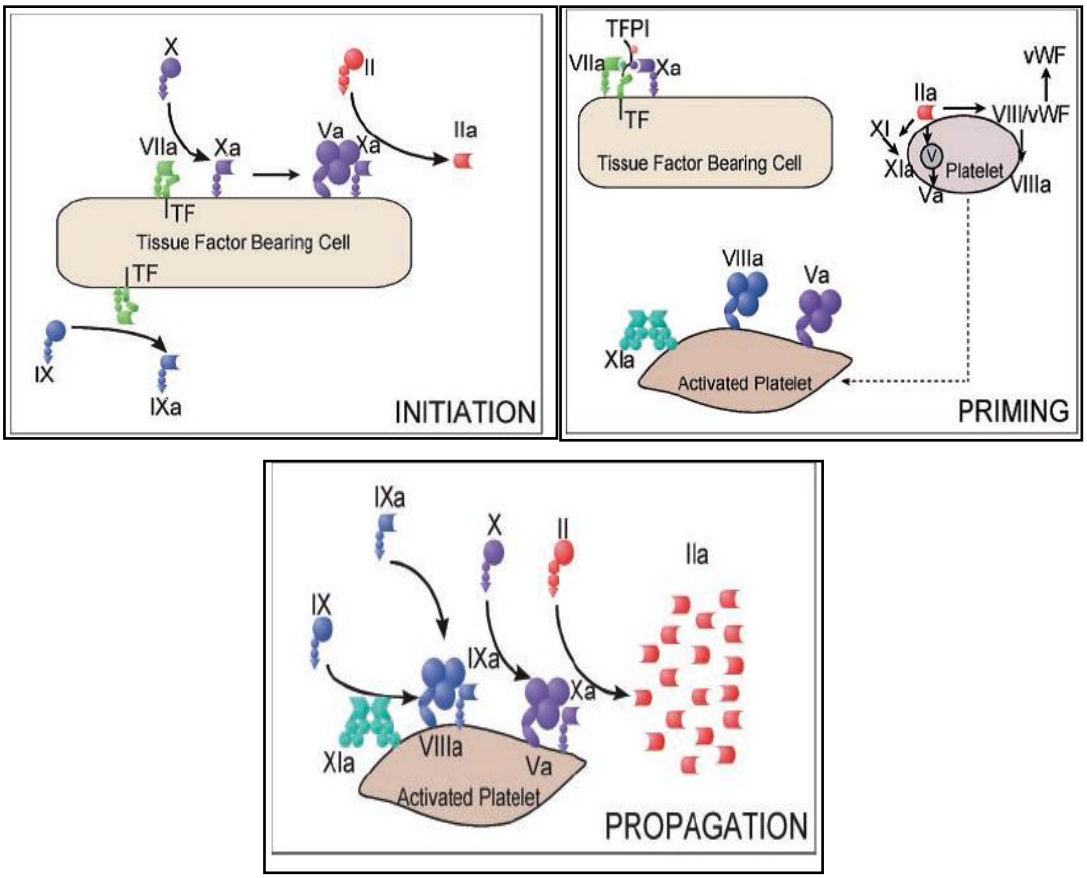

Figure 1. 15: Cell-based model of coagulation. In this scheme, only 3 phase of coagulation are shown and include initiation, priming, and propagation (Monroe *et al.*, 2002).

TF is expressed on smooth muscle cells and fibroblasts; however, it is not expressed on resting endothelium. TF is present in the membranes of cells surrounding the vascular bed but is normally not in contact with blood. It is exposed to the circulating blood by disruption of the endothelium or by activation of endothelial cells or monocytes. These membrane fragments derived from various cell types, white blood cells, endothelium, and platelets, play a more important role in pathological hemostasis (thrombosis) as opposed to normal clotting. It is best to consider this model as a process of interlinked phases as opposed to pathways. These phases of initiation, propagation, and termination illustrate the maintenance of vascular integrity (Loscalzo, 2003 and Riddel *et al.*, 2007). The 4 interlinking-phases of coagulation comprised in the current cell-based theory of coagulation are summarized in table 1.2.

Table 1. 2: Summary of the four phases of hemostasis, as proposed by the cell-based theory of coagulation (Riddel *et al.*, 2007).

Process Stages of Hemostasis			
Initiation	Amplification/priming	Propagation	Termination
Vascular endothelium and circulating blood cells are perturbed: interaction of plasma-derived FVIIa with TF	Thrombin activates platelet co-factors FVa and FVIII on the platelet surface, and FXI on the platelet surface	Results in the production of a significant level of thrombin activity, generation of a stable plug at the site of injury and cessation of blood loss	Clotting process is limited to avoid thrombotic occlusion in surrounding normal areas of the vasculature

The initiation phase is localized to the cells that express TF, which are normally found outside the vascular system. The FVIIa/TF complex activates small amounts of FIX and FX. FXa associates with its co-factor, FVa, and forms a prothrombinase complex on the surface of the TF-bearing cell (Hoffman, 2004 and Monroe *et al*., 1996). FV can be activated by FXa (Monkovic and Tracy, 1990) or by non-coagulation proteases (Allen and Tracy, 1995) to produce the FVa required for prothrombinase assembly (Hoffman, 2004 and Loscalzo, 2003). Low-level activity of the TF pathway occurs at all times within the extravascular space. Coagulation proteins percolate through tissues, leaving the vasculature, and are found in the lymph. It is likely that FVII is bound to extravascular TF even in the absence of an injury (Hoffman, 2004), and extravascular FX and FIX can be activated as they pass through the tissues. The coagulation process proceeds to the amplification phase only when damage to the vasculature allows platelets and FVIII bound to von Willebrand Factor (vWF) to move into the extravascular tissues and to adhere to TF-bearing cells at the site of injury (Loscalzo, 2003).

A small amount of thrombin generated on the TF-bearing cells has several important functions. A major function is the activation of platelets to expose receptors and binding sites for activated clotting factors. As a result of this activation, the platelets release partially activated forms of FV onto their surfaces. Another function of the thrombin formed during the initiation phase is the activation of the co-factors FV and FVIII on the activated platelet surface. In this process, the FVIII/vWF complex is dissociated, permitting vWF to mediate additional platelet adhesion and aggregation

at the site of injury. In addition, small amounts of thrombin activate FXI to FXIa on the platelet surface during amplification (Fig. 1.16) (Loscalzo, 2003).

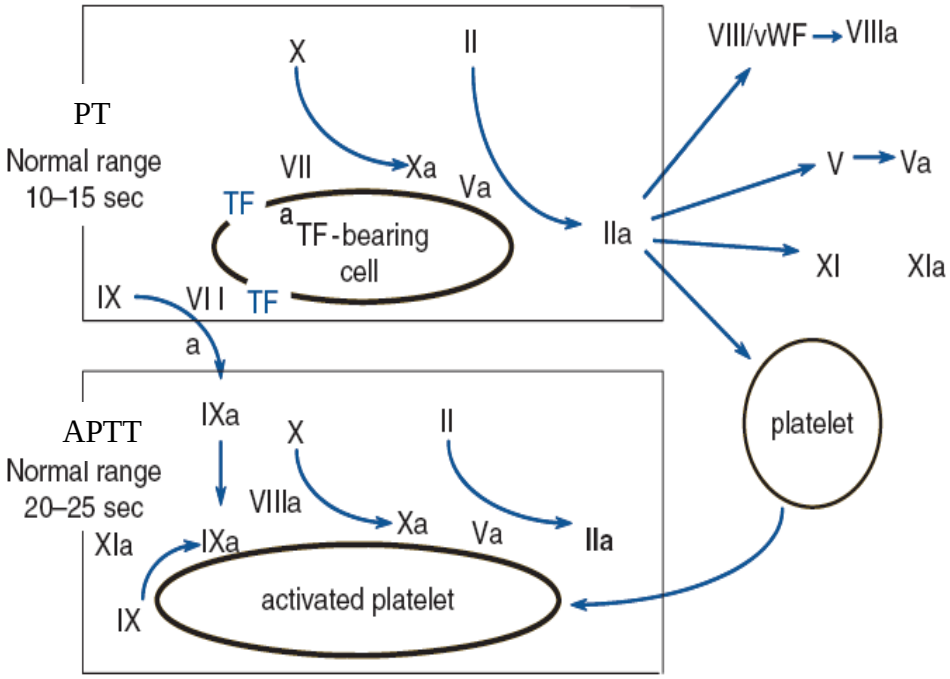

Figure 1. 16: Normal hemostasis depicting PT and APTT times (Hedner and Ezban, 2008).

The propagation phase of clot formation occurs on the surface of activated platelets which are recruited to the site of injury. FIXa is activated during the initiation phase and binds to FVIIIa on the platelet surface. Additional FIXa is supplied by platelet-bound FXIa; FXa cannot move effectively from the TF-bearing cell to the activated platelet and must be provided directly on the platelet surface by the FIXa/FVIIIa complex. FXa then rapidly associates with FVa bound to the platelet during the amplification phase. Finally the completion of the platelet prothrombinase assembly leads to a burst of thrombin generation of sufficient magnitude to convert fibrinogen to fibrin, leading to clot formation (Loscalzo, 2003) (Fig. 1.17).

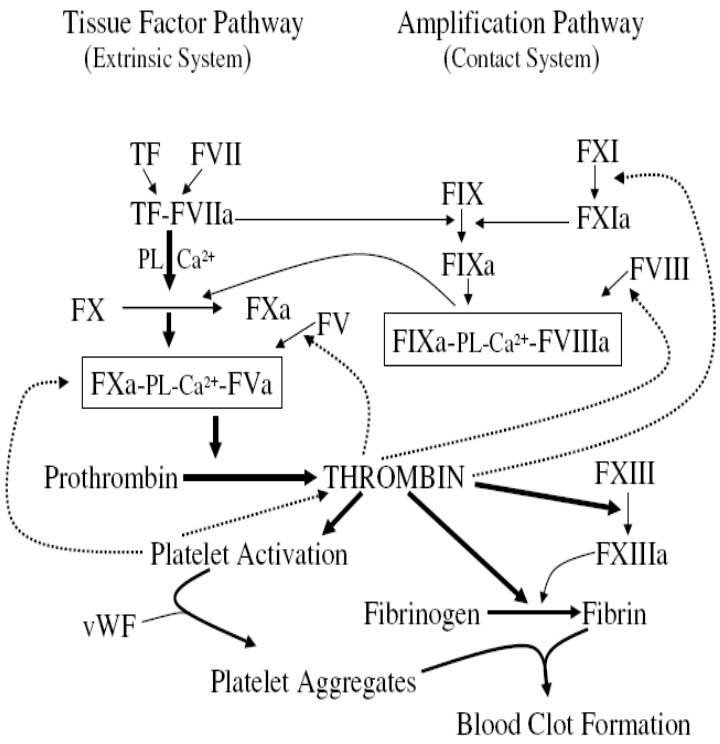

Figure 1. 17: Schematic diagram of the coagulation cascade. The initiation of blood clot formation occurs following vascular injury and the exposure of tissue factor (TF) to circulating blood. Thrombin exerts a positive feedback regulation (dotted lines) by activating platelets and other procoagulant proteins. Activated platelets provide a phospholipid (PL) surface that serves to enhance enzyme complex formation. Platelet aggregates are anchored to damaged endothelium by von Willebrand Factor (vWF). The majority of proteins are designated as factors (F) and Roman numerals (Gentry, 2004).

1.7.3. THE ROLE OF FIBRINOGEN

Plasma fibrinogen is an important component of the coagulation cascade, as well as a major determinant of blood viscosity and flow. In all species plasma fibrinogen is synthesized in the liver and is released into the circulation as a three non-identical polypeptide chains, Aα (66 062 kDa), Bβ (54 358 kDa), and γ (48 529 kDa), cross-linked by disulfide bridges. Although the synthesis of each chain is controlled by a separate gene, only the Aα chain appears to have structural diversity among species. All 3 polypeptide chains of fibrinogen are assembled with their N-terminals converged in a central 'E' nodule of the molecule. The C-terminal of the β and γ chains extended outward to form a 'D' nodule (Wolberg, 2007).

Increasing evidence from epidemiological studies suggests that elevated plasma fibrinogen levels are associated with an increased risk of cardiovascular disorders, including ischemic heart disease (IHD), strokes and other thrombotic events such as embolisms. An increase in plasma fibrinogen levels may promote a prothrombotic or

hypercoagulant state. An increase in plasma fibrinogen content in the blood is also influenced by age, body mass index, smoking, DM and menopause (Adams and Huntington, 2006).

1.7.4. FIBRIN CLOT FORMATION

Fibrin clot formation is initiated by the binding of thrombin to the E nodule of fibrinogen and by the removal of the N-termini of the Aα and Bβ chains (Fig. 1.18 i and ii). A two-step transition mechanism represents the fibrinopeptide cleavage and fibrin polymerization mechanisms. Cleavage of fibrinopeptide A (FpA) by thrombin reveals the 'A' site, a polymerization domain called. 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. Protofibrils result from the E: D interaction (Adams and Huntington, 2006 and Wolberg, 2007). Subsequent cleavage of fibrinopeptide B (FpB) triggers lateral association into fibrils that provide the scaffold for the growing thrombus. These newly generated fibrin polymers can then act as a co-factor for the thrombin activation of the transglutaminase FXIII, which cross-links the fibrin to strengthen the clot (Adams and Huntington, 2006). 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 can occur (Wolberg, 2007) (Fig. 1.19).

1.7.5. FIBRINOLYSIS

The action of two plasminogen activators namely, tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) forms plasmin, the main enzyme involved in fibrinolysis (Fig.1.18 iii). t-PA is primarily active on the vascular system and is the primary agent for the dissolution of thrombi via the activation of plasminogen to plasmin. u-PA is found in connective tissue, and plays a role in tissue remodelling and cell migration (Castãnon *et al*., 2007). The presence of fibrin enhances the activation of plasminogen to plasmin by t-PA (Fig.1.18).

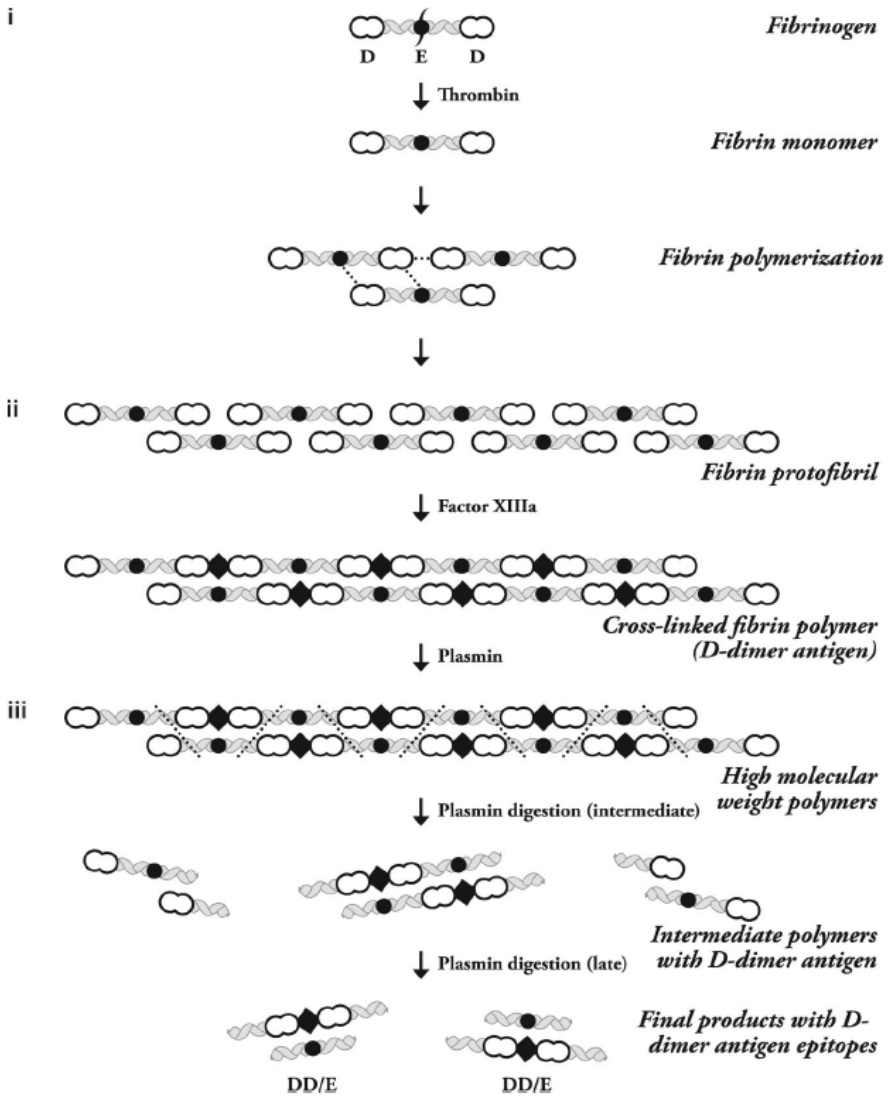

Figure 1. 18: Stepwise process of fibrin polymerization (i and ii) and degradation (iii). (DD/E, D-Dimer epitopes) (Adam *et al.*, 2009).

1.7.6. D-DIMER FORMATION

The first step of D-dimer formation is the cleavage of fibrinogen by thrombin. This exposes a polymerization site on fibrinogen that promotes the binding of either another fibrinogen or a monomeric fibrin molecule. Fibrin monomers then bind to one another in an overlapping manner to form 2 molecule thick protofibrils (Fig.1.19). Plasma remains fluid until 25 to 30% of plasma fibrinogen is cleaved by thrombin, allowing time for fibrin to polymerize while simultaneously promoting thrombin activation of plasma FXIII. Thrombin remains associated with fibrin, and as additional fibrin molecules polymerize, it activates plasma FXIII bound to fibrinogen. The complex between soluble fibrin polymers, thrombin, and plasma FXIII promotes

the formation of FXIIIa before a fibrin gel is detected. In the second step of D-dimer formation, FXIIIa covalently cross links fibrin monomers via intermolecular isopeptide bonds between lysine and glutamine residues within the soluble protofibrils and the insoluble fibrin gel. D-dimer antigen remains undetectable until it is released from cross-linked fibrin by the action of plasmin (Adam *et al*., 2009).

In the final step of D-dimer formation, plasmin formed on the fibrin surface by plasminogen activation cleaves fibrin at specific sites (Fig.1.19). Fibrin degradation products are produced in a wide variety of molecular weights, including the terminal degradation products of cross-linked fibrin containing D-dimer and fragment E complex (Fig. 1.18). It is uncommon to detect circulating terminal fibrin degradation products (D-dimer and E complex) in human plasma, whereas soluble high-molecular-weight fragments that contain the D-dimer antigen are present in patients with disseminated intravascular coagulation and other thrombotic disorders. These fragments may be derived from soluble fibrin before it has been incorporated into a fibrin gel, or alternatively may be derived from high molecular weight complexes released from an insoluble clot (Fig. 1.19) (Adam *et al*., 2009).

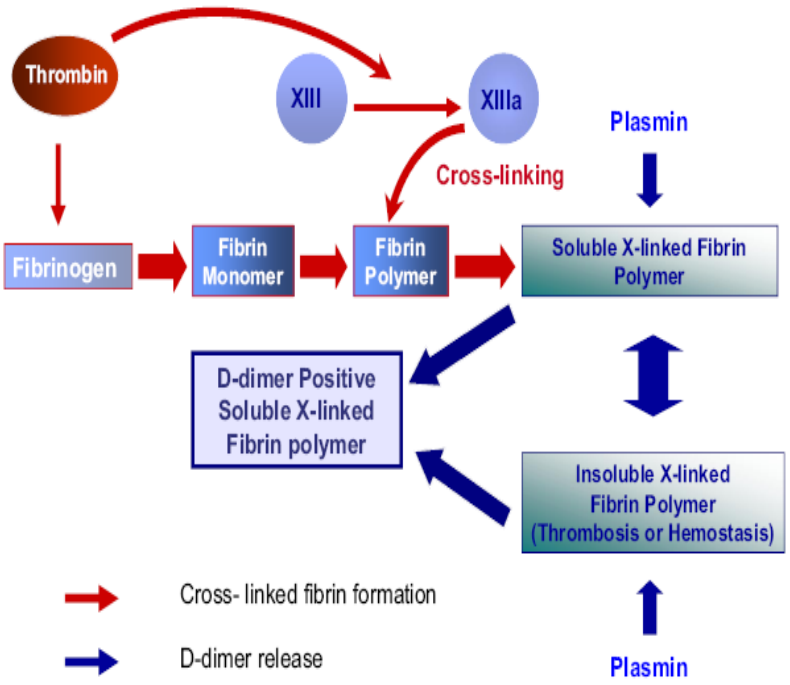

Figure 1. 19: Dynamics of D-dimer formation (Adam *et al*., 2009).

1.7.7. PLATELETS

17.7.1. STRUCTURE AND FUNCTION

The critical role played by platelets in hemostasis, thrombosis, vascular remodelling, and healing is related to their function as exocytotic cells that secrete important effector molecules at the site of vascular injury.

Resting platelets circulate as discoid structures (Fig.1.20), anuclear cells originating from megakaryocytes in the bone marrow, and have a life span of approximately 8–10 days (Willoughby *et al.,* 2002). Thrombopoietin is the major hormonal regulator of platelet production. Platelets contain a plasma membrane, internal membranes (open canalicular and dense tubular systems), a cytoskeleton (microtubules and microfilaments), mitochondria, glycogen granules, storage granules (α-granules and dense granules that are rich in serotonin, ADP and calcium), lysosomes, and peroxisomes (Willoughby *et al.,* 2002).

The platelet is surrounded by a plasma membrane containing phospholipids (negatively charged phosphatidylserine and phosphatidylinositol residues) that serve as substrates for phospholipases. Intrinsic glycoproteins such as glycoprotein (GP) II and IIIa extrude through this phospholipid bilayer and act as platelet receptors for activating and inhibiting agents (Willoughby *et al.,* 2002).

17.7.2. PLATELET ACTIVATION, AGGREGATION AND ADHESION

Platelets are activated by several physiological (thrombin, collagen, ADP, epinephrine, vasopressin, serotonin) and non-physiological (divalent cationophores, cyclic endoperoxide analogues) substances. When activated, they change shape, become spherical, and have extended long, spiky pseudopods (Fig1.20) (Willoughby *et al.,* 2002). Platelet aggregation is induced by various agonists, such as ADP, collagen and thrombin. Platelet aggregation depends on the release of ADP and prostaglandin (PG) H_2/thromboxane (TX) A_2. When platelets are activated, they change shape and secrete substances that activate and recruit more platelets to the site of injury.

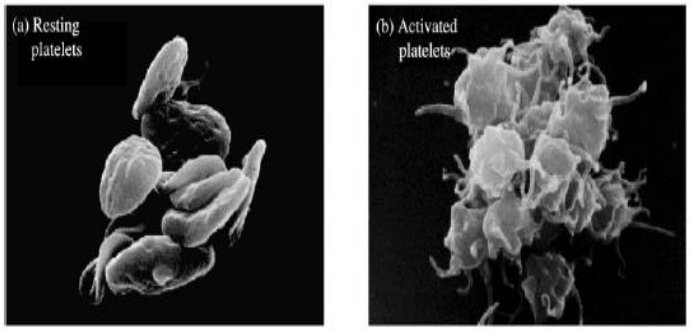

Figure 1. 20: Scanning electron microscopic photographs of (a) resting and (b) activated platelets (Willoughby *et al.*, 2002).

The central component in the platelet response is TXA_2, is an eicosanoid which is formed via a cyclooxygenase-TXA_2 synthase pathway. It is a powerful agonist of platelet activation and greatly contributes to thrombus formation. The binding of TXA_2 to a G-protein-coupled receptor induces phospholipase Cβ activation. An increase in $[Ca^{2+}]_i$ and PKC activation occurs due to the activation of phospholipase Cβ. The change in platelet shape and eventual adhesion of platelets to the site of injury is caused by this increase in $[Ca^{2+}]_i$ and PKC activation (Jin *et al*., 2007). The increase in $[Ca^{2+}]_i$ as a result in either influx of Ca^{2+} or secretion from intracellular stores play a major role in the response of platelets to various agonists (Jin *et al*., 2005).

The formation of the primary platelet plug is temporally and spatially coordinated with the activation of the blood coagulation system. Upon damage to the vascular wall, platelets undergo a series of events such as adhesion, aggregation, release of granule content, and morphological changes that lead to the formation of the platelet plug. Platelet adhesion is mediates by vWF, serving as a bridge between the tissue and the platelets, binding both to collagen exposed at sites of vascular injury and to the platelet membrane glycoprotein Ib-V-IX (GPIb-V-IX) (Dahback, 2005 and Sadler, 2005).

1.7.8. ALTERATIONS IN BLOOD COAGULATION SYSTEM IN PATIENTS WITH DIABETES

The abnormal metabolic state that accompanies diabetes causes arterial dysfunction. Relevant abnormalities include chronic hyperglycemia, dyslipidemia, and insulin resistance. These factors render arteries susceptible to atherosclerosis. Diabetes alters

the function of multiple cell types including the endothelium, smooth muscle cells, and platelets, indicating the extent of vascular disarray in this disease (Fig. 1.21).

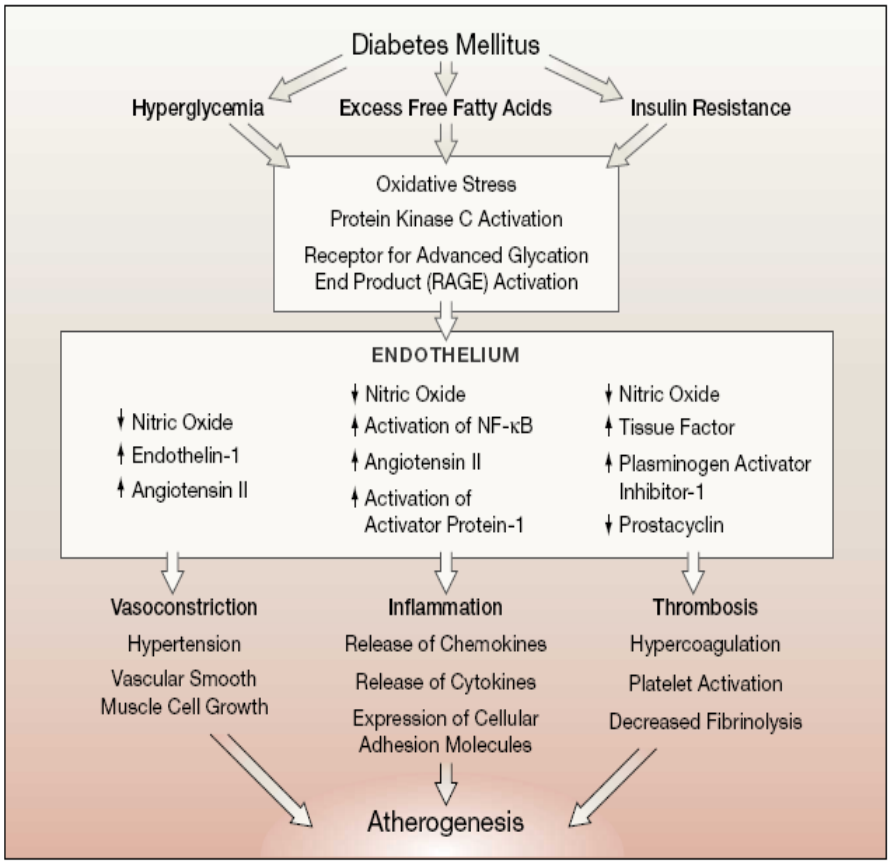

Figure 1. 21: In diabetes, hyperglycemia, excess free fatty acid release, and insulin resistance adverse metabolic events within the endothelial cell is engender. Activation of these systems impairs endothelial function, augments vasoconstriction, increases inflammation, and promotes thrombosis (Beckman *et al.*, 2002).

1.7.8.1. ENDOTHELIAL CELL DYSFUNCTION

A single layer of endothelial cells (EC) exists on the inner surface of all blood vessels. This layer of cells provides a metabolically active interface where the interaction between blood and tissue can occur. The endothelium represents the largest organ in the body covering an area of 1–7m^2. Endothelial cells protect blood vessels not only by providing a mechanical lining but also by controlling vascular tone through the release of vasodilators such as nitric oxide (NO) and prostacyclin (Ajjan and Grant, 2006). It has many additional functions, including the modulation of blood flow, nutrient delivery, coagulation, thrombosis and leukocyte diapedesis. It synthesizes important bioactive substances, including NO and other reactive oxygen species, which effect prostaglandins, endothelin, and angiotensin II, which regulate blood vessel function and structure. NO has many effects on the vessel wall including

vasodilatation and inhibition of inflammation and platelet aggregation. It limits inflammation by reducing leukocyte adhesion to EC and migration into the vessel wall and diminishes vascular smooth muscle cell (VSMC) proliferation and migration. Taken together, these properties inhibit atherogenesis and protect the blood vessel (Beckman *et al.*, 2002).

Reduced NO production has been associated with endothelial dysfunction, which may be due to reduced availability of substrate (L-arginine). A number of fundamental mechanisms contribute to the decreased bioavailability of endothelium-derived NO in diabetes (Ajjan and Grant, 2006). Hyperglycemia inhibits production of NO by blocking NO synthase (NOS) activation and increasing the production of reactive oxygen species (ROS), especially superoxide anion (O^{2-}), in endothelial and VSMC. Superoxide anion directly quenches nitric oxide by forming the toxic peroxynitrite ion, which uncouples NOS by oxidizing its cofactor, tetrahydrobiopterin, and causes NOS to produce O_2 (Beckman *et al.*, 2002). Another abnormality found in DM is a decrease in endothelium-derived NO. Inflammation and oxidative stress, which are commonly seen in atherosclerotic vessels, both affect NO production. This leads to impairment in vessel dilation consequently increasing physical stress on EC, thereby contributing to endothelial dysfunction. Also, decreased NO production results in proliferation of VSMC, promotion of platelet aggregation and increased leukocyte adhesion and infiltration, all of which contribute to the development of the atherosclerotic plaques (Ajjan and Grant, 2005; Beckman *et al.*, 2002).

Insulin resistance leads to excess liberation of free fatty acids from adipose tissue. These fatty acids activate the signalling enzyme PKC, inhibit PI-3K and increase the production of ROS (Beckman *et al.*, 2002).

1.7.8.2. IMPAIRED PLATELET FUNCTIONS

Platelets can modulate vascular function and participate significantly in thrombus formation. Abnormalities in platelet function may exacerbate the progression of atherosclerosis and the consequences of plaque rupture. Intra-platelet glucose concentration mirrors the extracellular concentration, since glucose entry into the platelet does not depend on insulin. In the platelet, as in EC, elevated glucose levels

lead to the activation of PKC, decreased production of platelet-derived NO, and increased formation of O^{2-}. In diabetes, platelets also show disordered calcium homeostasis. Disordered calcium regulation may contribute significantly to abnormal activity, since intra-platelet calcium regulates platelet shape change, secretions and aggregation, and TX formation (Beckman *et al.*, 2002).

Moreover, patients with diabetes have increased platelet-surface expression of glycoprotein IB (GPIb), which mediates binding to vWF, and GPII/IIIa, which mediates platelet fibrin interaction. These abnormalities may result from decreased endothelial production of the anti-aggregants NO and prostacyclin, increased production of fibrinogen, and platelet activators, such as thrombin and vWF. Taken together, diabetic abnormalities increase intrinsic platelet activation and decrease endogenous inhibitors of platelet activity (Beckman *et al.*, 2002).

17.8.3. ABNORMAL COAGULATION IN DIABETES

In addition to potentiating platelet function, diabetes augments blood coagulability, making it more likely that atherosclerotic plaque rupture or erosion will result in thrombotic occlusion of the artery. Most of the factors in the intrinsic pathway have been shown to be altered in diabetic patients (Ceriello *et al.*, 1990). The changes in plasma levels of glucose or insulin affect the plasma levels of the coagulation factors. There is evidence of a correlation between plasma levels of FpA, thrombin-antithrombin complex (TAT) and hyperglycemia. A rise in plasma glucose levels causes a rise in plasma levels of FpA and a decrease in plasma levels of TAT (Jorkl and Colwell, 1997). The inhibitors of the coagulation system in diabetic patients are depressed as the coagulant activity increases. These inhibitors include antithrombin III, a physiological inhibitor of FX and FII, and protein C, which inhibit FV and FVIII. The levels of TAT in plasma reflects the amount of thrombin formed, therefore, increased plasma levels of this complex suggests the activation of the coagulation cascade in the bloodstream. At the cellular level, platelets of diabetic patients show a greater procoagulant activity. As a result of hyperglycemia, non-enzymatic glycation may occur. The activators of plasminogen may therefore activate an impaired glycated plasminogen. In addition, glycation of the platelet membrane protein complex IIb-IIIa, which is the platelet receptor for fibrinogen, may account for the increased platelet aggregation in diabetic patients (Matsuda *et al*., 1996).

Throughout the last decade, the perception of T2DM has evolved from a focus on dysregulated glucose and insulin to encompass a global metabolic disorder characterized by dyslipidemia, hypertension and hypercoagulability in addition to hyperglycemia and hyperinsulinemia (Fig. 1.22). Each of these abnormalities plays an important role in cardiovascular disease (CVD) development and progression and provides targets for therapy (Beckman *et al.*, 2002).

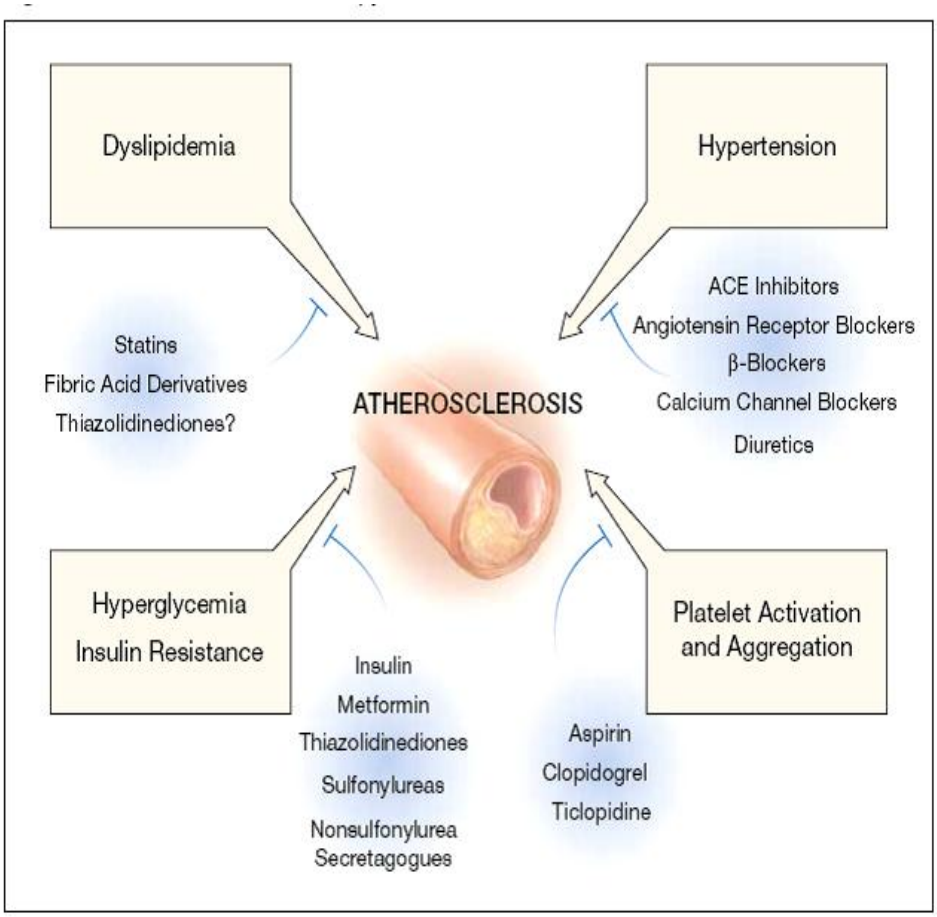

Figure 1. 22: Diabetic patients require therapy of each metabolic abnormality to attenuate atherogenesis (Beckman *et al.*, 2002).

CHAPTER TWO: AIM OF THE STUDY

Allium Sativum, a member of the *Alliaceae* family, has been found to have antidiabetic and anticoagulant activity. The aim of the study was to investigate the effects of the extracts derived from *Tulbaghia violacea*, also of the family *Alliaceae,* on diabetes and coagulation.

2.1. OBJECTIVES

- To determine the cytotoxicity of various *T. violacea* extracts on the INS-1 (insulin secreting), C2C12 (skeletal muscle), 3T3-L1 (adipose) and Chang liver cell lines.

- To determine the effect of the *T.violacea* extracts on glucose-stimulated insulin secretion (GSIS) in INS-1 cells using a radioimmunoassay (RIA) to measure insulin content.

- To determine whether organic *T. violacea* bulb extracts alter oxygen consumption in the INS-1 cells.

- To determine the effect of the organic *Tulbaghia violacea* bulb extracts on mitochondrial membrane potential in INS-1 cells.

- To determine whether organic *T. violacea* bulb extract affects the expression of the glucose transporter (Glut-2) levels in INS-1 cells using western blotting.

- To determine the effect of the *T. violacea* extracts on coagulation in an *in vitro* and *ex vivo* model using the PT, APTT, D-Dimer and Fibrinogen-C assay.

- To determine the effect of the *T. violacea* extracts on platelet function, namely, aggregation and adhesion, in an *in vitro* and *ex vivo* model.

- To determine the effect of the organic *T. violacea* bulbs extract on glucose uptake in C2C12, 3T3-L1 and Chang liver cells (peripheral tissue).

CHAPTER 3 MATERIAL AND METHODS

3.1. COLLECTION OF PLANT MATERIAL

T. violacea was collected from the Nelson Mandela Metropolitan University (NMMU) Herbarium and identified by the curator of the Herbarium in the Department of Botany, NMMU. The plant material was gently washed under running water to remove dust and other debris. It was then dissected into its various components, namely rhizomes, leaves and bulbs.

3.2. METHANOL AND AQUEOUS *T. VIOLACEA* EXTRACT PREPARATION

3.2.1. METHANOL *T. VIOLACEA* EXTRACTION

The plant components were dried in an oven at 40°C for 48 hrs and ground to a fine powder. Ten grams of the dried powder was mixed with 50 mL methanol and extracted overnight at 4°C. The crude *Tulbaghia violacea* extracts were centrifuged at 1500×*g* for 10 mins at 4°C. The supernatant was then filtered using a Whatman No 1 filter to remove all residual plant debris. The filtrate solvent (methanol) was removed in a rotary evaporator at 40°C, and the residues kept at 4°C. The subsequent residue was resuspended in 10 mL deionised distilled water and lyophinized. The dried extract was stored at 4°C in the dark. Extract was dissolved as a stock in dimethyl-sulfoxide (DMSO) as required (Serra *et al.,* 2005; Bungu *et al.,* 2008*)*.

3.2.2. AQUEOUS *T. VIOLACEA* EXTRACTION

The aqueous extraction was performed using the method described for the methanol extraction (section 3.2.1), but replacing methanol with saline (0.15 M NaCl) These extracts were centrifuged at 4500×*g* for 20 mins and the pellet was discarded. Acetone was added to the supernatant in an 80% v/v ratio to precipitate any protein. The resulting solution was centrifuged at 4500×*g* for 20 mins at 4°C. The supernatant was discarded and the pellet re-dissolved in 10 ml distilled water, frozen at -80°C and freeze dried. It was stored in the dark at 4°C (Bungu *et al.,* 2008). Table 3.1 summaries the various parts of *Tulbaghia violacea* that were extracted.

Table 3. 1: A summary of the keys used for subsequent experiments

Extract Code	***Tulbaghia violacea* extracts**	**Extraction method**	**Concentration range (µg/mL)**	**Concentration range (mg/mL)**
BO	Bulb organic	Methanol	0-20	0-50
LO	Leaf organic	Methanol	0-20	0-50
RO	Rhizome organic	Methanol	0-20	0-50
BA	Bulb aqueous	Acetone PPT	0-20	0-50
LA	Leaf aqueous	Acetone PPT	0-20	0-50
RA	Rhizome aqueous	Acetone PPT	0-20	0-50
CON	Control	Without extract	-	
PC	Positive Control: Metformin (glucose uptake) Acetylicylic Acid (anti-coagulation)	-	1 µM 50 mg/kg	

SECTION A: ANTI-DIABETIC STUDIES

3.3. MAINTENANCE OF THE CELL LINES

Routine maintenance of the various cell lines (INS-1, Chang liver, C2C12) (INS-1 cells were kindly donated by Prof M Donath), entailed culturing the cells in 10 cm cell culture dishes at 37°C with 5% CO_2, 90% humdity. Cells were subcultured when they reached 70-80% confluence. Subculturing was performed by removing media from the attached cells. Cells were washed twice with 7 mL phosphate buffered saline (PBS), and trypsinized by adding 1 mL trypsin (0.25% w/v) (Roche Biochemicals). After 10 seconds, 900 µl was removed and the cells were incubated with the remaining trypsin (100 µl) at 37°C for 5 mins. The trypsinized cells were resuspended in 1 mL of media, after which the cells were plated (1:5 ratio) into new culture dishes (Cellis, 1998).

3.3.1. RAT INS-1 CELLS

The pancreatic rat INS-1 cells were maintained in RPMI-1640 with Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1%), HEPES (1%), and β-mercaptoethanol (0.12%) (Gibco). INS-1 cells for experimental purposes were cultured in normoglycemic (11.1 mM glucose) or hyperglycemic (33.3 mM glucose) conditions (Choi *et al*., 2007).

3.3.2. 3T3-L1 PREADIPOCYTES AND ADIPOCYTES

3T3-L1 preadipocytes were maintained in DMEM media supplemented with 10% FBS. The differentiation of the preadipocytes into mature adipocytes was conducted using DMEM containing 10% FBS, 500 μM 3-isobutyl-1-methylxanthine 5 nM insulin and 100 nM dexamethasone over a period of 3 days (Hemati *et al*., 1997).

3.3.3. C2C12 MOUSE SKELETAL MYOBLASTS AND CHANG LIVER CELLS

C2C12 mouse skeletal myoblasts and Chang liver cells were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco).

3.4. 3-(4, 5-DIMETHYLTHIAZOL-2-YL)-2,5 DIPHENYLTETRAZOLIUM (MTT) CYTOTOXICITY STUDIES

Cell viability is often used to assess the rate of cell proliferation or cytotoxicity caused by drugs and cytotoxic agents. The MTT assay is a simple method for determining live cell numbers using a standard colorimetric assay reaction. MTT is a tetrazolium salt that is converted into a purple formazan product after reduction by mitochondrial enzymes that are only present in metabolically active cells. The amount of formazan product generated is proportional to the number of living cells in the sample. The formazan product can be solubilised using DMSO and spectrophotometrically quantified at 540 nm. The MTT assay was used to determine the cytotoxic effects of *Tulbaghia violacea* on the cell lines used in this study.

Confluent cells were replated in a 24-well microplates (Nunc). The INS-1 cells at 30 000 cells/mL, the C2C12 and Chang liver cells at 25 000 cells/mL, and the 3T3-L1 cells at 35 000 cells/mL. Cells were microscopically counted using a haemocytometer and trypan blue. Cells were allowed to attach overnight and exposed to a range of

extract concentrations: 0-50 µg/mL for the INS cells and 0-10 µg/mL for the C2C12, Chang liver cells and 3T3-L1 cells for 48 hrs. Media was aspirated and each well was incubated with 1 mL of media containing 0.5 mg/mL MTT (Sigma) for 4 hrs at 37°C. The media was then removed and the dye trapped within the cells was solubilized with 1 mL of DMSO. The extracted dye was read at 540 nm using a Biotek Powerwave XS microtiter plate reader (Analytical Diagnostic Products, South Africa).

3.5. DETERMINATION OF GLUCOSE-STIMULATED INSULIN SECRETION (GSIS)

To observe the effects the extracts had on insulin secretion under hyper/hypoglycemic conditions, confluent cells were seeded in a 24-well microplates at a density of 30 000 cells/mL and allowed to attach overnight. Once the cells had attached the culture media was changed and the cells were incubated with 11.1 mM glucose RPMI media (normoglycemic conditions) or 33.3 mM glucose RPMI media (hyperglycemic conditions) and exposed to experimental concentrations (table 3.1) of the extracts as determined in the cell viability studies for 48 hrs. After exposure, chronic insulin release, basal and stimulated insulin secretion, as well as insulin content were subsequently determined. Samples were assayed as described by Maedler (2001). The following method was applied.

After exposure, the culture media was transferred to 1.5mL eppendorf tubes and stored at -20°C (chronic insulin level). 1 x Krebs buffer (Annexure A), containing 3.3 mM glucose (1 mL) was added to each well and incubated at 37°C for 30 mins. This media was aspirated from each well and discarded. A second mL of 1 x Krebs containingg 3.3mM glucose solution was added to each well and incubated at 37°C for 1 hr. This media was transferred into 1.5 mL eppendorf tubes and stored at -20°C (basal insulin level). One mL of 1 x Krebs containing 16.7 mM glucose solution was added to each well and incubated at 37°C for an hour and transferred to 1.5mL eppendorf tubes and stored at -20°C (glucose-stimulated insulin level). To determine the insulin content, 1 mL 0.18 M HCl in 70% ethanol was added to each well, allowed to incubate for 1 hr and was then transferred to 1.5 mL eppendorfs tubes and stored at -20°C.

A number of extracts were initially tested to determine GSIS, including aqueous and organic extracts of the seeds, rhizomes, flowers, leaves and bulbs; however only those that showed a positive effect in the coagulation studies were further tested. The flowers, seeds and rhizomes had no effect on coagulation or GSIS and were subsequently eliminated from further studies.

3.6. INSULIN RADIOIMMUNOASSAY (RIA)

The insulin content of the GSIS samples was determined using a rat insulin radioimmunoassay (RIA) kit (Linco Research). In a RIA, a fixed concentration of labelled tracer antigen is incubated with a constant dilution of antiserum in which the concentration of antigen binding sites on the antibody is limited. When labelled antigen is added, there is competition between the labelled tracer and the unlabelled antigen for the limited and constant number of binding sites on the antibody. The amount of tracer bound to antibody will, therefore, decrease as the concentration of unlabelled antigen increases. This can be measured after separating antibody-bound from free tracer and counting either one, or both fractions. A standard log curve (Fig. 3.1) was constructed with increasing concentrations of standard unlabelled antigen (0.1-10 ng/mL). The amount of antigen in unknown samples was calculated from this curve. The rat insulin assay utilizes ^{125}I-labelled insulin and rat insulin antiserum to determine the level of insulin in culture media using the double antibody/polyethylene glycol (PEG) method. This technique involves non-specific protein precipitation with PEG to rapidly separate bound and unbound hormones at low cost and reduced experimental time (Peterson and Swerloff, 1979).

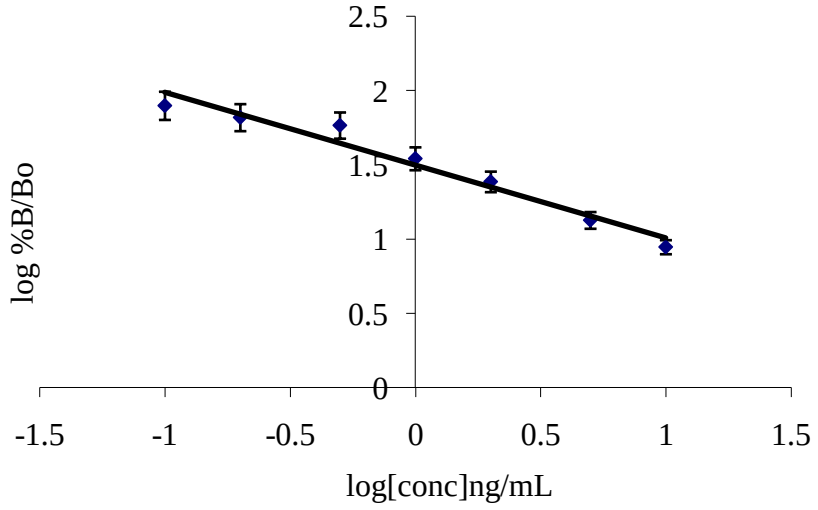

Figure 3. 1: Insulin standard curve. Each point is an average of triplicate values (n=3) (R^2=0.967).

The supernatants from the GSIS studies were analyzed for insulin using the protocol outlined in table 3.2.

Table 3. 2: RIA protocol for standard and test sample preparation (Linco RIA kit Manual Leaflet)

Day 1						**Day 2**
Tube number	**Assay buffer (µl)**	**Standards and samples**	**[^{125}I] Insulin tracer (µl)**	**Rat insulin antibody (µl)**		**Precipitating reagent (mL)**
1,2	-	-	100	-	Vortex, cover and incubate at 4°C for 24 hrs	-
3,4 (NBS)	200	-	100	-		1.0
5,6 (Bo)	100	100µl, 0.1 ng/mL	100	100		1.0
7, 8	-	100µl, 0.2 ng/mL	100	100		1.0
9, 10	-	100µl, 0.5 ng/mL	100	100		1.0
11, 12	-	100µl, 1 ng/mL	100	100		1.0
13, 14	-	100µl, 2 ng/mL	100	100		1.0
15, 16	-	100µl, 5 ng/mL	100	100		1.0
17, 18	-	100µl, 10 ng/mL	100	100		1.0
19 +n	-	100µl, of sample	100	100		1.0

After the addition of precipitating reagent, samples were vortexed, incubated 20 mins at 4°C and centrifuged for 20 mins at 4°C. The supernatant was discarded and the pellet was resuspended in 1mL liquid scintillation cocktail (BD Bioscience). A liquid scintillation analyser (TRI-CARB 2300TR, Beckman) was then used to count the radioactivity of the precipitated samples. The averages of the non-specific binding (NSB) tubes were subtracted from the average of each sample tube, except the total counts. The percent of tracer bound was calculated as follows:

$\%_B$ = {Total binding counts /Total counts} x 100
(Note: $\%_B$ should be in the range of 35-50%).
$\%_{B/Bo}$ = (sample or standard/Total binding) x 100
An average of 37% was calculated.

The insulin concentration for the samples was then calculated from the standard curve (Morgan an Lazarow, 1963).

All extracts were screened at a range of 0-10µg/mL to determine their effect on insulin secretion in INS-1 cells. The extracts that enhanced insulin secretion were used in subsequent experiments to determine if the effect was concentration dependent.

3.7. DETERMINATION OF GLUCOSE UTILISATION

The glucose uptake assay was performed using glucose reagent (phosphate buffer, phenol, 4-aminoantipyrine, peroxidise, ethylenediaminetetraacetate (EDTA) and glucose oxidase) (Annexure A: reagent composition). The principle of the glucose assay is based on the conversion of glucose to gluconic acid and hydrogen peroxide by glucose oxidase. Peroxidase then catalyses the reaction between hydrogen peroxide and a reduced dye, 4-aminoantipyrine (colourless in its reduced form), present in the reagent. This reaction produces an oxidized dye (reddish colour) and water. The colour compound is measured spectrophotometrically at 429nm using a Biotek Powerwave XS microtiter plate reader (Analytical Diagnostic Products, South Africa) (Keston, 1956). Glucose uptake studies were conducted on differentiated 3T3-L1 cells, C2C12 and Chang liver cells.

Control cells (CON) were represented by cells which were not exposed to extract and incubated in their respective culture media. The positive control cells (PC) were represented by differentiated 3T3-L1, C2C12 and Chang liver cells treated with 1 µM metformin (Sigma) in culture media.

Experimental cells were represented by differentiated 3T3-L1, C2C12 and Chang liver cells treated with the BO extract (0-10 µg/mL). 3T3-L1 preadipocytes were seeded (35 000 cells/mL) in 24-well microtiter plates (Nunc), allowed to reach confluence and differentiated over a period of 3 days (section 3.3.2). Thereafter, cells were incubated with 1 mL BO extract or PC for 48 hrs. C2C12 and Chang liver cells were seeded (25 000 cells/mL) and allowed to reach confluence over a 48 hr period, after which they were exposed to the BO extract for 48 hrs.

After 48 hrs exposure, glucose uptake was determined. Cells were incubated for 1 hr in glucose-free media, followed by incubation in culture medium containing 8mM glucose in the presence of 60 µU/mL insulin for 1 hr. Aliquots (50 µl) were removed

from each well and added to 150 µl distilled water. The diluted aliquot (50 µl) was added to 200 µl glucose reagent and incubated for 15 mins at 37°C, and the absorbance measured at 492 nm using a Biotek Powerwave XS microtiter plate reader (Analytical Diagnostic Products, South Africa). Cell numbers were normalised to 100 000 by determining cell viability using the MTT assay subsequent to glucose exposure. A glucose standard curve was constructed using triplicate samples (0.25 – 2 mM) (Fig. 3.2).

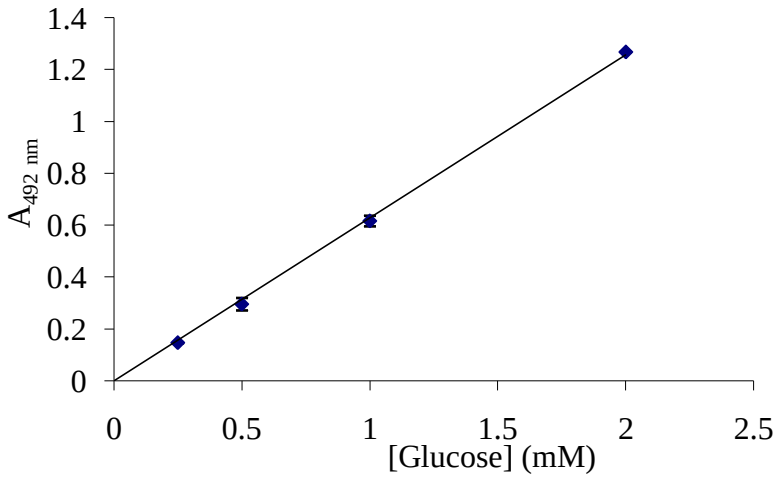

Figure 3. 2: Glucose standard curve of triplicate samples. Error bars indicate SEM (n=3) (R^2=0.999).

3.8. DETERMINATION OF OXYGEN CONSUMPTION STUDIES IN INS-1 CELLS

The Rank Brothers Oxygen Electrode, an electrochemical sensor which measures changes in oxygen concentration, was used to determine the oxygen consumption of the INS-1 cells. This electrode is very sensitive, and a range of 0.01 to 100% oxygen can be detected. Detection of the oxygen is possible with the use of two electrodes, a platinum (cathode) and silver electrode (anode) which is separated from the test media by an oxygen permeable teflon membrane. Conduction of a 3M KCl solution connects the two electrodes. When oxygen comes into contact with the platinum electrode, it is reduced to water. The reduction reaction is coupled to an oxidation reaction involving the silver electrode converting silver to silver chloride. The current produced from these reactions is related to the partial pressure of oxygen using the following formula (Hitchman, 1978):

$$Id = 4.Fc.Pm.A.\frac{P(O_2)}{b}$$

Where:

Id= current produced

Fc= Faraday's constant,

Pm = oxygen permeability of the teflon membrane

A = the surface area of the Pt electrode,

b = the thickness of the Teflon membrane

Cells ($1x10^6$/mL) in the appropriate media were placed within the incubation chamber (set at 37°C). The oxygen consumption of the cells was measured, captured and the data analyzed using Lab chart 6 software (Hitchman, 1978; Less and Tsao, 1979).

INS-1 cells were harvested after reaching confluence in 10 cm culture dishes. They were incubated with the BO extract (10 µg/mL) in either 33.3 mM glucose or 11.1 mM glucose concentrations for 48 hrs. Control cells (CON) were grown in either 33.3 mM glucose concentration or 11.1 mM glucose concentrations for 48 hrs and assayed without BO treatment. Oxygen consumption was performed after the cells were suspended via trypsinization. Cells were trypsinized and collected to a cell count of $1x10^6$/mL. Media was replenished in the reading chamber before each measurement to recalibrate the electrode to a 100% oxygen level. Once the cells were added to the chamber, oxygen consumption was measured for 500 seconds (basal). Oligomycin (1 mg/mL) was added and additional 200 seconds oxygen consumption was monitored. Oligomycin inhibits ATP synthase by blocking proton channels, thereby preventing oxidative phosphorylation of ADP to ATP and inhibiting or lowering oxygen consumption (leak) (Fig.4.14).

3.9. DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL IN INS-1 CELLS

The mitochondrial membrane potential ($\Delta\psi_m$) was determined using the JC-1 kit (BD bioscience) according to the manufactures instruction using flow cytometry (Beckman Coulter Cytomics FC 500).

Membrane-permeable lipophilic cationic fluorochromes are used as probes to measure $\Delta\psi$. They penetrate cells and their fluorescence is indicates the $\Delta\psi$. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) or 1st J-aggregate-

forming cationic dye is one such fluorochrome (Gravance *et al*., 2000). The fluorescence emission spectrum of JC-1 is dependent on its concentration which, is determined by the status of the $\Delta\psi$. JC-1 can exist in two different states, aggregates or monomers, each with a different emission spectra. JC-1 forms monomers at low dye concentrations and aggregates at higher concentrations. Both JC-1 aggregates and monomers exhibit fluorescence in the green end of the spectrum and can be measured in the Green (FL-1) channel in a flow cytometer. When live cells are incubated with JC-1, the fluorochrome penetrates the plasma membrane of cells as monomers. Uptake of JC-1 into mitochondria is driven by the $\Delta\psi$. The $\Delta\psi$ of normal, healthy mitochondria is polarized and JC-1 is rapidly taken up by such mitochondria. This uptake increases the concentration gradient of JC-1 leading to the formation of JC-1 aggregates (known as J-aggregates) within the mitochondria. These are detected by the red fluorescence or relative fluorescent intensity (RFI).

INS-1 cells were harvested after reaching confluence in 10 cm culture dishes. They were incubated with the BO extract (10 µg/mL) in either 33.3 mM glucose or 11.1 mM glucose for 48 hrs. Control cells (CON) were not incubated with extract. Membrane potential detection was performed after the cells were suspended via trypsinization. One million cells were counted using a haemocytometer. Cells were transferred to 15 mL sterile polystyrene centrifuge tubes and centrifuged at 400×*g* for 5 mins at RT. The supernatant was discarded. Pelleted cells were resuspended in the JC-1 working solution to disrupt cell-to-cell clumping, and incubated for 10–15 min at 37°C in a CO_2 incubator. One mL of assay buffer was added to the cells, which was centrifuged at 400×*g* for 5 min. Pelleted cells were resuspended in 1 ml of assay buffer and centrifuged at 400×*g* for 5 min. The cell pellet was resuspended in 0.5 mL 1×assay buffer and analyzed by flow cytometry (Beckman Coulter Cytomics FC 500).

3.10. IMMUNODETECTION OF GLUT-2

Cells were cultured in 10 cm culture dishes and exposed to the BO extract (10 µg/mL) in either 33.3 mM glucose or 11.1 mM glucose concentrations for 48 hrs. Cell lysis buffer (1mL) (50 mM Tris-HCl, pH 8; containing 150 mM NaCl; 0.02% NaN_3, 1% Triton X-100 and 1% protease inhibitor (Sigma) was added to the cells (Vivancos and Moreno, 2005). Cells were centrifuged at 12 000×*g* for 10 mins and the supernatant was transferred to a new collection tube and stored at -80°C. The protein content of

the samples was determined using the bicinchoninic acid (BCA) assay (section 3.10.1).

3.10.1. BCA ASSAY

BCA is a stable water-soluble compound. The method is based on the formation of an intense purple coloured complex between the copper ions and BCA. It is postulated that the copper ions (Cu^{2+}) arise from the oxidation of Cys, Tyr and Trp, which is a temperature-dependent reaction of peptide bonds with Cu^{2+}. The advantages of the BCA method include high sensitivity towards protein concentration ranges (0.5-1 μg/mL), ease and reduced sensitivity to interfering compounds (Walker, 1994 and Sheehan, 2009).

The working reagent is prepared by adding reagent B (4% $CuSO_4$) to reagent A (1% BCA-Na_2, 2% Na_2CO_3, 0.16% Na tartrate, 0.4% NaOH, 0.95% $NaHCO_3$, pH 11.25) using a ratio of 1:50. A range of bovine serum albumin (BSA) concentrations (0-1 mg/mL) was used to construct a standard curve (Fig.3.3). Ten μl of sample, standard or blank (ddH_2O) was added to 200 μl working reagent in a 96 well microtiter plate (Nunc). The solution was incubated at 37º C for 30 mins. The absorbance was measured at 540 nm using a Multiskan MS microtitre plate reader. All protein determinations were completed in triplicate.

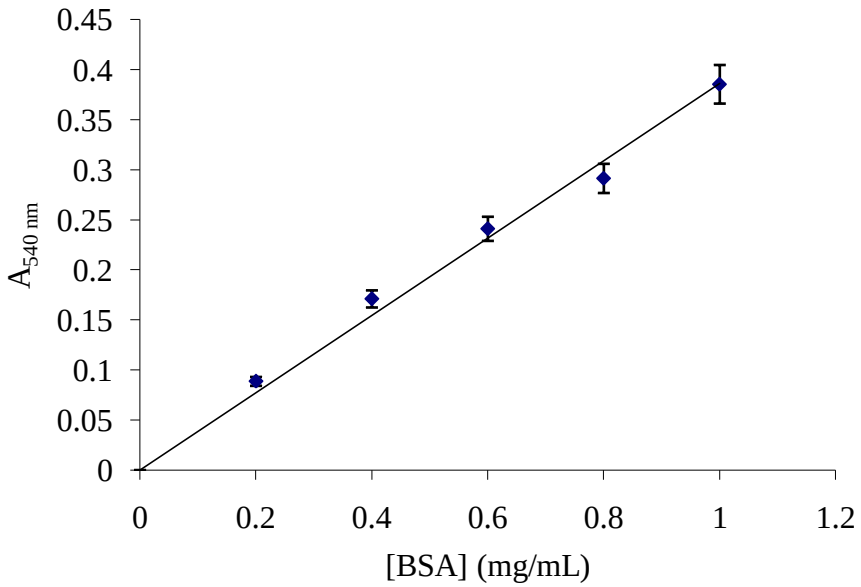

Figure 3. 3: Protein (BSA) standard curve representing triplicate samples. Error bars indicate SEM (n=3) (R^2=0.996).

3.10.2. SDS-PAGE AND PROTEIN TRANSFER

Proteins were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Discontinuous electrophoresis involves using two gels, viz. the stacking and resolving gels. The stacking gel (pH 6.9) concentrates the sample into thin bands that accumulate at the interface of the two gels as stacking gel has a higher electrical resistance and stronger electric field strength.

The resolving gel (pH 8-9) has a higher acrylamide concentration and ionic strength. This allows for separation of the stacked samples through the process of isotachophoresis (Sheehan, 2009), resulting in improved resolution.

Proteins may be electrophoresed in either their native or denatured state. Sodium dodecyl sulphate (SDS) is a detergent that binds to proteins, causing the dissociation of most oligomeric proteins into their monomer subunits and destroying the secondary structure. If disulphide bonds are present, a reducing agent (β-mercaptoethanol) is added and the sample is heated, resulting in the dissociation of the protein into smaller subunits. Therefore, migration depends only upon the molecular mass of the protein (Walker and Wilson 2005).

The protein lysate obtained from the INS-1 cells (50 µg) and a wide range molecular weight marker (Fermentas) were separated a 10% resolving gel. A voltage of 100 V (40 mA) was applied for 1.5hr (Laemmli, 1970). A standard curve was constructed using the molecular weight markers (pre-stained broad range (260-10 kDa) protein ladder and their relative migration (Fig. 3.4) to ensure the correct molecular weight of the desired band was observed.

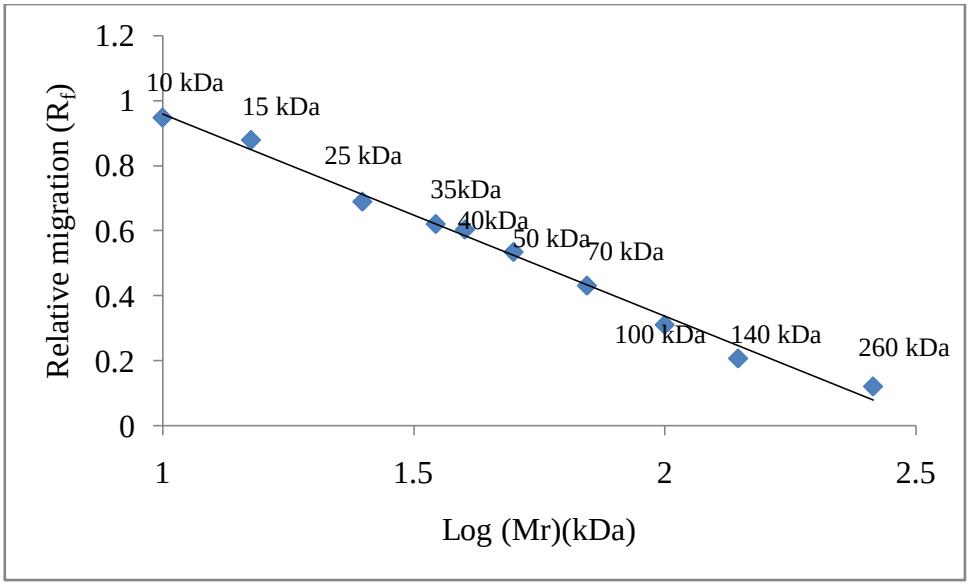

Figure 3. 4: Molecular weight standard curve (kDa) (R=0.995).

After electrophoresis, the separated proteins were transferred using a semi-dry electroblotting technique onto a polyvinylidene difluoride (PVDF) membrane. The membrane was pre-wetted in 100% methanol for 1-2 seconds and placed in ddH_2O for 5 mins to remove the methanol. The membrane, filter paper and gels were equilibrated in pre-chilled (4ºC) transfer buffer (50mM Tris-HCl at pH 8.30, containing 192 mM glycine and 20% methanol,) for 45 mins. The cassette was packed in layers, i.e. fibre pad, filter paper, gel, membrane, filter paper and lastly the fibre pad. All bubbles were removed to ensure a successful protein transfer. The transfer was performed at 25 V and 250 mA/cm^2 for 2 hrs (Bio-Rad instrumental manual).

3.10.3. DETECTION OF PROTEIN (ACTIN AND GLUT 2) EXPRESSION

After transfer, membranes were washed in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.4, containing 0.9% NaCl,) for 5 mins. This was repeated three time to ensure the removal of the transfer buffer. The membrane was blocked overnight in 2% skim milk powder which was dissolved in TBS. The membranes were washed 3x for 5 mins in TBS. Membranes were used to detect Glut-2 and actin levels.

The membrane was incubated for 2 hrs in 0.2% skim milk containing the primary antibody (actin rabbit polyclonal IgG Santa Cruz Biotechnology, 1:1000 dilution) and subsequently washed 3x for 5 mins in TBS. The secondary antibody (Anti-rabbit IgG alkaline phosphatase) (Sigma) was diluted (1:30 0000 in 0.2% skim milk and

incubated with the membrane for an hour. Thereafter, it was washed in TBS (4x). The secondary antibody containing the alkaline phosphatase enzyme was detected using 5 mL 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma). The reaction was stopped by placing the membrane in phosphate buffered saline (PBS) containing EDTA. Images were captured using an alpha imager 3400. Changes in band intensity were calculated using densitometry ratios of lysates of cells treated with extract versus untreated cells and one treatment versus another treatment.

The detection of Glut-2 was performed as described above, with the exception that the primary antibody, Glut-2 goat polyclonal IgG (Santa Cruz Biotechnology), was used at a 1:200 dilution and the secondary antibody, anti-goat IgG alkaline phosphatase (Biochem Biotech) was used at a 1:500 dilution.

SECTION B: COAGULATION

3.11. PT, APTT, D-DIMER AND FIBRINOGEN-C ASSAYS

Aspects of coagulation were targeted to determine the possible anticoagulant (APTT, PT) and fibrinolytic (D-Dimer, Fibrinogen-C) activity of *Tulbaghia violacea* extracts using *in vitro* and *ex vivo* models. The rat plasma was obtained by spinning the whole rat blood at 300xg for 10 minutes and collected the straw colour supernatant, otherwise calibration plasma was used. This was completed using the following common clinical tests:

3.11.1. Prothrombin Time (PT) test is a measure of the integrity of the extrinsic TF pathway and final common pathway of the procoagulant cascade. The PT represents the time, in seconds, for plasma to clot after the addition of calcium and an activator of the extrinsic pathway (thromboplastin) to plasma. The normal range for PT in human plasma is between 11 and 13.5 seconds. Thus, deficiencies or inhibitors of clotting factors within the extrinsic and final common pathways results in prolongation of the PT (Hood and Eby, 2008). PT reagents are sensitive to deficiencies of FVII, FII, FV and FX within the extrinsic pathway and less sensitive to deficiencies of factors within the final common pathway (FV, FX and FII and fibrinogen) (Verstraete and Wessler, 1992). The PT assay involved pipetting recomboplastin (200µl) into a plastic cuvette, and incubating it for 3 minutes. The extract (50µl) and calibration plasma (50µl) was incubated for 3 minutes in a separate cuvette. The cuvette

containing the recomboplastin was added to the extract-plasma mixture to initiate the reaction. The clotting time was measured in seconds.

3.11.2. Activated Partial Thromboplastin Time (APTT) is a measure of the integrity of the intrinsic (amplification) pathway, which includes factors I, II, V, VIII, IX, X, XI, and XII and final common pathways of the coagulation cascade. The APTT represents the time, in seconds, for plasma to clot after the addition of phospholipid, an intrinsic pathway activator (ellagic acid, kaolin, or micronized silica), and calcium. The normal range for APTT in human plasma is between 24 to 39 seconds. The APTT reagent is called partial thromboplastin because TF is not present in conjunction with the phospholipid as it is in the PT reagent. Formation of the prothrombinase complexes on the surface of the phospholipid enables prothrombin to be converted into thrombin, with subsequent clot formation (Verstraete and Wessler, 1992). Thus, deficiencies or inhibitors of clotting factors within the intrinsic and final common pathways result in prolongation of the APTT (Hood and Eby, 2008). The APTT assay involved pipetting recomboplastin (200µl) into a plastic cuvette, and incubating it for 3 mins. The extract (50µl) and calibration plasma (50µl) was incubated for 3 mins in a separate cuvette. The cuvette containing the recomboplastin was added to the extract-plasma mixture to initiate the reaction. The clotting time was measured, in seconds by measuring the light decreasing through the cuvette.

3.11.3. D-Dimer Test, the presence of D-Dimer confirms that both thrombin and plasmin have been generated since it can only be produced as the result of the plasmin degradation of cross-linked fibrin. This makes the test specific for fibrinolysis (Van Cott and Laposata, 2001). The assay uses spectrometric end point detection and can detect D-Dimer levels between 0 and 1000µg/mL (Shitrit *et al*., 2002). It is a specific marker for cross-linked fibrin and is often used as a marker for DIC. It is also a marker that effectively determines the presence of stabilized fibrin and thus the presence of microvascular thrombosis. The D-Dimer latex reagent is a suspension of polystyrene latex particles of uniform size coated with monoclonal antibody, highly specific for the D-Dimer domain included in fibrin soluble derivatives. D-Dimer was mixed with the latex reagent and the reaction buffer included in the kit

(Hemosil^{TM}, D-Dimer-0020008500, Instrumental Laboratory Company) and the coated latex particles allowed to agglutinate. The degree of agglutination is directly proportional to the concentration of D-Dimer in the sample and is determined by measuring the decrease of the transmitted light at 405 nm caused by the aggregate.

Measurements of the APTT, PT and Fibrinogen-C and D-Dimer levels were performed using a CL analyser (Instrumental Laboratory Corporation, Beckman) and an ACL-assay reagent kit (Instrumental Laboratory Corporation, Beckman). The *in vitro* tests were performed according to the package inserts using the *Tulbaghia violacea* extracts. The APTT, Fibrinogen-C and D-Dimer assays were performed as stipulated in table 3.3.

Table 3. 3: A summary of APTT, Fibrinogen-C and D-Dimer assays

Volume (µl)	***APTT***	***Fibrinogen-C***	***D-Dimer***
Reaction buffer	-	-	150
Calibration plasma	50	-	40
SynthAsil reagent	200	-	-
Factor diluents	-	100	-
Extract	50	100	10
Incubate for 3 min at 37°C			
$CaCl_2$ (0.15M)	100	-	-
Fibrinogen-C reagent	-	100	-
Latex D-Dimer reagent	-	-	200
Mix and measure the clotting time (sec)			

For the *ex vivo* model, the tests (PT, APTT, D-Dimer and Fibrinogen-C) were completed as in table 3.3 where rat plasma replaced the calibration plasma and no incubation was performed. No extract was included, since the animals were treated with the BO extract (50 mg/kg).

3.12. PLATELET AGGREGATION, ADHESION AND PROTEIN SECRETION INHIBITION USING HUMAN BLOOD

Platelet aggregation, adherence and secretion of proteins were monitored in this study using microscopy and flow cytometry.

3.12.1. MICROSCOPIC EVALUATION OF PLATELET AGGREGATION, ADHESION AND PROTEIN SECRETION

3.12.1.1. ISOLATION OF PLATELETS

Blood was obtained via venipuncture using vacutainer tubes containing 3.8% sodium citrate (1:9) from various healthy adult volunteers, free of medication. Ethical clearance was obtained in accordance with the guidelines set by the Human Ethics Committee of NMMU (Annexure B). Platelets were isolated by differential centrifugation of the blood (20 min at 200×*g*). Platelet-rich plasma was separated and centrifuged for 20 min at 1000×*g* to sediment the platelets. The resulting pellet was gently resuspended in Ca^{2+}/Mg^{2+}-free modified Tyrode's buffer (15 mM Tris/HCl, pH 7.4 containing 140mM NaCl and 10mM glucose). Platelets were washed three times in Ca^{2+}/Mg^{2+}-free Tyrode's buffer and suspended in Ca^{2+}/Mg^{2+}-free Tyrode's buffer at a final concentration of 10^9 platelets/mL (Fig. 3.5). Platelet-poor plasma was prepared by centrifugation of a fraction of the platelet-rich plasma at 1500×*g* for an additional 10 min. The platelet suspension was kept at 4°C and utilized within 1 hour (hr) of harvesting (Bellavite *et al*., 1993).

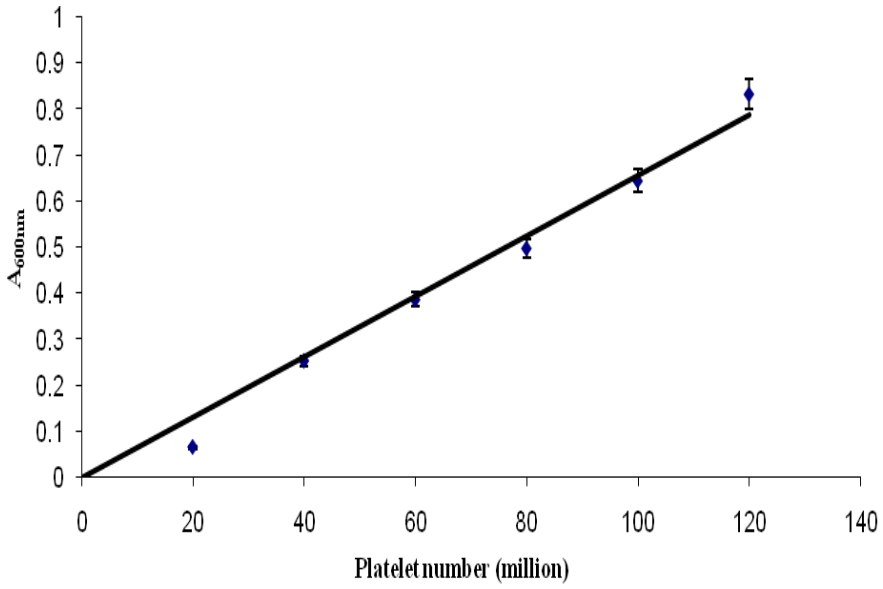

Figure 3. 5: A standard curve illustrating the number of platelet counts using a haemocytometer and microtiter plate reader (R^2=0.9812) (n=3).

3.12.1.2. PLATELET ADHESION AND AGGREGATION

Adhesion of the activated platelets to extracellular cell matrix coated culture plates (Nunc) was determined according to the method of Erikson and Whiss (2005). The isolated platelets obtained were incubated in the absence and presence of the extracts

at a concentration range of 0.25 - 1 mg/mL for 1 hr at 37°C. A volume of 1 mL of platelets (30 x 10^6 platelets/mL) was then activated using thrombin (Sigma) (0.25 U/ml, 30 min at RT). The platelet suspension was added to a 3 cm diameter culture plate and incubated for 1 hr at 37 °C without shaking to allow the platelets to adhere. The plate was microscopically examined (400x magnification using a Zeiss microscope). The same method was used to determine the effect of *T. violacea* extracts on platelet aggregation; however, the plates were replaced by culture plates free of any coating to allow platelet suspensions to aggregate unhindered (Erikson and Whiss 2005).

3.12.1.3. PROTEIN SECRETION ASSAY

Isolated platelets suspensions (1mL of 30 x 10^6 platelets/mL) were incubated in the absence and presence of the extracts at a range of 0.25-1 mg/mL for 1 hr at 37°C activated with thrombin (0.25 U/ml, 30 min at RT) and centrifuged (20 min at 1100× *g*). The level of proteins released to the supernatant by the activated platelets was determined using the BCA protein determination assay, with the use of a standard curve (Fig 3.6). The protein levels were expressed as mg/mL of platelet suspension. These were compared to the condition and the percentage of platelet protein secretion inhibition was determined (Erikson and Whiss 2005).

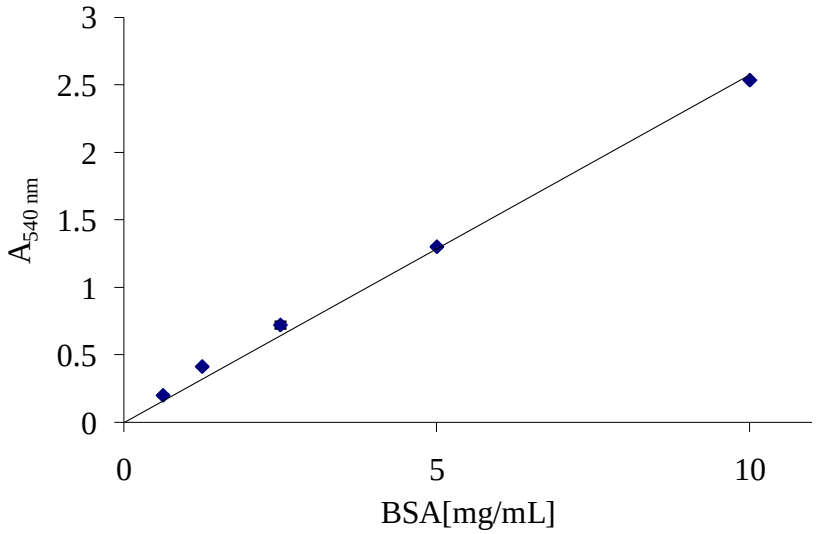

Figure 3. 6: Protein (BSA) standard curve. Error bars indicate SEM (n=3) (R^2=0.994).

3.13. *EX VIVO* RAT MODEL FOR COAGULATION AND PLATELET STUDIES

3.13.1. PLATELET AGGREGATION STUDIES USING FLOW CYTOMETRY

Many investigators have turned to flow cytometry to provide a method for platelet analysis in whole blood or in minimally purified platelet preparations. Due to their relatively high concentration, platelets can be rapidly examined by flow cytometry in very small blood volumes, allowing for the use of smaller test subjects such as mice and rats. The flow cytometry method allows for identification of platelet subsets based on antigen expression or studied markers such as P-selectin, a marker for platelet degradation, and PAC-1, a marker of GIIb/IIIa activation at rest or after stimulation with platelet agonists. Both activation state of circulating platelets and the reactivity of circulating platelets can be determined (Michalson *et al.*, 2000). Platelets in a sample are incubated with fluorescent probes and platelets drawn into the flow chamber and through the beam of the laser, thereby activating the fluorophore. The emitted fluorescence and light scatter properties of the platelet are detected. Due to the platelet's small size and unique receptor properties, the photomultiplier detectors on the flow cytometer must be set specifically for platelet fluorescence and light scatter (Michalson, 1996).

Male Wistar rats, 6 weeks old were randomly divided into 3 groups of 6 rats per group: the vehicle control (CON) groups receiving 1% Tween 80 dissolved in saline, positive control (PC) groups receiving 50 mg/kg Acetylicylic acid (Asprin) in 1% Tween 80 in saline and the experimental group receiving BO extract in 1% Tween 80 in saline, 50 mg/kg. The rats were fed on rat chow and allowed to acclimatise to their new environmental facility conditions (19–25°C and a 12 hr light/dark cycle) for 2 weeks. Water was available *ad libitum.* Rats were treated for 7 days (intraperitoneally injected with either vehicle, positive control or experimental. Rats were anesthetized with phenopontabarbital (1 mL). Blood was collected from the heart using a syringe with 3.8% sodium citrate (1:9) (Gardi *et al.*, 2009) and used for coagulation and platelet studies.

The method used to test platelet aggregation was adapted and optimised from Michalson *et al*. (1991). A number of controls were set, including the isotype of the dye, stained and unstained platelets, to ensure correct gating of the flow cytometer for

experimental platelet samples. Within 15 mins of drawing blood, it was centrifuged at 300× *g* to obtain platelet rich plasma, which was diluted with Tyrode's buffer to obtain $30x10^6$ platelets/mL. A volume of 40 µl was aliquoted into tubes containing 10 µl fluorescein isothiocyanate (FITC)-PAC-1 (BD Biosciences) probe (1 µg/mL), a monoclonal antibody that binds to fibrinogen-binding sites exposed by conformational changes that occur in the GPIIb/IIIa complex upon activation (Michalson *et al.*, 2000). A saturated concentration was used to ensure that all GPIIb/IIIa binding sites were bound. The platelets were activated with thrombin (0.25 U/ml, 15 min at RT). Platelets were fixed with a 2:1 ratio in 1% formaldehyde for 30 mins at room temperature. A threefold volume of Tyrode's buffer was added and the platelet mixture was immediately analyzed with a Beckman Coulter Cytomics FC 500 flow cytometer. Ten thousand events were counted. The excitation and emission wavelengths were 488 nm and 530 nm, respectively. The results were expressed as percentage inhibition in relation to the negative controls, which was comprised of the platelets from rats in the control group receiving the vehicle only:

$$\text{Percentage inhibition} = \left[\left(\frac{\Delta\, PAC{-}1\ POSITIVE\ CELLS[CONTROL]}{\Delta PAC{-}1\ POSITIVE\ CELLS\ [TEST\ SAMPLE]}\right) \times 100\right]$$

3.13.2. *EX VIVO* COAGULATION STUDIES

Rat plasma was obtained as described in section 3.12.1.1 and standard coagulation tests were performed, as described in section 3.11 (APTT, PT, Fibrinogen-C and D-dimer). Ethical clearance was obtained in accordance with the guidelines set by the Animal Ethical Committee of NMMU (Annexure C).

3.14. STATISTICAL ANALYSIS

All experimental values reflect an average of a minimum of 3 experiments, each at least completed in triplicate. Error bars indicate standard error of mean (SEM) unless otherwise specified. Statistical significance was evaluated by the paired Student *t*-test. Values of $p \leq 0.01$ (*) or $p \leq 0.05$ (#) were considered to be statistically significant.

CHAPTER 4: RESULTS

SECTION A: ANALYSIS OF ANTIDIABETIC STUDIES

4.1. MTT CYTOTOXCITY OF *T. VIOLACEA* EXTRACTS

4.1.1. CYTOTOXICITY IN INS-1 CELLS

To determine whether any of the *Tulbaghia violacea* extracts were cytotoxic to the INS-1 cells, the MTT cell viability assay was performed using a 0-50 µg/mL concentration range of each extract. These results are shown below in Fig. 4.1.

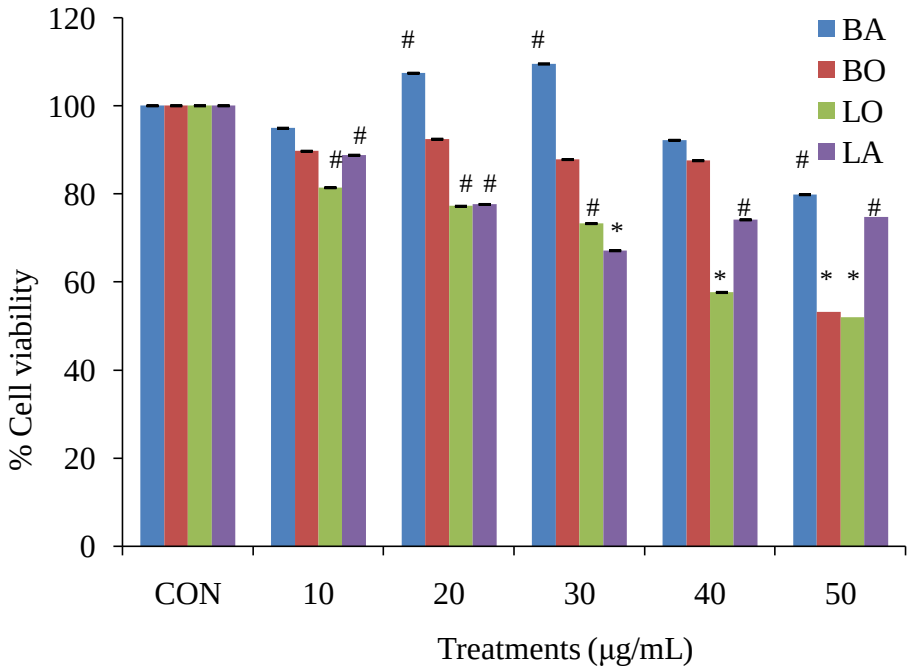

Figure 4. 1: Cytotoxicity of the various *T. violacea* extracts in INS-1 cells (n=3). # p<0.05 and * p<0.01 relative to the control (CON).

With the majority of the extracts, there was a decrease in cell viability of the INS-1 cells in response to the treatment. However, at 20 and 30 µg/mL the BA extract produced a significant cell proliferative effect ($p<0.05$). At higher concentrations of the extract, there was a significant decrease in the number of viable cells. The lowest concentrations of the extracts, were not toxic to the INS-1 cells, as 80% cell viability was noted. A range of 0-10 µg/mL was used for all subsequent experiments based on the data above. BO 10-40 µg/mL maintained ± 90 % cell viability and was used for subsequent assays.

4.1.2. MTT CYTOTOXICITY STUDIES IN C2C12, 3T3-L1 AND CHANG LIVER CELLS USING THE BO EXTRACT

To ascertain whether the optimal concentration range of the BO extract used in INS-1 cells, exerted any cytotoxic effects on the various cells lines (C2C12, 3T3-L1 and Chang liver), a MTT cytotoxicity assay was performed (Fig 4.2). After 48 hrs exposure, cell viability was measured spectrophotometrically.

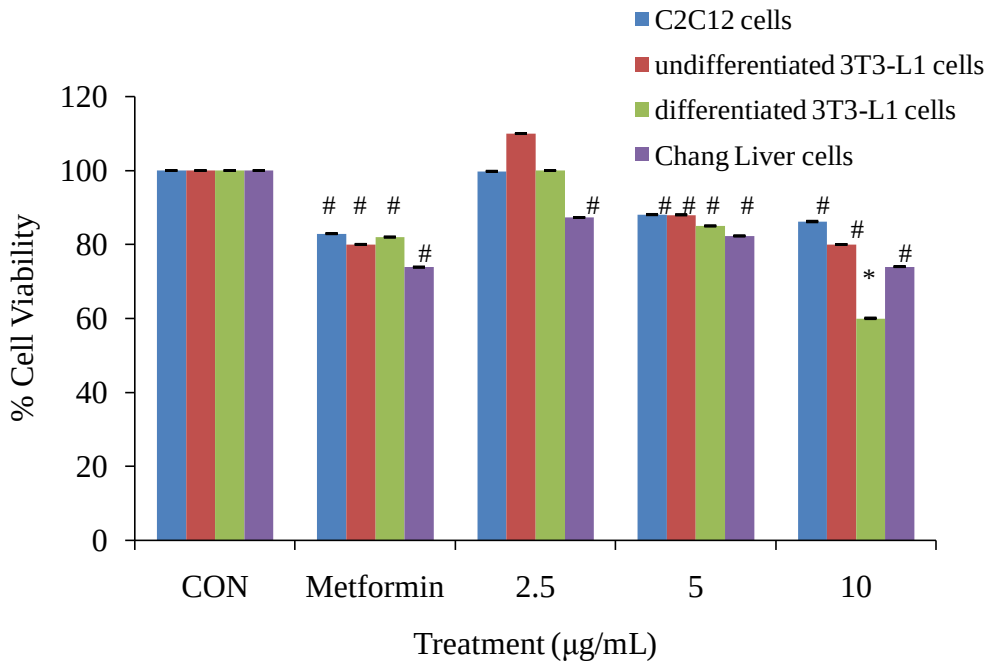

Figure 4. 2: The cytotoxic effect of the BO extract on the peripheral cells (C2C12, differentiated and undifferentiated 3T3-L1) and the Chang liver cells (n=3) # p<0.05 and * p<0.01 relative to the CON.

It was observed that the BO extract was slightly toxic to the various cell lines (C2C12, 3T3-L1 and the Chang liver cells) at 10 µg/mL. The toxicity displayed was different to what was observed in Fig. 4.1, due to the fact that different cells have different toxicity tolerance levels. Metformin (PC) displayed a degree of toxicity in all the cell lines, with cell viability ranging between 75-80%. The concentration of metformin (1 µM) used was consistent with that routinely reported in literature. The observed toxicity could be due to the high passage number of cells used to conduct these experiments. The MTT assay was completed on the experimental cells to normalize to 100 000 cells, across all conditions tested in the glucose utilisation assay.

4.2. SCREENING OF *T. VIOLACEA* EXTRACTS ON GSIS.

4.2.1. CHRONIC INSULIN RELEASE

INS-1 cells were cultured and maintained at normo (11.1 mM) and hyperglycemic (33.3mM) levels for 48 hrs in order to determine the chronic levels of insulin released by the cells exposed to the extracts (Fig. 4.3). Exposure of the INS-1 cells to the bulb (BO, BA) and leaf (LO, LA) extracts, showed that the majority of the extracts maintained at both the normoglycemic (11.1 mM glucose) and hyperglycemic (33.3 mM glucose) levels resulted in a significant decrease in insulin secretion and β-cell function. Conversely, the LO (5 µg/mL) at the hyperglycemic level and the BA and LA (5 µg/mL) at the normoglycemic level produced a significant ($p< 0.01$) increase in insulin secretion compared to the control (CON). Cells exposed to BA (2.5µg/mL) had similar insulin secretion levels to control cells at normoglycemic levels.

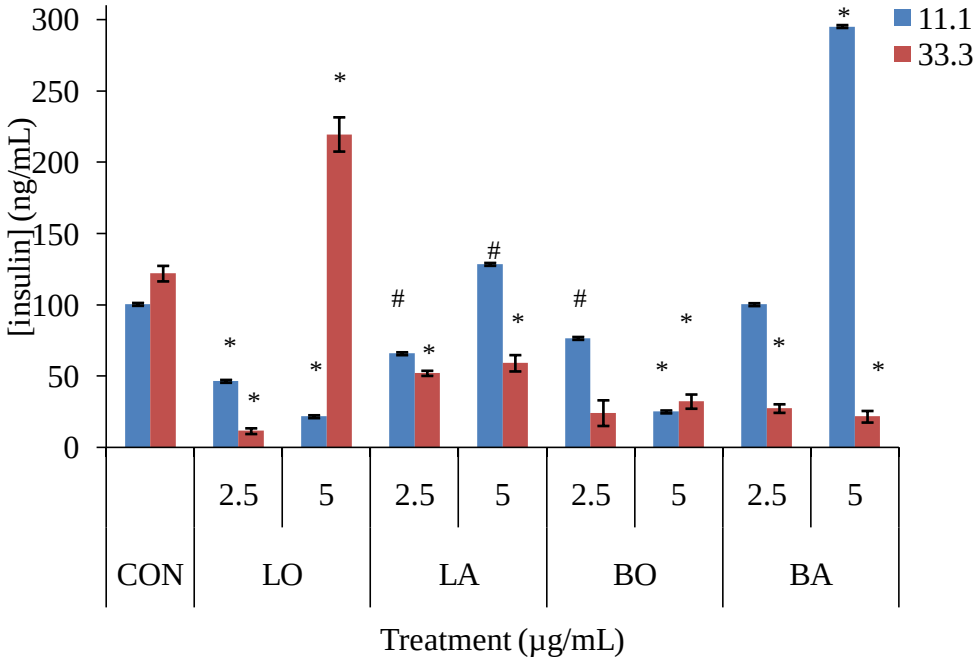

Figure 4. 3: Chronic insulin secretion levels of INS-1 cell exposed to *T. violacea* extracts after 48 hrs exposure of the cells in 11.1 and 33.3 mM glucose (n =4). $^{\#}p<0.05$ and * $p<0.01$ relative to the relevant the control (CON).

4.2.2. BASAL INSULIN SECRETION

Once chronic insulin levels had been assayed, cells were exposed to 3.3 mM glucose to ascertain basal insulin secretion levels in the presence of the extracts. The LA and LO (5 µg/mL), significantly increased insulin secretion ($p<0.01$) at the normoglycemic level (Fig 4.4) in a concentration dependent manner. At the hyperglycemic level all extracts significantly inhibited insulin secretion, generally in a

concentration dependent manner. It was expected that the 11.1 mM control would have a higher insulin secretion than that of the 33.3 mM glucose control, however this was not observed.

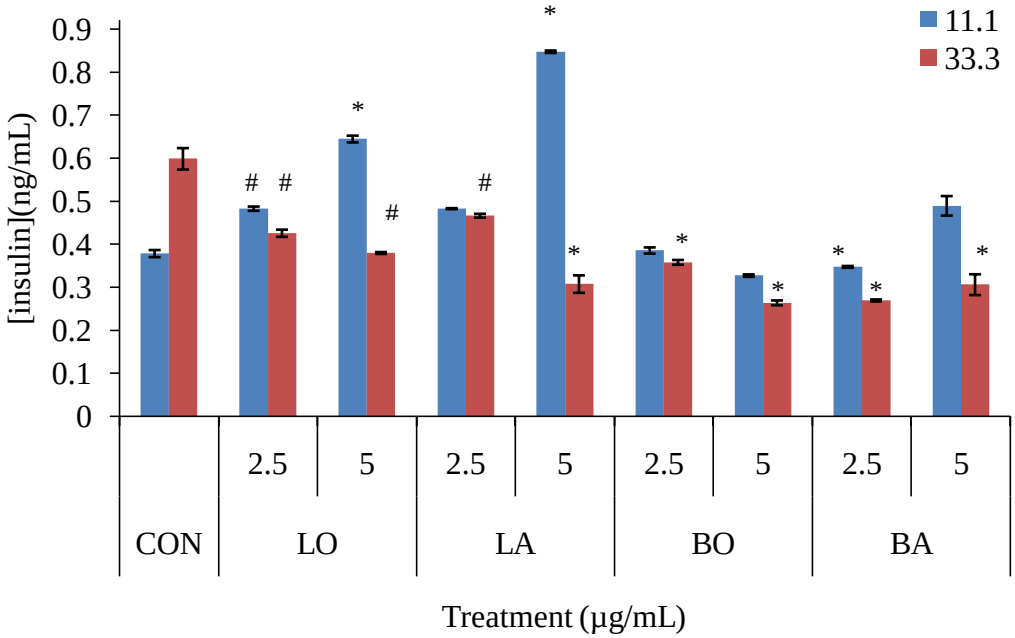

Figure 4. 4: Basal insulin secretion levels of INS-1 cells exposed to *T. violacea* extracts after 48 hour in 11.1 and 33.3 mM glucose (n =4). #p<0.05 and * p<0.01 relative to the control (CON).

4.2.3. STIMULATED INSULIN SECRETION

Once basal insulin secretion levels had been assayed, the cells were incubated with a higher glucose concentration, 16.7 mM, to determine the stimulatory effect on insulin secretion. At the hyperglycemic level only the BO extract (2.5 and 5 µg/mL) stimulated insulin secretion significantly relative to the control (Fig.4.5). Conversely, the remaining extracts, LO (2.5 and 5 µg/mL), LA (2.5 µg/mL) and BA (5 µg/mL), significantly decreased insulin secretion ($p<0.05$). At the normoglycemic levels, all extracts produced significantly higher levels of insulin stimulation than the relevant controls. Treatment with the BO (2.5 µg/mL), LO (2.5µg/mL) and LA (5 µg/mL) had the highest stimulatory effect on the INS-1cells.

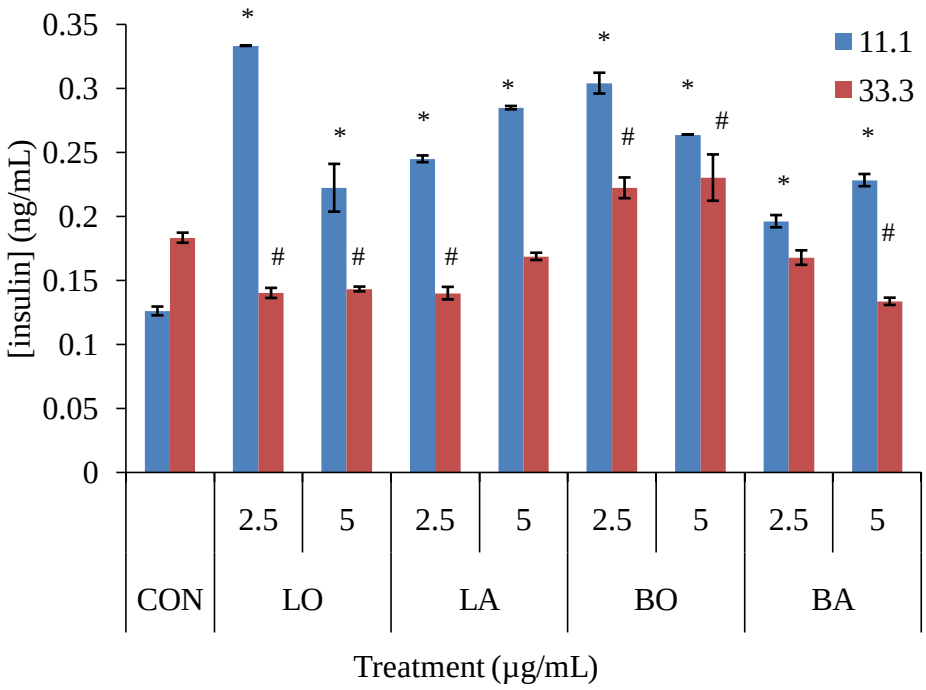

Figure 4. 5: Glucose-stimulated insulin secretion levels of INS-1cells exposed to *T. violacea* extracts after a 48 hour exposure in 11.1 mM and 33.3 mM glucose (n =4). $^{\#}p<0.05$ and * $p<0.01$ relative to the control (CON).

4.2.4. INSULIN CONTENT

At the hyperglycemic level, all the extracts significantly reduced the insulin content of the β-cells (Fig. 4.6). This reduction was found to be concentration dependent for all extracts except BA. At the normoglycemic level, all the extracts (apart from BA and LA, both at 5 µg/mL) significantly reduced the insulin content of the β-cell compared to the control.

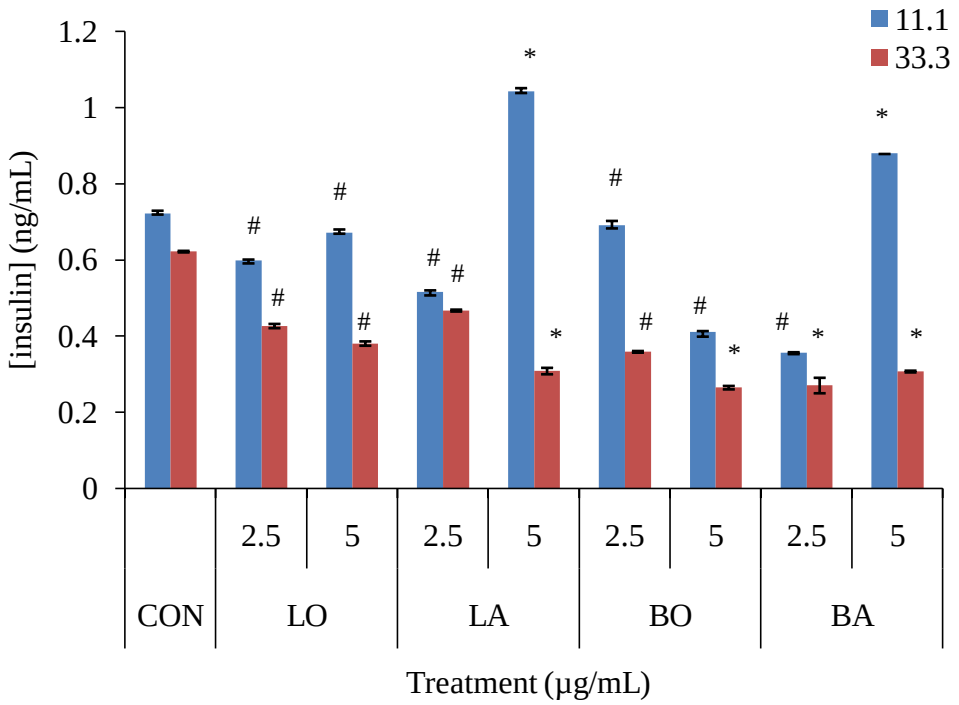

Figure 4. 6: The insulin content of INS-1 cells exposed to *T. violacea* extracts after 48 hrs in 11.1 mM and 33.3 mM glucose (n =4). #p<0.05 and * p<0.01 relative to the control (CON).

4.2.5. STIMULATORY INDEX

The stimulatory index is an indication of the INS-1 cell sensitivity to glucose stimulation (Fig. 4.7). At the normoglycemic level, only the LO (2.5 µg/mL) and the LA (5 µg/mL) (2-fold) stimulated insulin secretion significantly ($p<0.05$). The BO in a concentration dependent manner stimulated insulin secretion 2.6 fold, none of the other extracts showed the capacity to have a stimulatory effect. At the hyperglycemic level the BO (2.5 and 5 µg/mL) showed a 2 and 2.6 fold increase in insulin secretion, respectively. BA (2.5 and 5 µg/mL) at the hyperglycemic level displayed a significant effect on insulin secretion to the control ($p<0.01$). Both the controls show low insulin stimulation due to high glucose exposure. Generally the insulin secreted by the 33.3mM control stimulated cells should be similar or slightly lower than the insulin secreted by the control cells at the basal level as seen in Fig 4.4. These results indicate that the cells were leaky during the basal phase and secreted most of the insulin at this stage leaving only a negligible amount of insulin in the cells when content was measured.

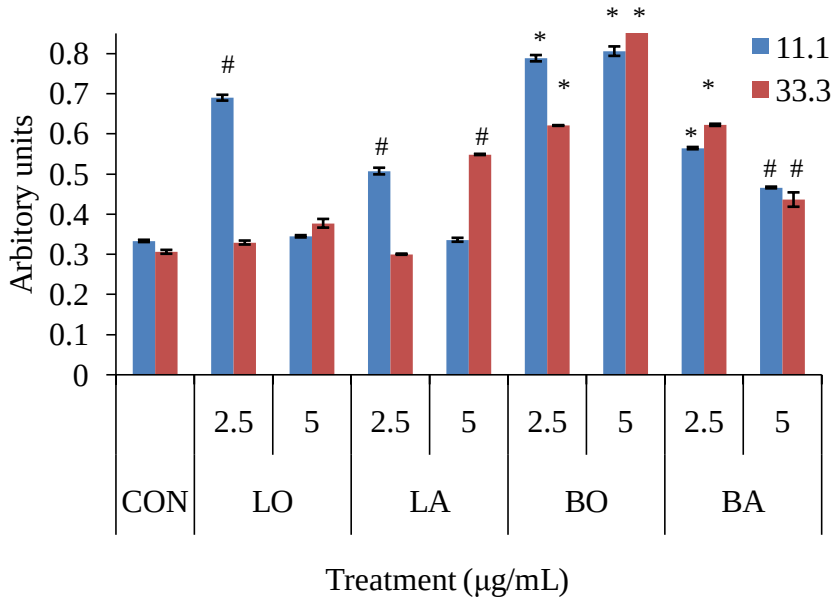

Figure 4. 7: Stimulatory index of INS-1 cells exposed to *T. violacea* extracts after 48 hrs in 11.1 mM and 33.3 mM glucose (n =4). $^{\#}p<0.05$ and * $p<0.01$ relative to the control (CON).

Based on the screening results of the coagulation (section 4.4) and GSIS (section 4.2) studies, the BO extract was selected for subsequent experiments to determine its secretagogue potential.

4.3. ANALYSIS OF INSULIN SECRETION BY BO EXTRACTS

4.3.1. CHRONIC INSULIN RELEASE

It was found that insulin secretion decreased significantly due to exposure to BO under both hyperglycemic and normoglycemic levels (Fig.4.8) relative to the control. This inhibition was evident at all concentrations apart from the highest concentration (10 µg/mL) at the hyperglycemic level which produced a significant increase in insulin secretion compared to the control. Further evaluation of the results suggests that at hyperglycemic levels, BO may have had an inhibitory effect on insulin secretion up to a concentration of 5 µg/mL, but stimulatory effect at higher concentrations.

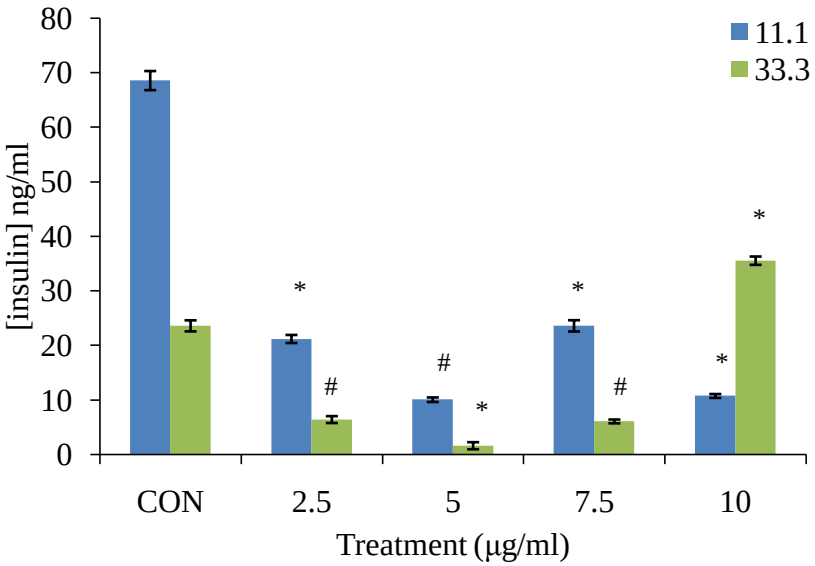

Figure 4. 8: Chronic insulin secretion levels of INS-1 cells exposed to BO extracts after 48 hrs in 11.1 mM and 33.3 mM glucose (n =4). #p<0.05 and * p<0.01 relative to the control (CON).

4.3.2. BASAL INSULIN SECRETION

All the extract concentrations significantly decreased insulin secretion at both the hyperglycemic and normoglycemic levels (Fig. 4.9). At the hyperglycemic level, a concentration dependent decrease in the release of insulin by the β-cells was observed from 2.5-7.5 µg/mL of the BO extract. A concentration dependent decrease was also observed at the normoglycemic level over the entire concentration range tested, although the decrease was not as pronounced as in the hyperglycemic conditions.

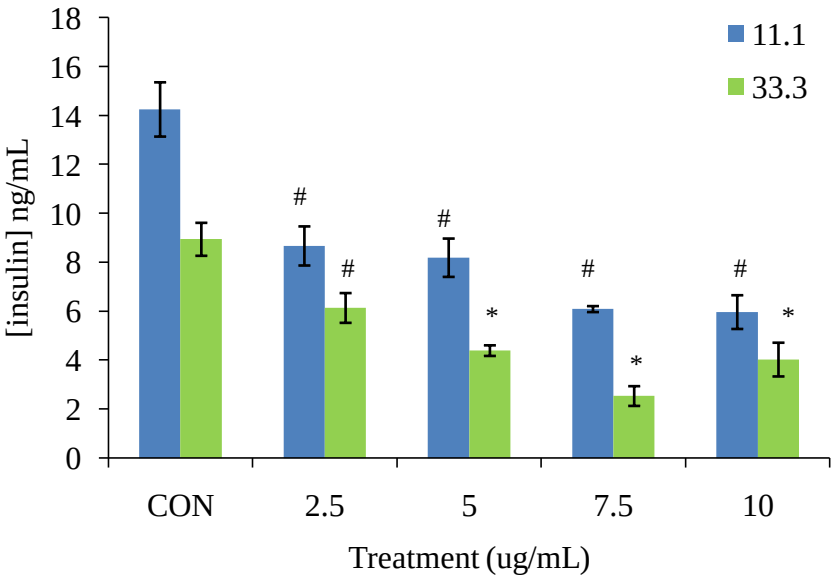

Figure 4. 9: Basal insulin secretion levels of INS-1cells exposed to BO extracts after 48 hrs in 11.1 and 33.3 mM glucose (n =4) #p<0.05 and * p<0.01 relative to the control (CON).

4.3.3. GLUCOSE-STIMULATED INSULIN SECRETION

At the hyperglycemic level the BO extract had no effect on insulin secretion relative to the control; however, at the normoglycemic level it produced a significant reduction in insulin secretion (Fig. 4.10). It can be observed that the stimulated levels are lower than the basal levels (Fig. 4.9), indicating that the cells were not responding to glucose.

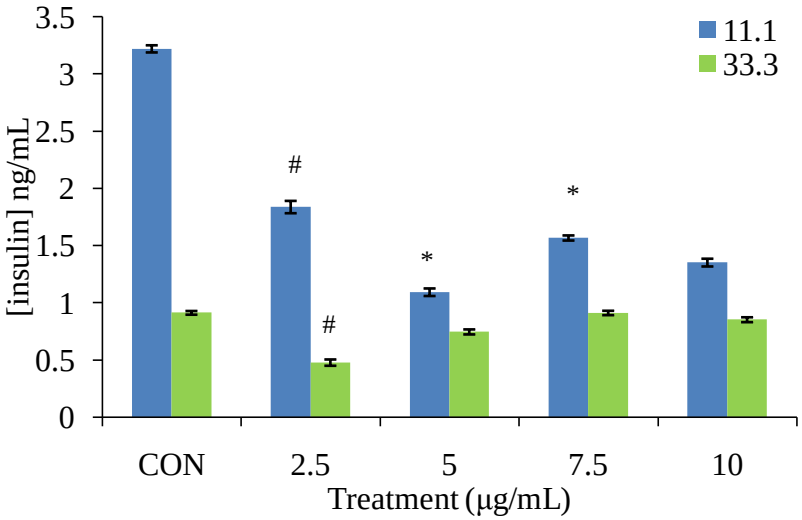

Figure 4. 10:Glucose-stimulated insulin secretion levels of INS-1 cells exposed to BO extracts after 48 hrs in 11.1 and 33.3 mM glucose. (n =4) #p<0.05 and * p<0.01 relative to the relevant control (CON).

4.3.4. INSULIN CONTENT

At the normoglycemic level, low concentrations (2.5 and 5µg/mL) of the BO extract increased the insulin content of the INS-1 cells, but at higher concentrations (7.5 and 10 ug/mL) the insulin content decreased (Fig. 4.11).

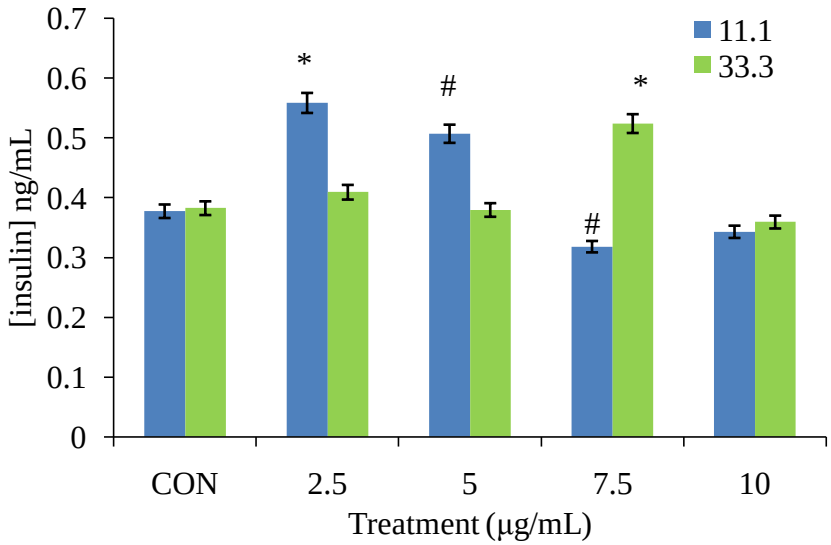

Figure 4. 11:The insulin content of INS-1 cells exposed to the BO extracts after 48 hour in 11.1 and 33.3 mM glucose.(n =4) #p<0.05 and * p<0.01 relative to the control (CON).

At the hyperglycemic level, the insulin content increased only after exposure to 7.5 mg/mL BO extract. Insulin content should be approximately 10 times the basal levels. The insulin content level (Fig. 4. 11) is very low relative to the basal level (Fig. 4.9), which is indicative of insulin leakage at the basal level.

4.3.5. STIMULATORY INDEX

The BO (7.5 and 10 µg/mL) extract displayed a significant 3- and 2-fold increase, respectively, in the stimulatory index compared to the controls for the hyperglycemic levels. However, in the normoglycemic level the extent of insulin secretion at 2.5 and 10 µg/mL BO was equivalent to untreated cells. An elevation in insulin secretion was noted for the 7.5 µg/mL BO at normoglycemic levels (Fig. 4.12).

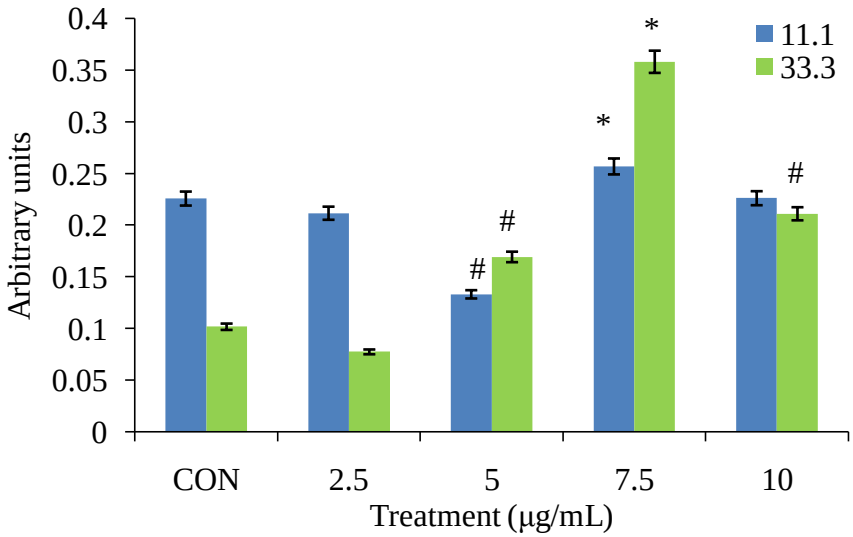

Figure 4. 12: The stimulatory index of INS-1 cells exposed to BO extracts after 48 hrs in 11.1 and 33.3 mM glucose (n =4). #p<0.05 and *p<0.01 relative to control CON.

4.4. GLUCOSE UPTAKE IN C2C12, 3T3-L1 AND CHANG LIVER CELLS

Cells were exposed for 48 hours to the BO extract to determine its effect on glucose utilisation in normal cells (Fig. 4.13). The extract significantly enhanced glucose utilisation in the Chang liver cells. The effect was observed to be concentration dependent, with the percentage glucose utilisation increasing from 122 to 163% as the concentration of the BO extract increased (2.5-10 µg/mL). The highest concentration of the BO extract (10 µg/mL) increased glucose uptake equivalent to that of metformin (1 µM). Metformin is known to improve glucose utilisation by increasing glucose uptake into hepatocytes; this is one of the known mechanisms of action of this drug (Mithieux *et al.*, 2006).

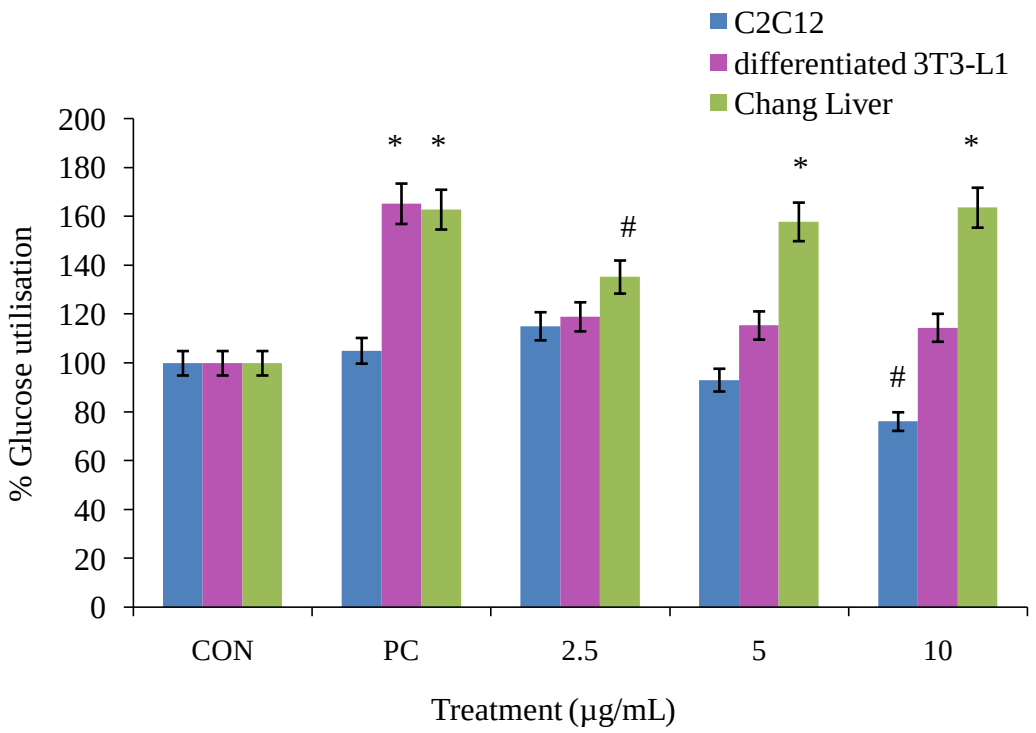

Figure 4.13: Effect of metformin (1µM) and BO extracts on glucose uptake in C2C12, 3T3-L1 and Chang liver cells (n=3) $^{\#}p<0.05$ and $*p<0.01$ relative to the relevant CON.

No significant effect on glucose utilisation was noted for the C2C12 and 3T3-L1 cells, with none of the concentrations promoting glucose utilisation higher than the metformin (PC). The PC in the differentiated adipocytes (3T3-L1) was effective at enhancing glucose utilisation; however this was not observed in the C2C12 cells, where an insignificant increase in glucose utilisation was noted. This is contrary to reports on the effect of metformin on skeletal muscles. In the C2C12 cells, the BO extract appeared to cause a suppression of glucose utilisation in a concentration dependent manner. No effect was observed for the 3T3-L1 cells, with uptake being higher than CON cells, but lower than the PC cells. The increase observed was not found to be stastically significant.

4.5. THE INFLUENCE OF *T. VIOLACEA* BULB ORGANIC EXTRACTS ON OXYGEN CONSUMPTION/RESPIRATION IN INS-1 CELLS

Based on the GSIS results, the 10 µg/mL BO extract concentration was selected to assay oxygen consumption, performed at normo- and hyperglycemic levels. Oxygen consumption studies were performed on INS-1 cells. A typical trace is shown in Fig 4.14.

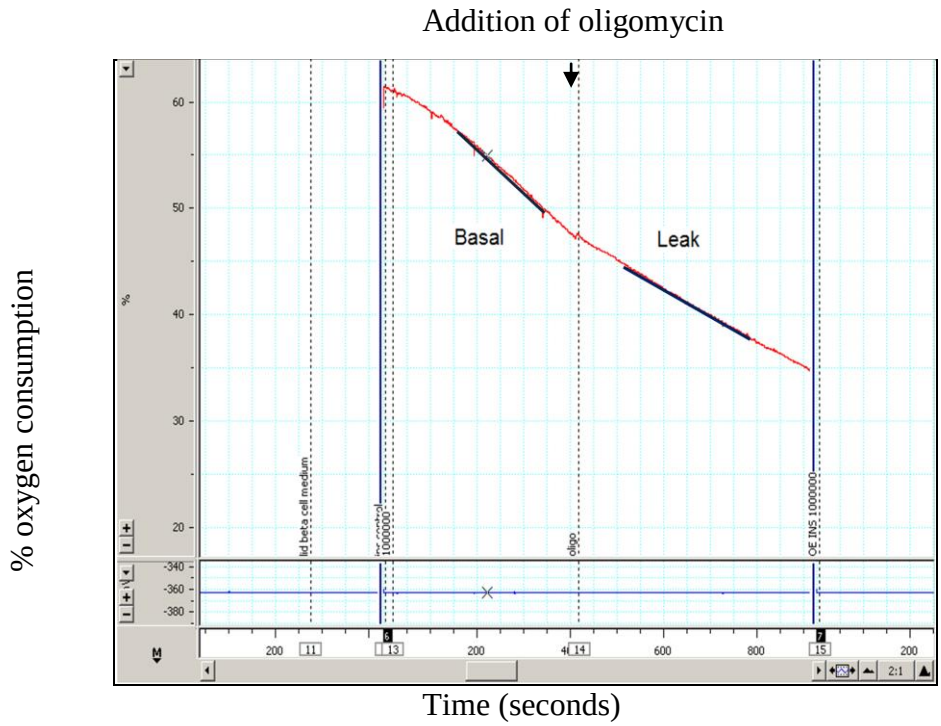

Figure 4. 14: Respiration trace of INS-1 cells before and after the addition of oligomycin. The slope of the trace indicates the respiration rate or oxygen consumed by the cells.

At the normoglycemic level, there was a significant (6-fold) increase in oxygen consumption relative to the control cells when exposed to the BO extracts (Fig.4.15). No significant difference between the control cells and the cells exposed to the extract was observed for oxygen consumption upon the addition of oligomycin. Cells exposed to the extract hyperglycemic conditions resulted, in a 4.5-fold increase in oxygen consumption relative to the control. In the hyperglycemic conditions, the addition of oligomycin to the INS-1 cells showed a decrease in oxygen consumption due to BO extract when compared to the control; however, this was not significant. A similar number of passages for the INS-1 cells was used for GSIS and as a result there could have been alterations in the cell lines. Oligomycin had no effect on respiration. As stated in section 4.3 the INS-1 cells appear to be leaky which could account for the ineffectiveness of oligomycin oxygen consumption resulting from the inhibition of ATPase. Despite these cells being leaky, it appears as if the extracts are exerting an effect on oxygen consumption.

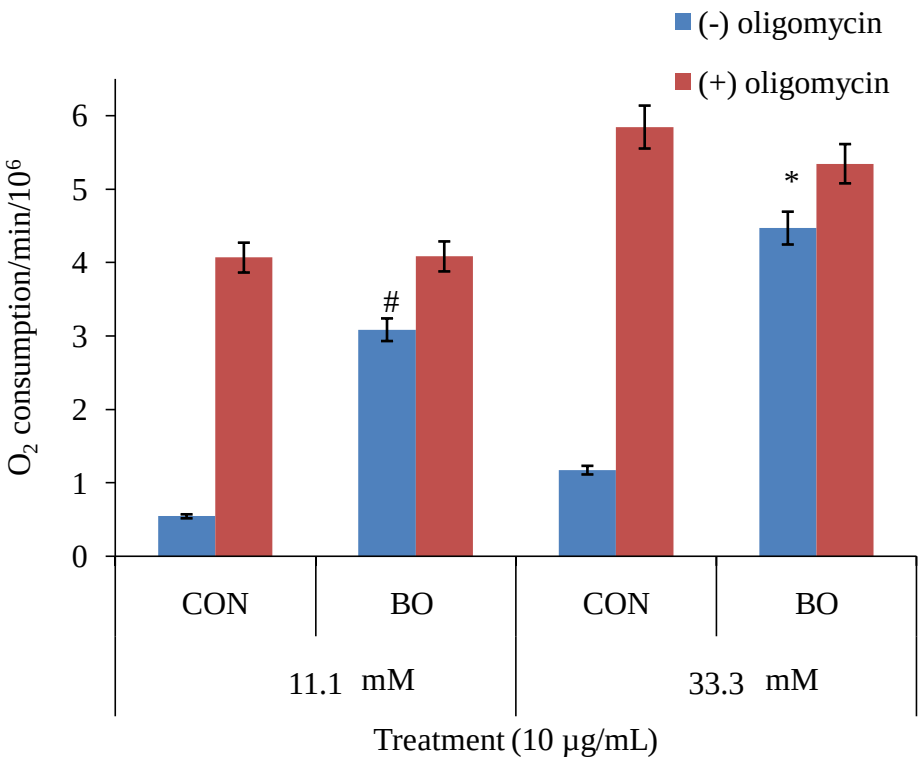

Figure 4. 15: Effect of the BO extract on oxygen consumption in the INS-1 cells at 11.1 and 33.3mM glucose (n=3). # p<0.05 and * p<0.01 relative to the relevant CON.

4.6. MITOCHONDRIAL MEMBRANE POTENTIAL IN INS-1 CELLS

Based on the stimulatory index results of GSIS the 10 µg/mL BO extract concentration was selected for mitochondrial membrane potential determination. This was performed at normo- and hyperglycemic levels. Changes to the mitochondrial membrane potential were detected via fluorescence intensity plots (Fig.4.16), as well as mean fluorescence values for the FL2 channel (Fig. 4.16).

The mean fluorescence allowed for the extent of mitochondrial polarisation to be measured and alludes to the assumed increase of insulin release. It was observed that at the normoglycemic level, there was a significant decrease in polarization of the mitochondrial membrane when cells were exposed to the extract, with a 1.8-fold decrease noted. However, the opposite was observed when cell were exposed to the extract at hyperglycemic levels, where a significant increase in polarization and a 1.6-fold increase was observed. An increase in insulin secretion can be linked to a possible increase in the ATP/ADP ratio.

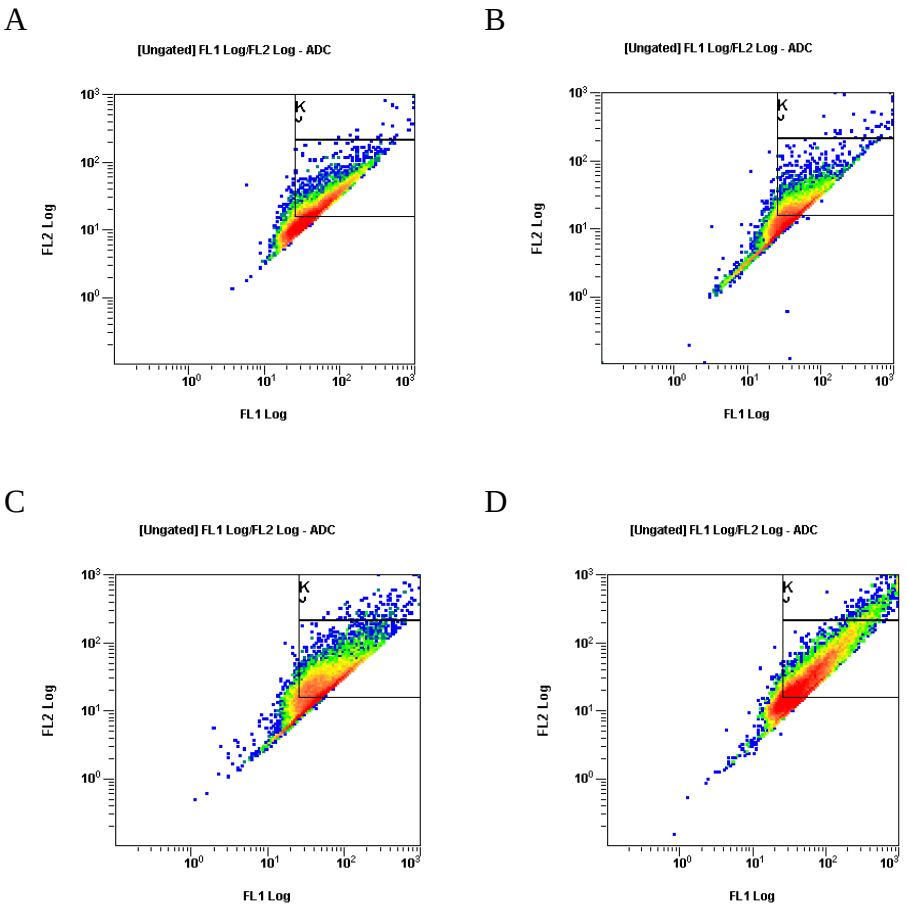

Figure 4. 16: Typical flow cytometry scatter plots of the INS-1 cells (A) untreated normoglycemic INS-1(CON) (B) BO treated (10 µg/mL) at 11.1 mM glucose concentration (C) untreated hyperglycemic INS-1(CON) (D) BO treated (10 µg/mL) at 33.3 mM glucose concentration.

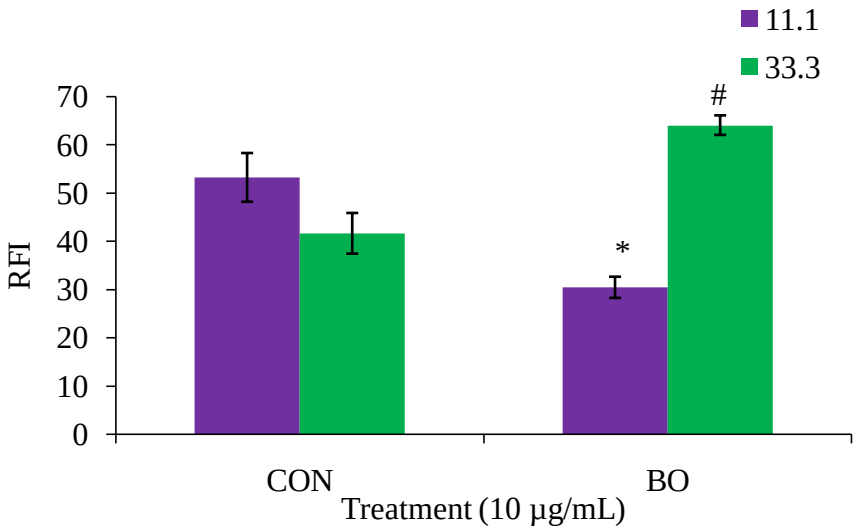

Figure 4. 17: Effect of BO (10 µg/mL) on the mitochondrial membrane potential of INS-1 cells at 11.1 and 33.3mM glucose (n=3). * p<0.01 and # p <0.05 relative to the relevant CON.

4.7. WESTERN BLOT ANALYSIS

4.7.1. SDS-PAGE GEL ANALYSIS

Band separation, as shown in Fig. 4.18, showed protein bands of 42 kDa and 57 kDa corresponding to actin and Glut-2, respectively.

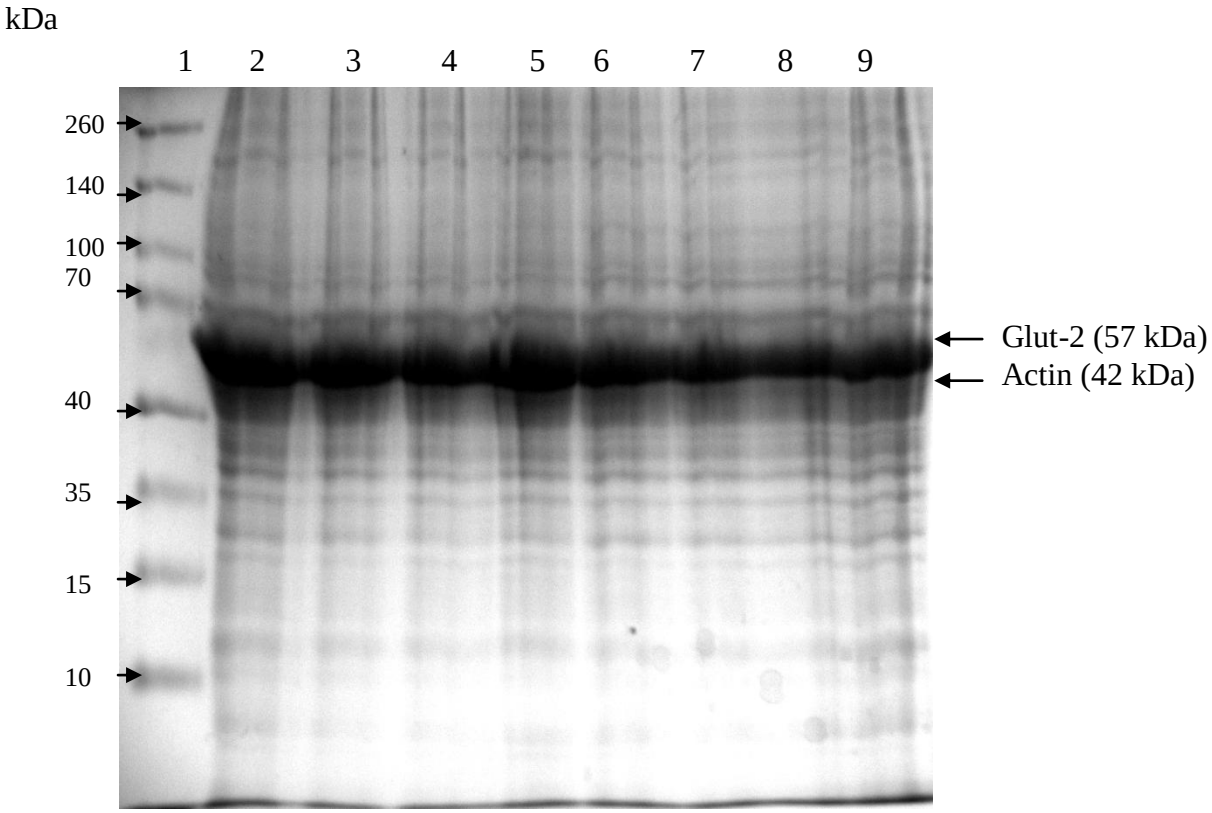

Figure 4. 18: SDS-PAGE (10%) analysis of INS-1 cell lysates (50µg) exposed to BO (10 µg/mL) extract in either 11.1 or 33.3 mM glucose concentrations for 48 hrs (1)Prestained broad range (260-10 kDa) protein ladder (Fermentas), (lanes:2 and 3) CON in 11.1 mM glucose (lanes:4 and 5) 10 ug/mL BO extract in 11.1 mM glucose, (lanes:6 and 7) CON in 33.3 mM glucose and (lanes:8 and 9) 10 ug/mL BO extract in 33.3 mM glucose.

4.7.2. ACTIN EXPRESSION

Actin was used as a control. However, using densitometry it was found that at the hyperglycemic level (lane 4 and 5) actin expression was enhanced, showing a 2-fold increase relative to the expression in cells at normoglycemic level (lane 2) (Fig. 4.19). Actin levels within the respective glycemic conditions were constant between the extract and the control at the relevant level. This implies that the extract did not enhance actin expression in the cells, however glucose enhanced actin expression.

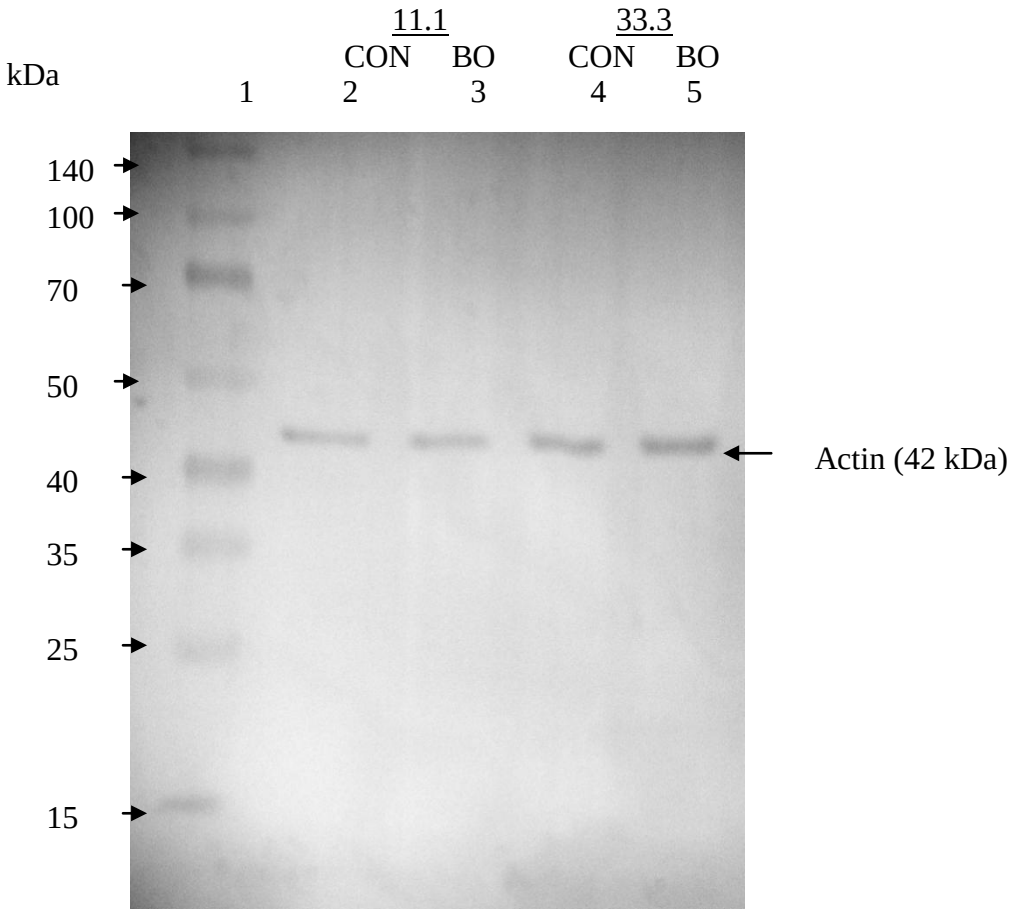

Figure 4. 19: A typical set of Western blot results of the INS-1 cell exposed to BO (10 µg/mL) extract in either 33.3 mM glucose or 11.1 mM glucose concentrations for 48 hrs for a loading control. Actin was detected using an actin rabbit polyclonal antibody. The bands observed correspond to a molecular weight of approximately 42 kDa. Lanes: (1) Prestained broad range (260-10 kDa) protein ladder (Fermentas), (2) control and (3) 10 ug/mL BO extract in 11.1 mM glucose, (4) control and (5) 10 ug/mL BO extract in 33.3 mM glucose.

4.7.3. ANALYSIS OF GLUT-2 EXPRESSION IN INS-1 CELLS

The BO (10 µg/mL) extract reduced Glut-2 expression by 2-fold at normoglycemic conditions (lane 3) compared to the CON (lane 2). At the hyperglycemic conditions, the BO (10 µg/mL) (lane 5) extract effected a 1.4 fold increase in Glut-2 expression relative to the CON (lanes 4) based on densitometric analysis. The increase in the Glut-2 transporter expression can be related to chronic insulin secretion (Fig. 4.8), where the BO extract increased insulin secretion at the hyperglycemic level using 10 µg/mL.

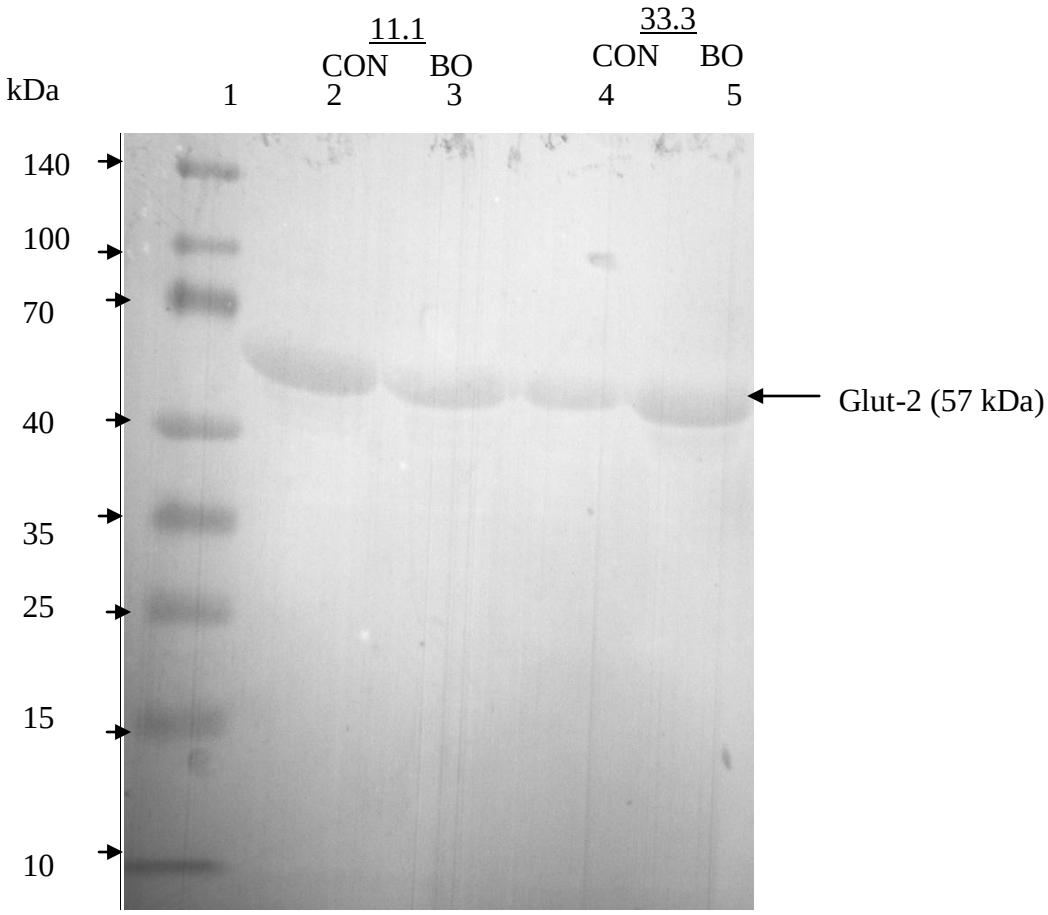

Figure 4. 20: Typical set of Western blot results of the INS-1 cell exposed to BO (10 µg/mL) extract in either 11.1 mM glucose or 33.3 mM glucose concentration for 48 hrs. Glut-2 polyclonal antibody was used to detect the Glut-2 transporter. The bands observed correspond to a molecular weight of approximately 57 kDa. Lanes: (1) Broad range (260-10 kDa) protein ladder (Fermentas), (2) CON in 11.1 mM glucose, (3) 10 ug/mL BO extract in 11.1 mM glucose, (4) CON in 33.3 mM glucose and (5) 10 ug/mL BO extract in 33.3 mM glucose.

SECTION B: COAGULATION STUDIES

4.8. SCREENING OF *TULBAGHIA VIOLACEA* EXTRACTS

Extracts were tested at a range of concentrations to determine the effect of *T. violacea* on the PT (Fig. 4.21) and APTT (Fig 4.22). No significant effect on the clotting times was detected at any of the tested concentrations.

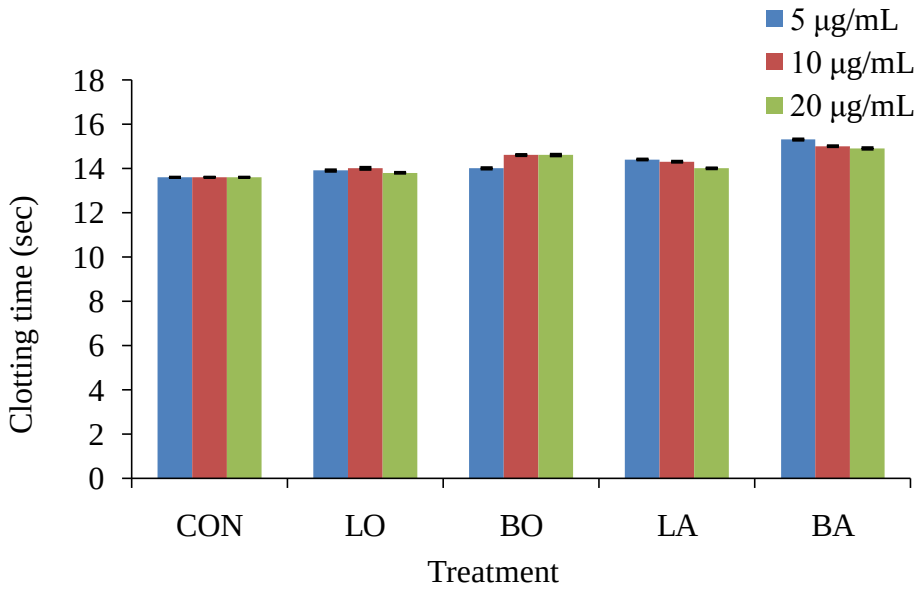

Figure 4. 21: Effect of *T. violacea* extracts on the PT *in vitro* model (n=5).

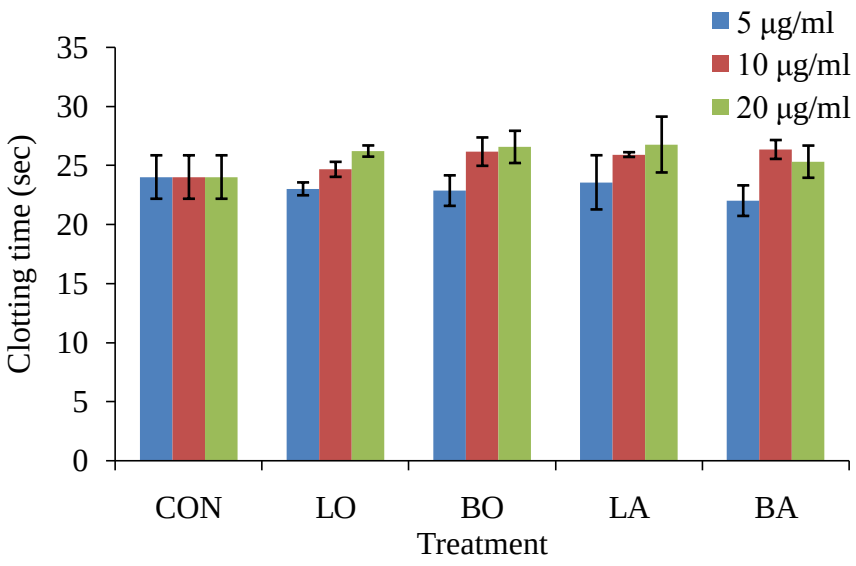

Figure 4. 22: Effect of *T. violacea* extracts on the APTT *in vitro* (n=5).

The D-Dimer (Fig 4.23) and Fibrinogen-C tests (Fig. 4.24) were performed using the *Tulbaghia violacea* extracts. At 20 and 10 µg/mL of the BO extract (Fig. 4.23), there was an increase in the D-Dimer released relative to the control. BO extract at 20

µg/mL produced a 1.4-fold increase in D-Dimer concentration. A similar result was obtained for Fibrinogen-C, with 20 µg/mL BO extract producing a 1.25-fold in Fibrinogen-C concentration.

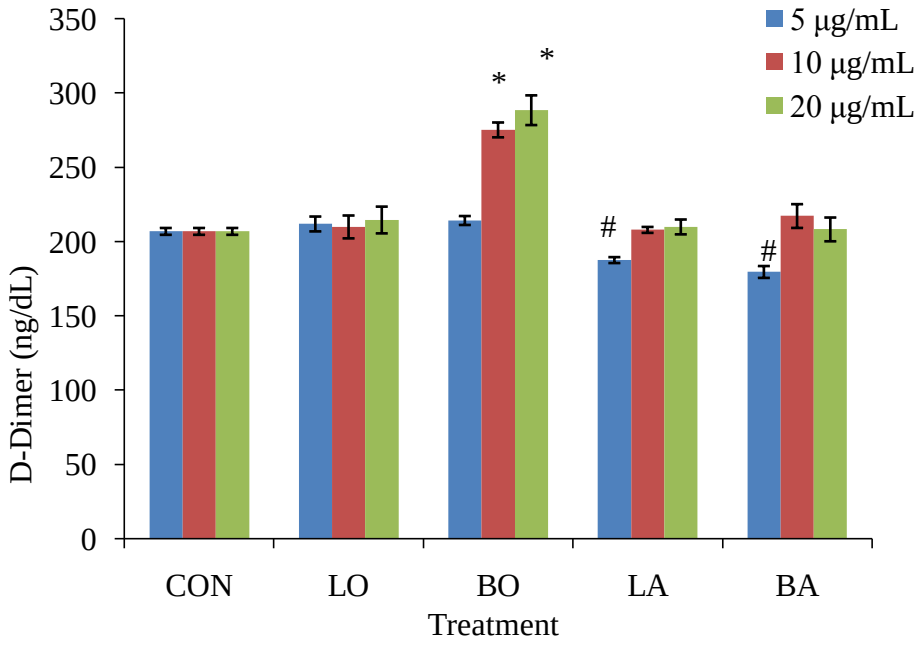

Figure 4. 23: Effect of *T. violacea* extracts on D-Dimer formation *in vitro* (n=5). * p<0.01 and #p<0.05 relative to the CON

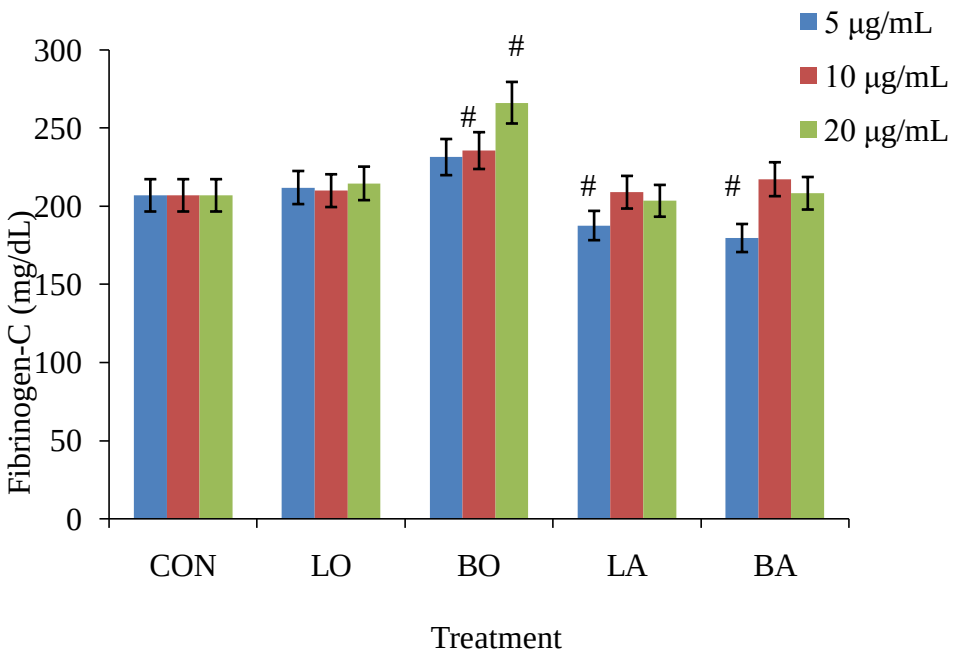

Figure 4. 24: Effect of *T. violacea* extracts on Fibrinogen-C formation in *vitro* (n=5). #p<0.05 and * p<0.01 relative to the CON

A significant decrease (p<0.05) in D-Dimer and Fibrinogen-C was observed in the presence of the aqueous extracts (LA and BA) at the lowest concentration (5 µg/mL).

A 0.8-fold decrease was noted for both the D-Dimer and Fibrinogen-C assays, respectively.

The effect of the extracts on the properties of platelets was monitored, subsequent to activation with thrombin, to determine possible anti-aggregation and anti-adhesion properties (section 3.12.1.2).Most of the extracts (1 mg/mL) were able to inhibit platelet aggregation and adhesion (table 4.1). The BO extract displayed the highest inhibition of aggregation and adhesion (Fig. 4.25 and 4.26, respectively), however, it should be noted that these tests were subjective. The microscopic images provided a cheap, convenient and qualitative method of assessing the effects of the extracts on platelet adhesion and aggregation. Fig. 4.25 and 4.26, show typical results that were observed. The results for the LO, RO, BA, LA and RA are summarised in tables 4.1 and 4.2 using the scale as indicated below each table.

Table 4. 1: Thrombin-induced platelet aggregation of various *T. violacea* extracts

Concentration (mg/mL)	Treatment					
	BO	LO	RO	BA	LA	RA
1	++++	+++	++	+++	++	++
0.5	+++	++	++	+	+	++
0.25	++	+	+	+	+	+

++++70-89% inhibition, +++ 50-69% inhibition, + + 20-49% inhibition, + 0-19% inhibition

Table 4. 2: Platelet adhesion inhibition of various *T. violacea* extracts

Concentration (mg/mL)	Treatment					
	BO	LO	RO	BA	LA	RA
1	++++	++++	+++	++	+	+++
0.5	++++	+++	+++	+ +	+	++
0.25	++++	++	+	+	+	+

++++70-89% inhibition, +++ 50-69% inhibition, + + 20-49% inhibition, + 0-19% inhibition

Examples of the microscopic images of platelet aggregation as a result of exposure to a concentration range of the BO extracts are given in Fig. 4.25. With an increase in concentration of the BO extract (0.25-1 mg/mL), there was a decrease in the ability of the platelets to aggregate. BO extract (1 mg/mL) inhibited platelet aggregation equivalent to that of the non-aggregated platelets or non-thrombin induced platelets, where platelets were observed to be floating (Fig. 4.25 c).

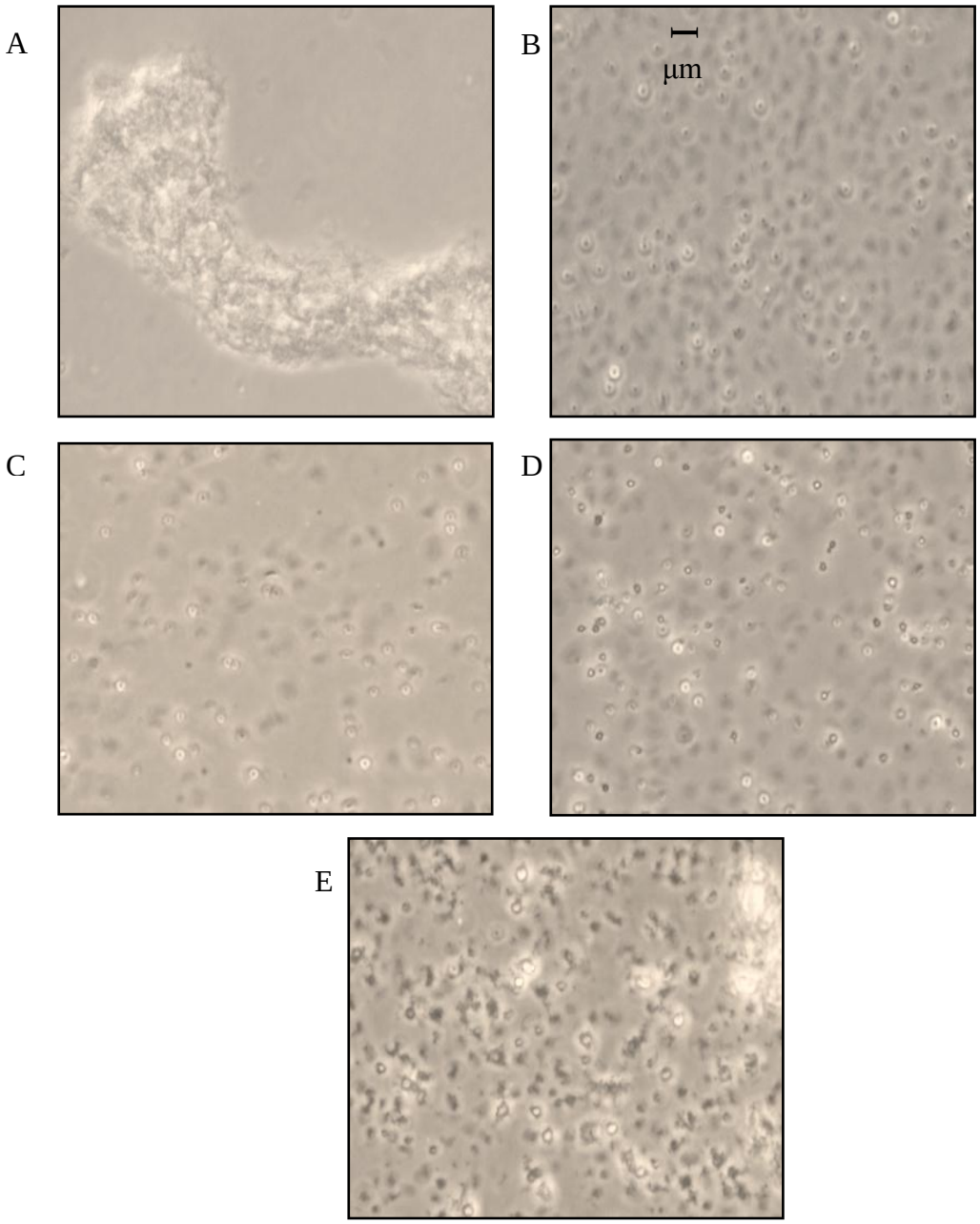

Figure 4. 25: Example of aggregated platelets, activated with thrombin (0.25 U/ml) evaluated microscopically. (A) Positive control, activated platelets in the absence of any *T.violacea* extracts, acting a control of platelet aggregation (PC); (B) Control, non-aggregated platelets (C), Activated platelets in the presence of 1 mg/mL of BO extract; (D), 0.5 mg/mL BO extract and (E), 0.25 mg/mL BO extract. 400X magnification.

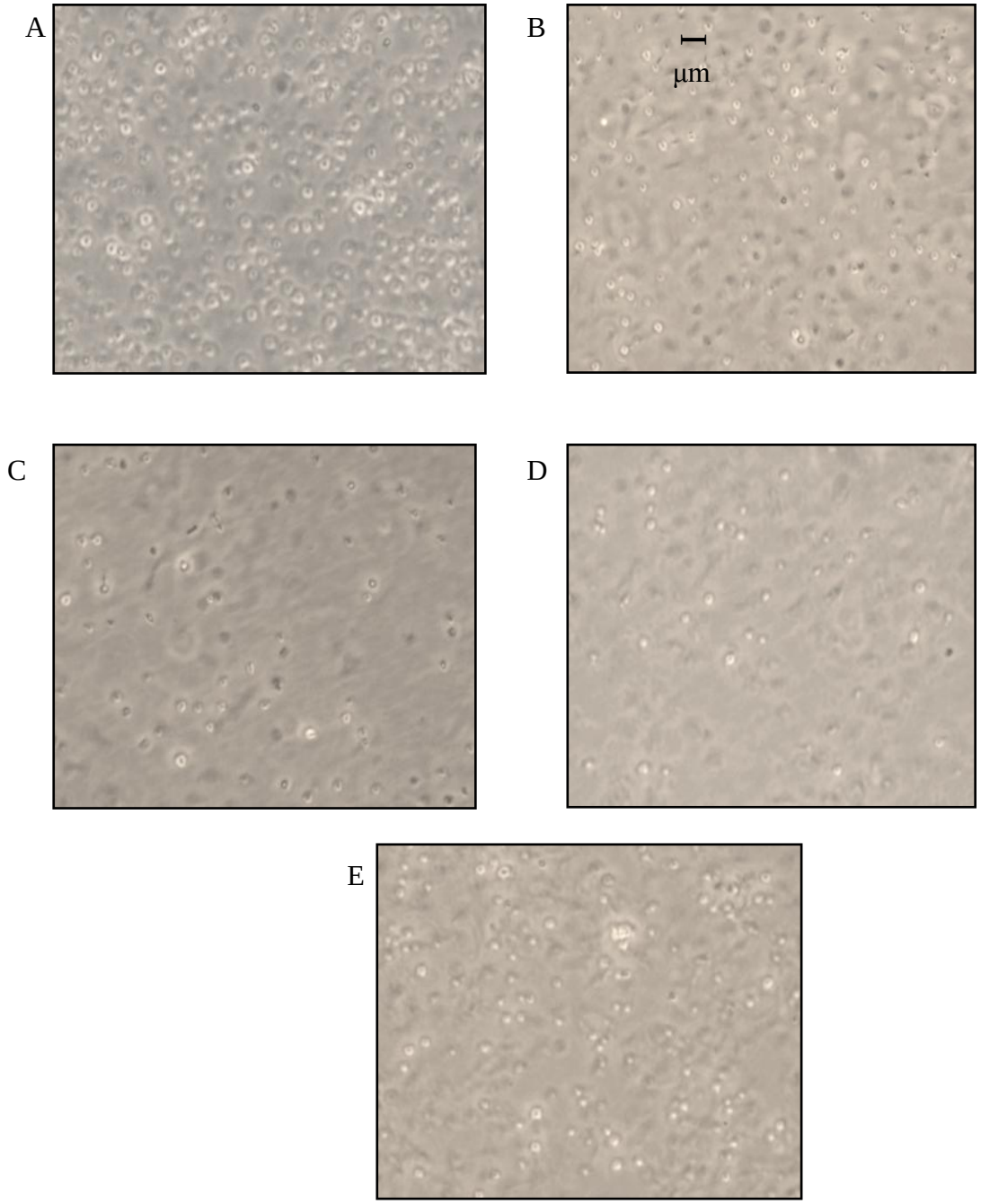

Figure 4. 26: An example of adherent platelets, activated with thrombin (0.25 U/ml) evaluated microscopically. (A) Positive control, activated platelets in the absence of any *T. violacea* extracts, acting as control of platelet aggregation (PC); (B) Control, non-aggregated platelets (C), Activated platelets in the presence of 1 mg/mL of BO extract; (D), 0.5 mg/mL BO extract and (E), 0.25 mg/mL BO extract. 400X magnification.

From the aggregation and adhesion studies, it was noted that the BO extract displayed the highest anti-platelet activity.

Protein secretion results obtained (Fig. 4.27) for the different treatments on the platelets, indicate that most of the extracts (aqueous and organic), at the various concentrations significantly inhibited the protein secretion relative to the control. The organic extracts showed a concentration dependent increase in inhibiting protein secretion with the BO extract (35 to 53%) having superior inhibition ability, compared to the RO extracts (31 to 39%) and LO extracts (38 to 43 %). The BO extracts inhibited protein secretion by 53% (1 mg/mL) supporting the results observed for platelet adhesion and aggregation (Fig. 4.25 and 4.26). The aqueous extracts also displayed protein inhibition ability, with the RA (37 to 45%) having the highest concentration dependent inhibition of protein secretion compared to the LA extracts (39%) and BA extracts (36%).

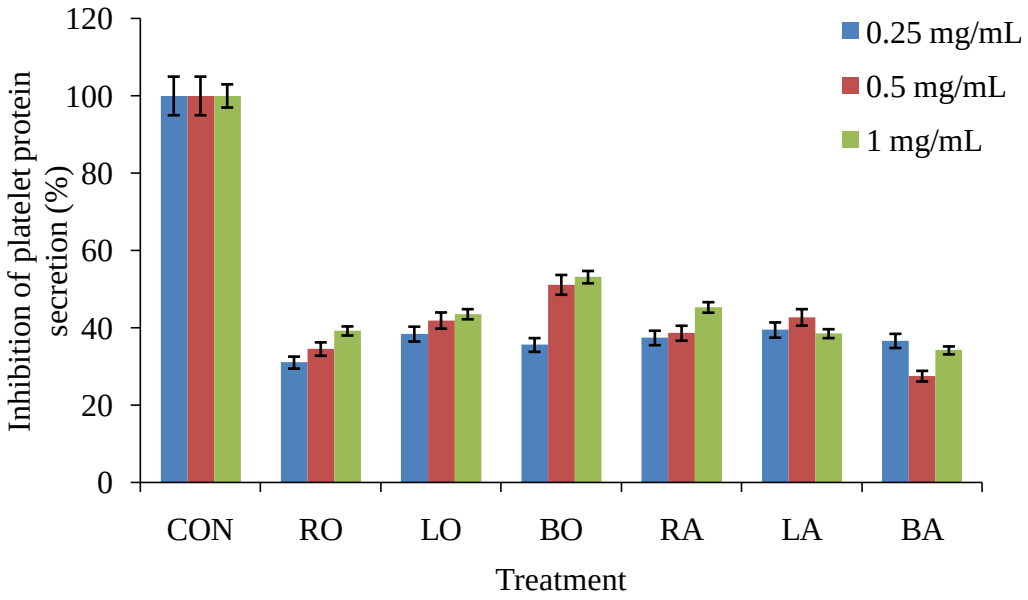

Figure 4. 27: Effects of *T. violacea* extracts, at different concentrations, on the release of protein secretion from the thrombin-activated platelets (n=3). All values were found to be are statistically different from the control (CON).

The BO extracts displayed concentration dependent results for the coagulation tests, D-Dimer and Fibrinogen-C. Concentration dependent results for the BO extract were also seen for platelet aggregation, adhesion and protein secretion inhibition. Based on these screening results, the BO extract was selected for further testing in an *ex vivo* model.

4.9. *EX VIVO* RAT MODEL USING BULB ORGANIC *TULBAGHIA VIOLACEA* EXTRACTS

When a platelet is activated, a conformational change occurs on the platelet surface membrane receptor, GIIb/IIa. A fibrinogen binding site is activated and this

conformational change can be detected by the monoclonal antibody (FITC-PAC-1) probe to determine aggregation and can be visualised as histograms (Fig. 4.28).

In the *ex vivo* rat model, it was observed that the BO extracts decreased thrombin-induced platelet aggregation significantly by 50%. Aspirin inhibited platelet aggregation by 30%. It is a known platelet aggregation inhibitor at the concentration tested. These results indicate that the BO extract is 1.67-fold more efficient as a platelet aggregation inhibitor than aspirin (Fig.4.29).

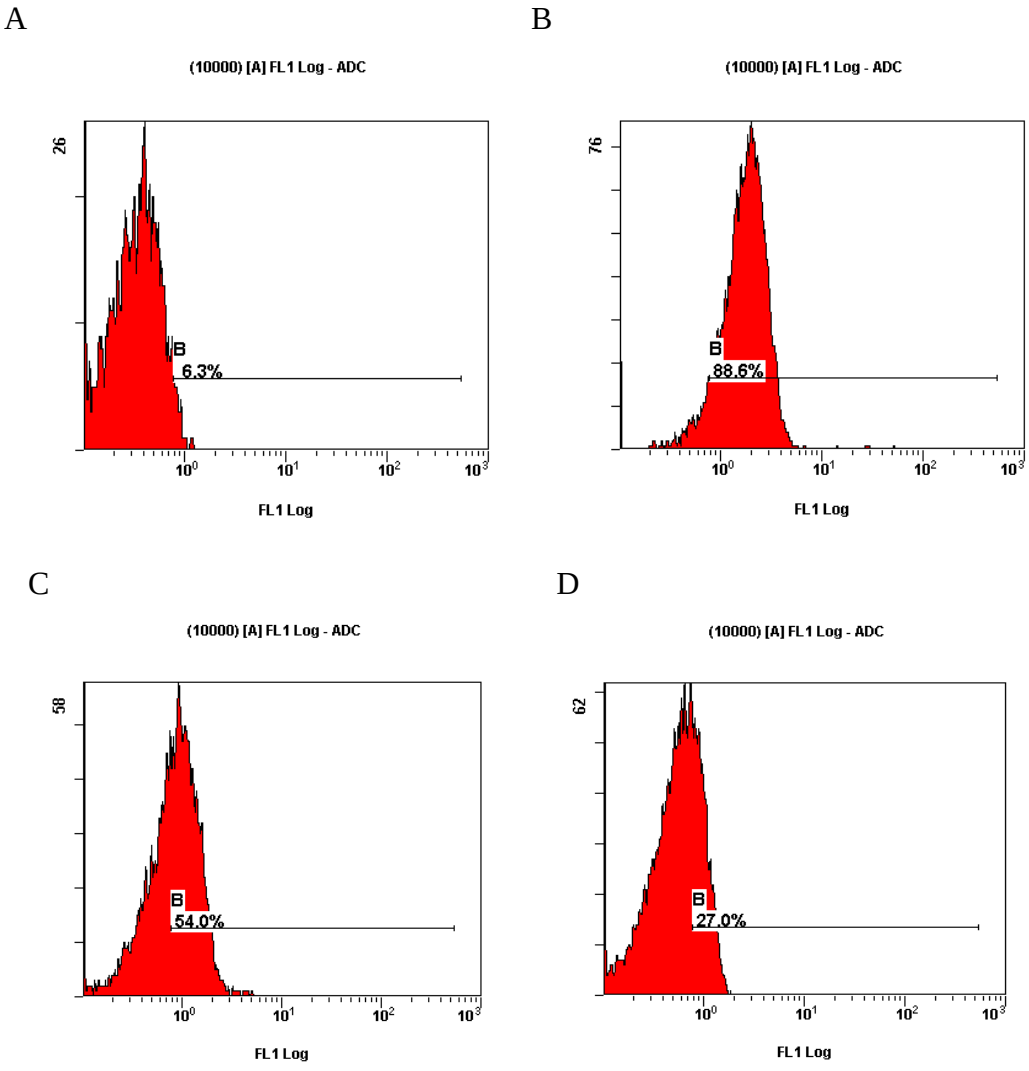

Figure 4. 28: An example of one of the replicates of the flow cytometry histograms of rat platelet aggregation, and the percentage of aggregated platelets (A) Untreated control where no platelet aggregation was initiated (B) activated platelets obtained from untreated rats (C) platelets from aspirin-treated rats (PC) activated with thrombin (D) platelets from BO (50 mg/kg) treated rats activated with thrombin.

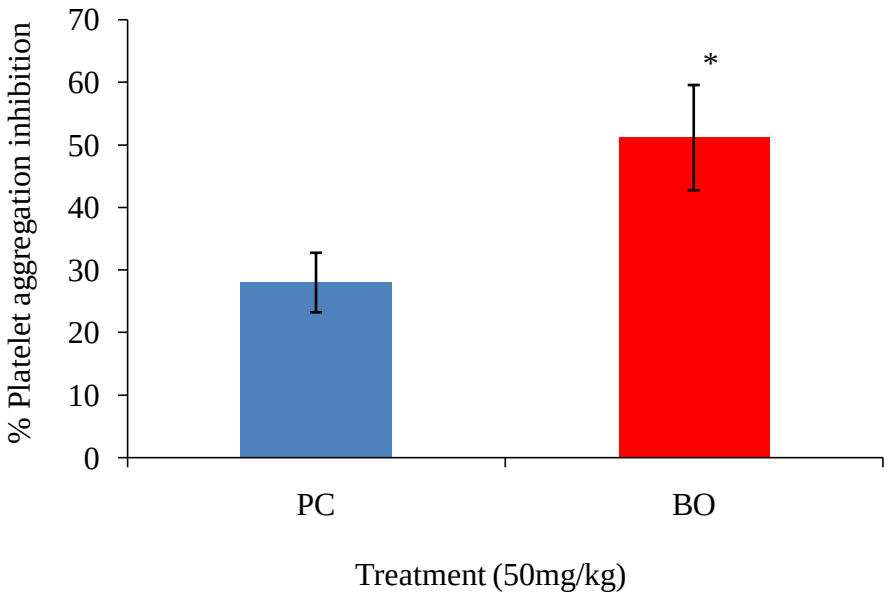

Figure 4. 29: Effect of the BO extracts on platelet aggregation in an *ex vivo* rat model (n=5) *p<0.05 relative to the PC.

The *ex vivo* model was used to determine the effects of the BO extract on APTT, PT and fibrinolysis (D-dimer, and Fibrinogen-C). Based on the short comings of the *in vitro* results, which could account for the cell-based coagulation model, the *ex vivo* model substantiated the *in vitro* coagulation results.

It was observed that BO decreased the PT clotting time 2-fold relative to both the PC and CON (Fig 4.30). The result of aspirin (PC) was expected as it acts on the platelet surface and not on the clotting factors. In the PT test the recomboplastin mimics the TF-bearing cells and the phospholipids in APTT mimics the platelets, thus the APTT reflects the processes occurring on platelet surfaces.

In contrast, the BO extract produced a significant 1.5-fold increase in APTT clotting time relative to CON and a 1.2-fold increase relative to the PC (Fig. 4.31). These results account for the platelet interaction and are consistent with those observed in the flow cytometry platelet aggregation results (Fig. 4.29).

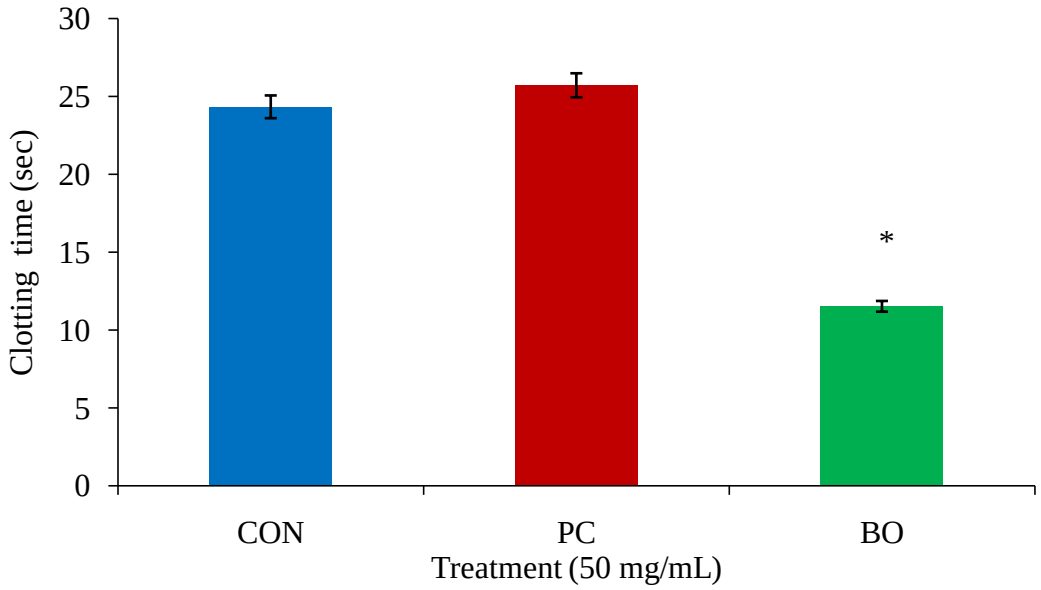

Figure 4. 30: Effect of BO extracts on the clotting time of the PT test in an *ex vivo* model (n=6) *p<0.01 relative to the CON

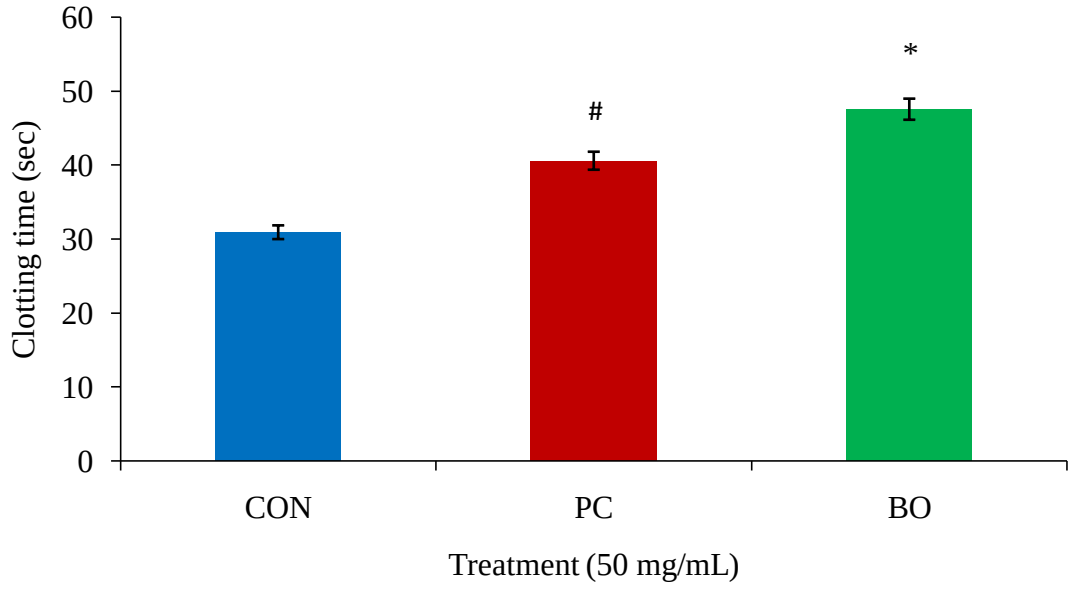

Figure 4. 31: Effect of BO extracts on the clotting time of the APTT test in an *ex vivo* model (n=6) # p<0.05 and *p<0.01 relative to the CON.

The D-Dimer and Fibrinogen-C tests provide an indication of the effect by the BO extracts on fibrinolysis in the *ex vivo* model (Fig. 4.32 and 4.33). It was observed that the BO extract produced a significant 2.6-fold and 3-fold decrease in Fibrinogen-C relative to the CON and PC, respectively. No effect was noted for the BO extract on the D-Dimer assay, which could be attributed to the low Fibrinogen-C levels; alternatively, it may be due to a lack of sensitivity in detection by the kit used.

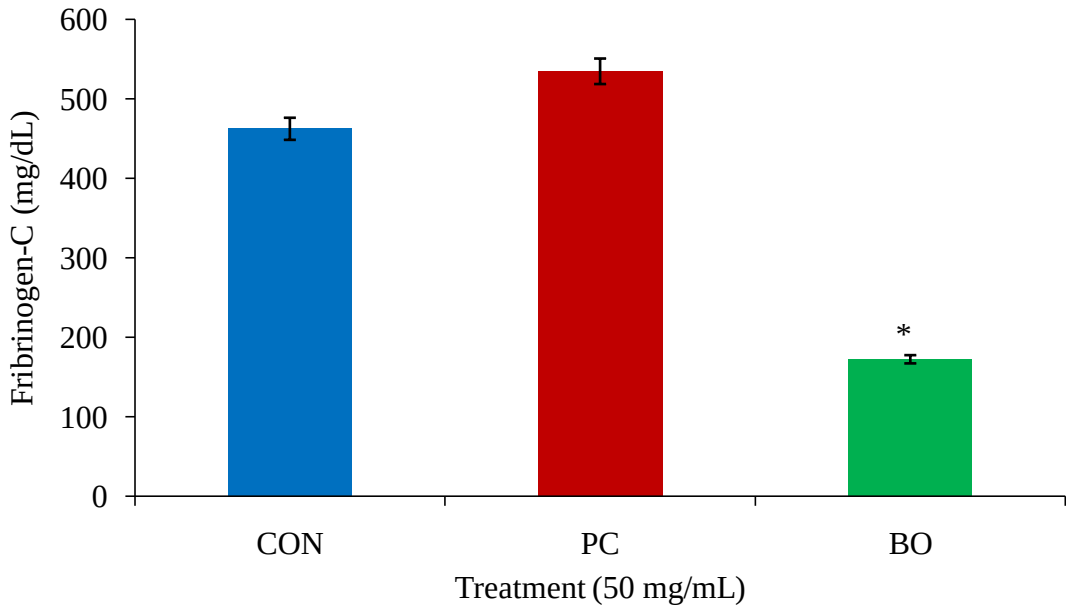

Figure 4. 32: Effect of aspirin (PC) and extract BO on the clotting time of the Fibrinogen-C test in an *ex vivo* rat model (n=6) # p<0.05 and *p<0.01 relative to the CON.

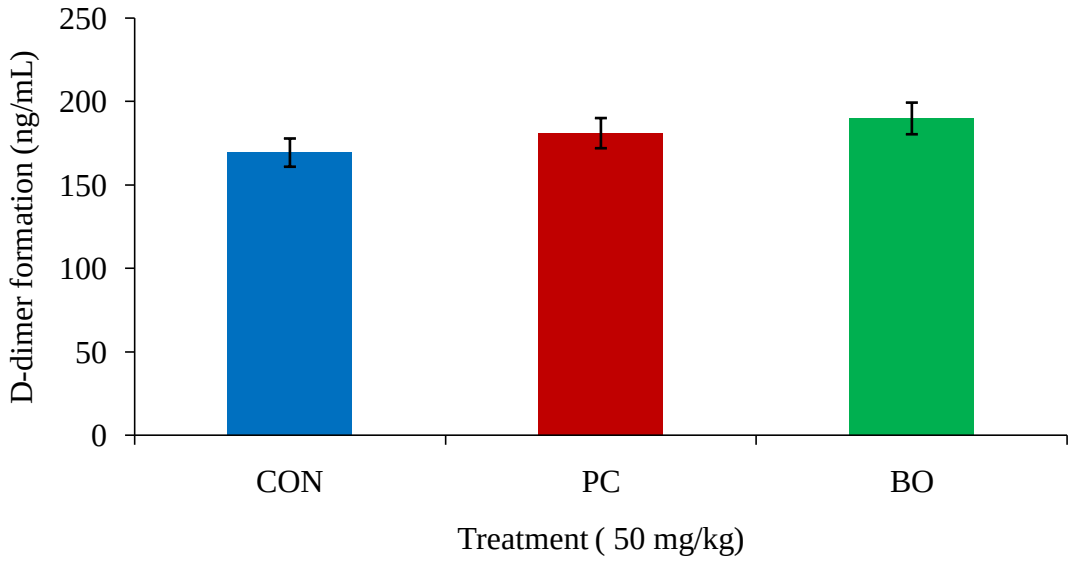

Figure 4. 33: Effect of aspirin (PC) and extracts (BO) on D-Dimer formation in an *ex vivo* rat model (n=6).

CHAPTER 5: DISCUSSION

SECTION A: ANTIDIABETIC STUDIES

Conventional antidiabetic agents can affect several pathways of glucose metabolism, such as insulin secretion, glucose uptake by targeting organs as well as nutrient absorption. Insulin is a key hormone involved in blood glucose homeostasis and its deficiency contributes to the pathogenesis of DM. The biochemical mechanisms underlying glucose-stimulated insulin secretion from pancreatic β-cells are not completely understood; however this study has shown a link between insulin secretion, membrane potential and oxygen consumption in INS-1 cells.

β-Cells are designed to sense blood glucose and other secretagogues and adjust insulin secretion according to the needs of the organism. Rather than activating specific receptor molecules, glucose is metabolized to generate downstream signals that stimulate insulin secretion. Glucose enters β-cells by facilitated diffusion through the glucose transporter (GLUT-2 in rodents; mainly GLUT-1 in humans) and is retained inside the cell through its phosphorylation by glucokinase. This step is essential in determining the rate of glycolysis and pyruvate generation. ATP synthesized in the mitochondrial matrix is transported to the cytosol in exchange for cytosolic ADP via adenine nucleotide translocators. The resulting increase in the cytosolic ATP/ADP ratio inhibits the opening of the plasma membrane K_{ATP} channel. Closure of K_{ATP} channels depolarizes the plasma membrane, triggering the opening of voltage-dependent Ca^{2+} channels. As in other exocytotic fusion events, Ca^{2+} (cytosolic) is an essential trigger of insulin granule exocytosis and the amount of insulin released. The Ca^{2+}-signal resulting from mitochondrial activation completes the chain of events between glucose uptake into the β-cells and insulin exocytosis (Wiederkehr and Wollheim, 2006).

New opportunities have arisen in the study of the mechanisms of insulin secretion and β-cell dysfunction due to the advancements made in cultured cell lines. These cell lines have led to the establishment of more appropriate methods in the study of these mechanisms, which are often the target of studies involving natural products. The most widely used insulin-secreting cell lines are RIN, HIT, beta-TC, MIN6 and INS-1 cells (Poitout *et al*., 1996). These cell lines release insulin and small amounts of

glucagon and somatostatin. None of these cell lines mimic primary β-cell physiology perfectly they are however, extremely valuable tools for the study of molecular events underlying β-cell function (Poitout e*t al*., 1996 and Affourtit and Brand, 2008).

A number of antidiabetic studies have been conducted on garlic, onion and coriander, which are major members of the *Liliaceae* family. This suggests that the mechanisms and properties discovered in one member are possibly consistent with other members of the family. *T. violacea*, another member of the *Liliaceae* family, is the focus of this study.

It has been proposed by Benavides *et al*. (2007) that insulin secretion is enhanced as a result of allicin, the possible active ingredient in garlic. It is believed that it competes with insulin for hepatic insulin-activating sites leading to enhanced insulin levels. Another proposed mechanism of insulin secretion is that garlic can effectively combine with compounds like cysteine and enhance serum insulin (Mathew and Augusti, 1973). Jain and Vyas (1975) proposed that garlic can act as an antidiabetic agent by increasing either the pancreatic secretion of insulin from the β-cells or its release from bound insulin. Kumari and Augusti (2002) reported that S-methylcysteine sulfoxide, isolated from onion, had antihyperglycemic and antioxidant effects. The probable mechanism of action of the compounds may be partly due to the stimulation of insulin secretion.

In this study INS-1 cells were used to screen the different effects of *Tulbaghia violacea* extracts on insulin secreting ability. It was found that the BO extracts stimulated insulin secretion in a concentration dependent manner.

During the study, the cell laboratory experienced several episodes of contamination. This reduced the primary stocks which lead to an unavoidable increase in the passage number of the cells. The INS-1 cells were affected by the high passage number used. New cells were ordered from the ATCC, unfortunately these were not delivered by the time this study was completed. The INS-1 cells appeared leaky as suggested by the high basal insulin levels found under normoglycemic control conditions. This further impacted on the stimulatory effect being impaired, leaving the cells with very low insulin content. The trends obtained from these experiments showed that the BO

extracts increased insulin secretion at both the normoglycemic and hyperglycemic levels. However, once a lower passage number of cells are received it is advisable that these experiments are repeated to obtain a clearer understanding of the effects of the extracts on INS-1 cells.

Glucose is a primary source of energy that is utilised by mammalian cells. It is tightly regulated in the blood and has a narrow range of 5-6 mM in humans. The peripheral tissues, such as skeletal muscle (myoblasts or C2C12) and fat (adipocytes or 3T3-L1) are the major insulin-sensitive tissues (Lanner *et al.,* 2008). Glucose transport is facilitated by glucose transporters, each with different tissue distributions, kinetic properties and sugar specificities. Currently there are 13 members of this family. Glut-2 transporter isoforms are primarily expressed in β-cells and in the liver, and have a relatively low affinity for glucose (Khan and Pessin, 2002).

There are three major pathways for the cellular fate of glucose, including: 1) oxidation to pyruvate, which may undergo further oxidation in the citric acid cycle; 2) storage as the polysaccharide glycogen for rapid utilization at a later time; and 3) conversion to other sugars and intermediates essential for other important biosynthetic and/or metabolic pathways, including the generation of glycerol 3-phosphate used in triglyceride and phospholipid synthesis, a major cellular fate of glucose in adipose, muscle, and liver tissues. Glucose is an efficient fuel in that more ATP is produced per O_2 molecule when compared with oxidation of fat and other fuel sources. Moreover, glucose is unique in that its metabolism can furnish ATP even in the absence of oxygen (Bouchè *et al.,* 2003*)*.

The study by Thomson *et al*. (2006) showed that when aqueous extracts of garlic were used at a high concentration (500 mg/kg), there was a significant decrease in the serum-glucose concentration. However, no significant change in the level of glucose was observed in the serum of rats receiving low doses (50 mg/kg) of garlic. They also found that high doses of boiled garlic extract had no hypoglycemic effect on the serum glucose level in rats. These results suggest that boiling garlic destroys the active, volatile, and chemically unstable ingredient in this herb that may be responsible for lowering the blood sugar level. It is well established that some saponins have hypoglycaemic activity, which may be due to the inhibition of liver

gluconeogenesis or glycogenolysis. Therefore, the presence of these constituents may explain the hypoglycaemic activity of *Tulbaghia violacea* BO extract on the Chang liver cells (Sheweita *et al*., 2002).

In this study it was found that the BO extract increased glucose utilisation in the Chang liver cells, but decreased it in the C2C12 cells. No effect on the 3T3-L1 cells was observed.

Diabetes is a metabolic disturbance that gradually affects the function of various systems in the body. Poorly controlled blood glucose is believed to be the most important factor in the development of diabetic complications in both T1DM and T2DM (American Association of Diabetes Educators, 2002). The number of studies investigating the hypoglycemic effect of garlic is limited, and the findings of such studies are inconsistent.

Recently, it was reported that garlic juice reversed hyperglycemia and alleviated oxidative stress and damage in liver and kidney in alloxan-induced diabetic rats (El-Demerdash *et al*., 2005). In diabetic patients, it was reported that garlic oil can correct hyperglycemia (Duncan, 1999). In addition, a precursor of various allyl sulphide constituents of garlic oil, S-allyl-cysteine sulphoxide (alliin), was shown to have a hypoglycemic effect similar to that of glibenclamide (Sheela and Augusti, 1992). However, Swanston-Flatt and co-workers (1990) failed to find a hypoglycemic effect for garlic powder in animals with streptozotocin (STZ)-induced diabetes. Similarly, Baluchnejadmojarad and Rohgani (2003a and b) found no hypoglycemic effect of an aqueous extract of garlic in STZ-induced diabetic rats, although they did find a significant effect of garlic on vascular reactivity. These inconsistent results could in part be due to the differences in preparations or derivatives of garlic used in the different studies. No standardisation of the different extracts was employed across the different studies. The chemicals present in a garlic product are largely dependent on the processing conditions, such as temperature, the duration of preparation, and the extraction solvents used (Staba *et al*., 2001).

The results of the study by Lui *et al.* (2006) showed that long-term treatment with garlic oil can improve oral glucose tolerance and renal function and suggest that garlic

oil may be a useful supplemental remedy in diabetes. This effect could not be attributed to diallyl disulphides for the effect of garlic oil on diabetes, and the results of the study by Lui *et al.* (2006) suggest that high doses of diallyl disulphides may further exacerbate the metabolic disturbances in diabetes.

In the 1970s, Jain *et al.* (1973) and Jain and Vyas (1975) investigated the hypoglycemic effect of extracts of garlic with water or several different organic solvents on oral glucose tolerance in normal and alloxan-induced diabetic rabbits. It was found that all the garlic preparations possessed an acute hypoglycemic effect, with the ethyl ether extract having a comparable affect to that of tolbutamide. Subsequent studies, reported a hypoglycemic effect of garlic oil in diabetic animals (Anwar and Meki, 2003 and Lui *et al.,* 2005*).* The hypoglycemic effect of garlic has also been shown in other non-diabetic hyperglycemic animal models (Kasuga *et al*., 1999; Tahiliani and Kar, 2003)

In the study by Lui *et al.* (2005), garlic oil significantly lowered the oxidation rate of exogenous glucose in the presence of both 10 and 100 AU/mL of insulin. The rate of conversion of glucose to glycogen in the presence of 100 AU/mL of insulin was significantly higher in rats treated with garlic oil and diallyl trisulphides.

Metformin is an anti-hyperglycemic agent used in the treatment of non-insulin dependent T2DM. This may involve a direct action of metformin on the pathways of insulin action distal to insulin-receptor binding, and indirect effects due to a general improvement in the metabolic environment. Metformin reduces fasting plasma insulin and improves insulin-stimulated glucose metabolism (Bailey and Turner, 1996). It acts in part by decreasing endogenous glucose production. Although its mechanism of action is not completely understood, it is postulated that it may involve the inhibition of glucose-6-phosphatase activity, decreasing hepatic glucose production by a sparing effect and increasing muscle insulin sensitivity. Currently, metformin is considered the first-line of therapy for most patients with T2DM (St. Onge *et al*., 2009).

In adipocytes metformin increased insulin-stimulated translocation of Glut-4 and Glut-1 into the plasma membrane and in cultured muscle cells metformin increased

the abundance of Glut-1 in the plasma membrane without increasing the total cellular complement of these transporter isoforms in either tissue (Thomas *et al.*, 1998).

The design of the β-cell suggests a central role for mitochondria in the coupling of glucose metabolism to insulin secretion. This is underlined by the efficient glucose oxidation in this cell type. More than 80% of the utilized glucose is oxidized. Glucose sensing is achieved through the expression of specific glucose transporters (for example Glut-2), which are key metabolic enzymes that efficiently stimulate the process of oxidative phosphorylation.

Oligomycin is a mitochondrion-targeting agent that inhibits F_0F_1- ATPase and ATP synthase by blocking the proton channels that are essential for the oxidative phosphorylation of ADP to ATP. Despite inhibition of electron transport there is an increase in the proton gradient, and ultimately electron transport is halted due to the steep gradient created and the inability to pump more electrons against this gradient (Nakata *et al.*, 1995), which normally leads to a reduction in oxygen consumption by the cells. Our findings indicated that oligomycin had no effect on the INS-1 cells used. No known literature is available on the effects of garlic or *Tulbaghia violacea* on oxygen consumption in INS-1 cells. In this study it was noted that the BO extract increased oxygen consumption at both the hyperglycemic and normoglycemic levels.

The change in actin levels between 11.1 and 33.3 mM glucose could possibly be due to the link between insulin secretion and changes in the cytoskeleton to facilitate exocytosis of secretory vesicles. These are carried from the *trans*-Golgi network via the microtubules that form part of the cytoskeleton of the β-cell. The cytoskeleton of the β-cell is an important component of insulin secretion. This network consists of polymerized structures of actin filaments and microtubules that form an important bridge between the endoplasmic reticulum and the Golgi apparatus and the plasma membrane. The microtubules consist of polymerized tubulin, and the application of glucose to the cells is known to increase the amount of polymerized tubulin in the β-cell (Montague *et al*., 1976). The polymerization of tubulin and mobilization of vesicles through the cytoskeleton network is regulated by proteins that bind to tubulin, known as microtubule-associated proteins. The microtubule-associated proteins are

believed to be phosphorylated by cAMP-responsive protein kinases (Easom, 2000). Likewise, the amount of polymerized actin in islet cells increases from roughly 40% to about 70% upon glucose-stimulated insulin secretion. The force generating microtubule-associated ATPase, kinesin, has been identified as important in the mobilization of insulin secretory vesicles (Doyle and Egan, 2003).

Glut-2 is the primary hepatic liver transporter. It is a low affinity, high capacity transporter expressed in high levels on the sinusoidal membranes of hepatocytes, the basolateral membrane of intestinal epithelial cells, renal proximal tubule cells, and pancreatic β-cells. In non-fed, low blood glucose states, Glut-2 is present in large amounts on the hepatocytes plasma membrane, and facilitates the net hepatic efflux of glucose (Eisenberg *et al.,* 2005). In this study Glut-2 expression in INS-1 cells was enhanced (1.4 fold) at the hyperglycemic level and reduced (2-fold) at the normoglycemic level by the BO extract, which could potentially correlate with the observed improvement in glucose stimulated insulin secretion (GSIS).

SECTION B: ANTICOAGULANT STUDIES

Thrombus formation and degradation is regulated by a hemostatic mechanism consisting of the vascular platelet system, coagulation system and the fibrinolytic system. A normal state of blood circulation is therefore maintained to confer resistance to abnormal clot formation by the coagulation and platelet systems and to promote acceleration of clot degradation by the fibrinolytic system. All these systems are referred to as the antithrombotic function. The blood fibrinolytic system is activated t-PA which converts zymogen plasminogen to plasmin. Plasmin specifically degrades fibrin, the main component of a thrombus, in a process called fibrinolysis or thrombolysis. This system is inhibited by type-1 plasminogen activator inhibitor (PAI-1). Both t-PA and PAI-I are secreted from vascular endothelial cells. These cells also contribute to the regulation of other systems in hemostasis, including the coagulation system and antithrombotic system.

The role of garlic in preventing cardiovascular diseases has been studied extensively over the last decade (Brace, 2002; Rajaram, 2003; Borek, 2006). Several studies have indicated that preparations of garlic increase fibrinolytic activity but inhibit platelet

aggregation, as well as lowering blood pressure and cholesterol in humans. These effects are advantageous in preventing or ameliorating cardiovascular disorders, such as acute myocardial infarction caused by occlusion of the blood circulation by blood clots formed in response to damaged or dysfunctional vascular endothelial cells (Brace, 2002; Rajaram, 2003; Borek, 2006).

In clinical tests of blood coagulation, PT is used to evaluate the overall efficiency of the extrinsic clotting pathway. A prolonged PT indicates a deficiency in coagulation factors V, VII and X. On the other hand, APTT is a test of the intrinsic clotting activity. A prolonged APTT usually represents a deficiency in factors VIII, IX, XI, XII and vWF (Laffan and Bradshaw, 1995). In both the *in vitro* PT (Fig. 4.21) and APTT (Fig. 4.22) tests no significant effect on clotting time was observed due to any of the extracts at the concentrations tested. Bungu (2005) showed a prolongation of the clotting time from 28 to 36 seconds using aqueous extract infusions of *T. violacea* leaves (30 μg/mL) and bulb (40 μg/mL). However these extract concentrations were not tested. Bungu *et al*., (2008) also reported that the effectiveness of the antithrombotic activities of *T. violacea* extracts was seasonal. Several researchers (Rahman and Billing, 2000; Lanzotti, 2006) report that investigating the health promoting effects of garlic are problematic and proposed that the method of extraction can eliminate or decreases the effectiveness of the active components and chemical composition of the garlic extract.

In the *in vivo* study by Chan *et al.* (2007), PT and APTT were not altered by garlic oil feeding. Furthermore, they observed that diallyl trisulphide rich garlic oil could prolong BT (bleeding time) and TT (thrombin time), as well as enhance the activity of AT-III and protein C in rats. The researchers propose that the prolonged BT might result from an impaired activity of thrombin rather than altered activity of coagulation factors in both the extrinsic and intrinsic clotting pathways. In their study, the increased fibrinogen level did not accelerate blood coagulation, but instead, led to a prolonged TT. This indicates that anticoagulation factors may play an important role in counteracting hypercoagulation (Chan *et al.*, 2007).

Our findings showed that, in the *ex vivo* model, the BO extract resulted in a 2-fold decrease of the PT clotting time relative to both the positive control and the control

(Fig 4.30). Aspirin acts on the platelet surface and not on the clotting factors; hence there is a lack of deviation between the control and the positive control. Elevations in the normal clotting times was observed for both the positive control and control in rats (Fig. 4.30) compared to humans (Fig. 4.21). This elevation can be attributed to the differences in the normal clotting times of rat (26.07 ± 0.04s) and human (13.2 ± 0.9s) plasma (García-Manzano *et al.*, 2001) making comparison between the *ex vivo* and *in vitro* model difficult. Although a difference is observed between the *in vitro* and *ex vivo* models in the control of the APTT it is well known that rat plasma usually clots faster (16.5 ± 0.4s) than human plasma (33.8 ± 2.3s) (García-Manzano *et al.*, 2001). However, the appropriate controls were used for each experiment and account for this variation in clotting times between the species.

In the *ex vivo* model for the APTT assay the BO extract produced a 1.5-fold increase in the clotting time relative to the control and a 1.2-fold increase relative to the positive control (Fig.4.31). The APTT data reflects account for the platelet interaction. These results are consistent with those observed in the flow cytometry platelet aggregation results (Fig.4.29).

In the *in vitro* model, the D-Dimer (Fig. 4.23) and Fibrinogen-C test (Fig. 4.24) were performed using the various *Tulbaghia violacea* extracts. Of the numerous extracts investigated, the BO extracts was found to increase the amount of D-Dimer released and Fibrinogen-C formation. A concentration dependent increase was found, with the highest concentration displaying a 1.4-fold and a 1.25-fold increase for D-Dimer and Fibrinogen-C assays, respectively. These results indicate that the BO extracts may enhance fibrinolysis and are similar to other studies using garlic extracts that indicate that fibrinolysis is enhanced by garlic extracts, resulting in dissolution of clots and thrombi (Rahman and Lowe, 2006).

The D-Dimer and Fibrinogen-C test provide an indication of the effect on fibrinolysis by the BO extracts in the *ex vivo* model. The BO extract produced a 2.6-fold and 3-fold decrease relative to the control and positive control, respectively, in the Fibrinogen-C assay. No effect of BO extract was noted for the D-Dimer assay. However, in comparison to the *in vivo* model the controls differ from the *ex vivo* model which had a lower fibrinogen concentration. This is due to the range of

fibrinogen in rats being lower than in humans. No effect was observed in the D-Dimer formation. This could be attributed to the low Fibrinogen-C (Fig. 4.32) obtained and thereby affecting the formation of D-Dimers.

Generally saponins have a positive effect on the prevention of platelet aggregation, blood coagulation and fibrinolysis (Lanzotti, 2006). Based on the D-Dimer and Fibrinogen-C results, the BO extract *in vitro* had an effect on the rate of fibrinolysis is consistent with results published for garlic extracts, however this was not observed for the concentrations tested in the *ex vivo* model. The presences of saponins in *Tulbaghia violacea* especially in organic extracts have been identified and could account for this enhanced fibrinolytic ability *in vitro* (Burton, 1990).

Several platelet inhibitors have been isolated and characterized from garlic and onion and their effects on platelet aggregation and TX formation have been studied (Allison *et al*., 2006; Chan *et al.,* 2003). Platelet aggregation and subsequent thrombus formation are significantly reduced by garlic and its constituents with both aqueous and organic extracts. This is thought to be due to inhibition of ADP- and platelet-activating factor (PAF)-induced platelet aggregation. Alternative mechanisms of inhibition of platelet aggregation by garlic constituents are via the inhibition of calcium mobilization and sodium 2-propenyl thiosulfate to modulate cyclooxygenase activity in platelets, thus preventing their aggregation (Rahman and Lowe, 2007; Apitz-Castro *et al.,* 1992).

The main proposed mechanism of inhibition of platelet aggregation by garlic's constituents is via inhibition of calcium mobilization and inhibition of several steps of the arachidonic acid pathway in platelets (Allison *et al.,* 2006; Bordia *et al*., 1998). In a study by Allison *et al.* (2006) it was found that AGE interfered with Ca^{2+} mobilization. Calcium mobilization is a critical step in various aspects of platelet activation such as aggregation, shape change, and secretion. Stimulation of human platelets with various agonists elevates Ca^{2+} in 2 ways, namely the release of Ca^{2+} from intracellular stores and the activation of Ca^{2+} entry through plasma-membrane channels. AGE is proposed to suppress calcium mobilization either by blocking the influx of this ion into the platelet or by its chelation within the platelet cytosol. Whether *T. violacea* extracts also affect calcium should be explored. Jastrzebski *et al.*

(2007) showed that boiled garlic loses its anti-platelet activity. This may be because heating the garlic can destroy the enzyme, allinase, which is responsible for it converting alliin to allicin. Allicin is the anti-platelet component of the raw garlic extract. The results in this study showed that garlic can be beneficial for preventing thrombosis if taken in a raw rather than a boiled or processed form.

An additional study by Chan *et al.* (2003), indicated that diallyl disulphides and diallyl trisulphide, two major organosulphur compounds derived from garlic, could inhibit platelet TX formation, and hence platelet aggregation. Ajoenes and sodium 2-propenyl thiosulfate, garlic derivatives, modulate COX-1 activity in canine platelets, thereby inhibiting platelet aggregation *in vitro* (Rahman and Lowe, 2006). Further studies confirm that garlic and onion inhibit platelet aggregation *in vitro,* moreover, some studies report that extracts of garlic inhibits platelet aggregation and TX formation *in vivo* and *ex vivo* (Ali *et al.,* 2000;Willoughby *et al*., 2002; Fukao *et al.*, 2007). Bordia *et al.* (1996) showed that garlic inhibited platelet aggregation in an *in vivo* human study where an organic extract (1g/day) was administered over a period of 5 days. It was found by Bordia *et al.* (1996) that when rats were given aqueous extracts of garlic and onion, orally or intraperitoneally daily for a period of 4 weeks the TXB_2 levels in the serum of rats treated with the low dose of an aqueous garlic extract (50 mg/kg) was significantly inhibited regardless of the mode of administration and at the high dose of garlic and onion (500 mg/kg), TXB_2 levels in the serum of the rats were further reduced.

An *ex vivo* study completed by Chan *et al.* (2003) observed that diallyl disulphides and diallyl trisulphide could effectively protect platelets against oxidation and ADP-induced aggregation, in which diallyl trisulphide was superior to diallyl disulphides. However, garlic oil feeding did not affect *in vivo* ADP-induced platelet aggregation. Garlic has also been found to be effective against blood clots (anti-platelet action) due in part to the compounds alliin and ajoenes, which have fibrinolytic activity. Ajoene inhibits TX synthesis through the inhibition of the COX-1 and lipoxygenase enzymes (Chan *et al.*, 2003).

In this study all the extracts tested were able to inhibit protein secretion and platelet aggregation and adhesion, in a concentration dependent manner, with the organic

extracts having the greatest degree of inhibition *in vitro*. The BO extract displayed the highest degree of inhibition of all the extracts. Most of the extracts inhibited the protein secretion by more than 50%. The BO extracts inhibited protein secretion by 53% (1 mg/mL) supporting the results observed from the microscopic evaluation of the extracts on platelet adhesion and aggregation. The reactive compounds in *T. violacea* extract tested are yet to be fully identified. The BO extract predominantly modulates its antithrombotic effect by preventing platelet aggregation. In the *ex vivo* rat model it was observed that the BO extracts decreased thrombin-induced platelet aggregation by 50%. This was significant when compared to the effects of aspirin, a known platelet aggregation inhibitor at the concentrations tested which inhibited platelet aggregation by 30%. Numerous *in vitro* studies have confirmed the ability of garlic to reduce parameters associated with cardiovascular disease, which are consistent with the results obtained for the *T. violacea* extracts tested.

Aspirin was used as a positive control as it has known anti-platelet aggregation properties. Aspirin blocks the action of both COX-1 and COX-2. Aspirin acts on COX-1 by irreversibly acetylating serine 530, resulting in the inhibition of TX A_3 release from platelets and PGI_2 from EC. Inhibiting COX-1 leads to inhibition of platelet adhesion and aggregation, and aborts the malignant cascade that is triggered by plaque rupture (Michalson, 2004). COX-2 inhibition, with its anti-inflammatory effects, decreases vascular inflammation at the site of the plaque, and that, in turn, reduces mononuclear cell infiltration and enhances plaque stability. In addition to anti-platelet activity, aspirin seems to have an effect on clotting factors, which may be another mechanism for its anti-thrombotic properties. Aspirin can acetylate fibrinogen, which facilitates clot lysis and results in a decreased polymerisation rate and increased fibrin gel porosity. Effects on fibrinolysis may be due to the high affinity of acetylated fibrin clots to plasminogen. Changes in fibrin structure have been attributed to acetylation of lysine residues in the fibrinogen molecule. This is particularly important in diabetic individuals who may have glycated fibrinogen at the lysine residue due to the high glucose levels (Ajjan and Grant, 2006).

A number of studies on *T. violacea* have focused on its effects on cystolic blood pressure and its ACE inhibitor properties both using organic and aqueous extracts. Perhaps these outcomes may be based partially upon its anti-platelet abilities observed

in this study (Duncan *et al*., 1999; Serra *et al.*, 2005; Mackraj *et al.*, 2007; Mackraj *et al.*, 2008*).* The BO extract should be analysed for its possible effect on blood pressure and presence of ACE and COX-1 inhibitors.

Many reviewers believe that not all the clinical trials employing garlic for therapeutic purposes have adequate methodological quality (Alder *et al*., 2003). It should be noted that various garlic products may differ widely in their biochemical composition, and as a result may possess different pharmacological properties, depending upon the method of processing or preparation. This accounts for the accompanying differences in efficacy (Duke *et al*., 2003; McKenna *et al*., 2002; Kasuga *et al*., 2001; Munday *et al.*, 1999).

CHAPTER 6: CONCLUSION AND FUTURE STUDIES

The aim of the present study was to investigate the anticoagulant and antidiabetic properties of *T. violacea. T. violacea* is widely used as a medicinal plant for many ailments, however little or few studies have been conducted to determine its biological activities and the efficacy of its use. The close relationship of *T.violacea* to garlic would indicate that it might share similar biological properties. To date nothing has been reported on *T. violacea's* effects on platelet function and its possible antidiabetic effects. However, a number of studies on garlic have demonstrated that garlic possesses both these properties. It is postulated that the biological activities present in *T. violacea* are similar to those in garlic. A number of studies on *T. violacea* have focused upon compounds that are present. The compounds that have been identified include: three substituted s-substituted cysteine sulphoxide derivative; flavones such as kaempferol and quercetin, 2, 4, 5, 7-tetrathioctane-2-2 dioxide and 2, 4, 5, 7-tettrathioctane. Other studies have focused on the antifungal and anticancer activities in various cell lines. The most important of the studies conducted has been on its ACE inhibitor and antithrombin properties (Table 1.1).

In the first part of the study, various extracts were isolated from *Tulbaghia violacea* to determine their effect as anti-diabetic agent. It was found that the BO extract enhanced GSIS and it was selected for concentration dependent testing. Due to the high passage number of the INS-1 cells, it is advisable to repeat the GSIS experiments with lower passage number cells and check insulin secretion in these cells. The effect of the BO extract on proliferation should be conducted with the use of proliferation markers such as Ki-67, present in the active cycle and absent in resting cells. Thus an increase in expression of Ki-67 is a reflection of cell proliferation (Honegger *et al*., 2003).

The BO extract had a significant effect on enhancing glucose uptake in the Chang liver cells, the effect was shown to be concentration dependent. No effect on glucose utilisation was seen for 3T3-LI cells, while the BO extract reduced glucose utilisation in the C2C12 cells. This indicates that improved glucose utilisation by the BO extract is limited to the Chang liver cells. The main glucose transporter in the liver is Glut-2 which has a very low affinity for glucose. This could explain the relatively slight

increase in glucose absorption in the liver after treatment with the BO extract. Future studies investigating the effect of the BO on glucose uptake should be performed on insulin resistant cell lines which are more indicative of the diabetic state; these experiments should be conducted at different glycemic levels.

The constituents of the BO extract should be elucidated and characterised. This can be achieved through analytical methods such as mass high performance liquid chromatography mass spectrometry (HPLC-MS). The separated and/or characterised compounds found can then be used on the different cell lines and their effects on parameters such as glucose utilisation or insulin secretion can be observed to determine which compound or synergy of compounds contribute to the effects observed in this study.

Further studies in an insulin resistant model should be completed. This can be performed in insulin resistant cell lines (3T3-L1, C2C12 and Chang liver) to establish the BO effects under these conditions. It should also be determined whether the increase in insulin secretion as observed is due to an increase in Glut-2 expression, an increase in the ATP/ADP ratio or an increase in Ca^{2+} levels. Once the efficacy of these compounds has been tested, its action on the insulin pathway can be elucidated. An appropriate animal model should be used to evaluate the anti-diabetic effects of *Tulbaghia violacea* to determine whether these effects are present in a complete physiological system. The ability of BO to act as a secretagogue should be investigated further, using primary rat islets.

Oxygen consumption studies were performed in INS-1 cells with BO extract (10 µg/mL) in 33.3 and 11.1 mM glucose. An increase in oxygen consumption relative to the control was observed. Glut-2 protein expression increased in hyperglycemic conditions, but decreased in normoglycemic conditions, when treated with the BO extract. This indicates that the BO extract promoted Glut-2 expression at high glucose levels. With cells cultured at the normoglycemic level, a decrease in polarization of the mitochondrial membrane was noted. However, the opposite is observed with exposure of the cells cultured at hyperglycemic levels, where a significant increase in polarization was observed indicating that the BO extract enhanced insulin secretion and glucose uptake (Fig. 4.15).

RT-PCR studies should be conducted to determine the effect of the BO extract on gene expression in INS-1 cells and other peripheral cell lines of particular interest. The change in expression of markers such as IRS-1 and Glut-2 in the INS-1cells and IRS-1 and Glut-4 expression in the C2C12, 3T3-L1 and Chang liver cells should be investigated.

The second part of the study focused on screening different extracts to determine their effects on coagulation and platelet function. It has been reported in numerous articles and literature reviews that garlic and *T. violacea* have seasonal variation. In addition, plant part and method of extraction can affect both the activity and the compounds present in the extract. It was found that none of the extracts had any significant effect on the coagulation pathway *in vitro* (PT, APTT); however, the BO had a significant effect on fibrinolysis (the rate of dissolution of a clot once formed) (D-Dimer and Fibrinogen-C). The effect of the extracts on platelet function was subsequently investigated. It was found that most of the extracts had an effect on platelet adhesion, aggregation and protein release; with up to 50% inhibition being noted. The BO extract had the most significant ($p<0.05$) effect on platelets function and this was observed to be concentration (0.25-2 mg/mL) dependent.

The BO extracts displayed concentration dependent results for the coagulation tests, D-Dimer and Fibrinogen-C. A concentration dependent result for the BO extract was seen for platelet aggregation, adhesion and protein secretion inhibition. Based on these screening results the BO extract was selected for further testing in an *ex viv*o model to determine it properties in a physiological model. The BO extract decreased thrombin-induced platelet aggregation by 50%. This is significant when compared to aspirin which inhibited platelet aggregation by 30% relative to the negative control. This indicates that the BO is 20% more potent than aspirin, confirming the results obtained in the *in vitro* model. The PT and APTT results obtained in the *in vitro* and *ex vivo* model did not correlate. In the *ex vivo* model the BO extract had an effect on fibrinolysis, by reducing the Fibrinogen-C test result by up to 70%, however no effect was observed for the D-Dimer test. This indicates that BO extract of *T. violacea* may modulate antithrombin activity as an antiplatelet agent.

The role thrombin plays in the pathogenesis of thrombosis and arthrosclerosis makes it an important target for antithrombotic agents. Its structure of two positively charged regions, plays an important part in its specificity towards macromolecular substrates (Fibrinogen) and co-factors in the coagulation pathway, as well as inhibitors such as heparin. For future anticoagulant studies, the mechanism by which *T.violacea* inhibits platelet aggregation and adhesion can be elucidated by determining where in the platelet activation pathway the extract exerts its effect. It is thought that garlic affects coagulation in its ability to inhibit ADP- and PAF-induced platelet aggregation via the inhibition of calcium mobilization and sodium 2-propenyl thiosulfate, to modulate COX activity in platelets (Jeng *et al.,* 2007). The effect of *T.violacea* on these parts of the coagulation pathway should be investigated.

Future work on the activities of *T. violacea* should focus on its possible abilities to lower serum cholesterol and triglycerides and increase the amount of high-density lipoproteins (HDL) in an *in vivo* model. Atherosclerosis can be tested for by using flow cytometry to detect specific markers, such as platelet p-selectin and CD61 (Henry *et al.,* 2009). Dietary-induced (high cholesterol feed or beef lard) atherosclerosis was significantly reversed in rabbits when fed garlic consistently for a few weeks, whether this holds for *T. violacea* requires investigation.

CHAPTER 7: REFERENCES

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ANNEXURE A: REAGENTS

GLUCOSE OXIDASE REAGENT PREPARATION.

Add the following reagents to 100 ml of phosphate buffer

0.028 g phenol (Sigma, USA)

0.008 g 4-aminoantipyrine (Sigma, USA)

0.074 g EDTA (Fluka, USA)

65 µl glucose oxidase (Sigma, USA)

0.001 g peroxidise (Sigma, USA)

Phosphate Buffer:

Solution A: Dissolve 7g NaH_2PO_4 in 100ml water.

Solution B: Dissolve 18.4g Na_2HPO_4 in 400ml water

Add 10ml solution A to 40ml solution B and dilute with water to a final volume of 1.3l and use for the glucose utilization study.

10X KREBS RINGER BUFFER (BUFFER REQUIRED IN GSIS)

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

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

ANNEXURE B: HUMAN ETHICS APPROVAL

Nelson Mandela Metropolitan University

for tomorrow

Chairperson of the Research Ethics Committee (Human)
NMMU
Tel . +27 (0)41 504-2499 Fax. +27 (0)41 504-2770
Rosa.DuRandt@nmmu.ac.za

Ref: N 01/11/03/07 [H06SB-003/Approval]

Contact person: Mrs U Spies

12 September 2006

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

Dear Dr Frost

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

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

The Committee approved the above mentioned application.

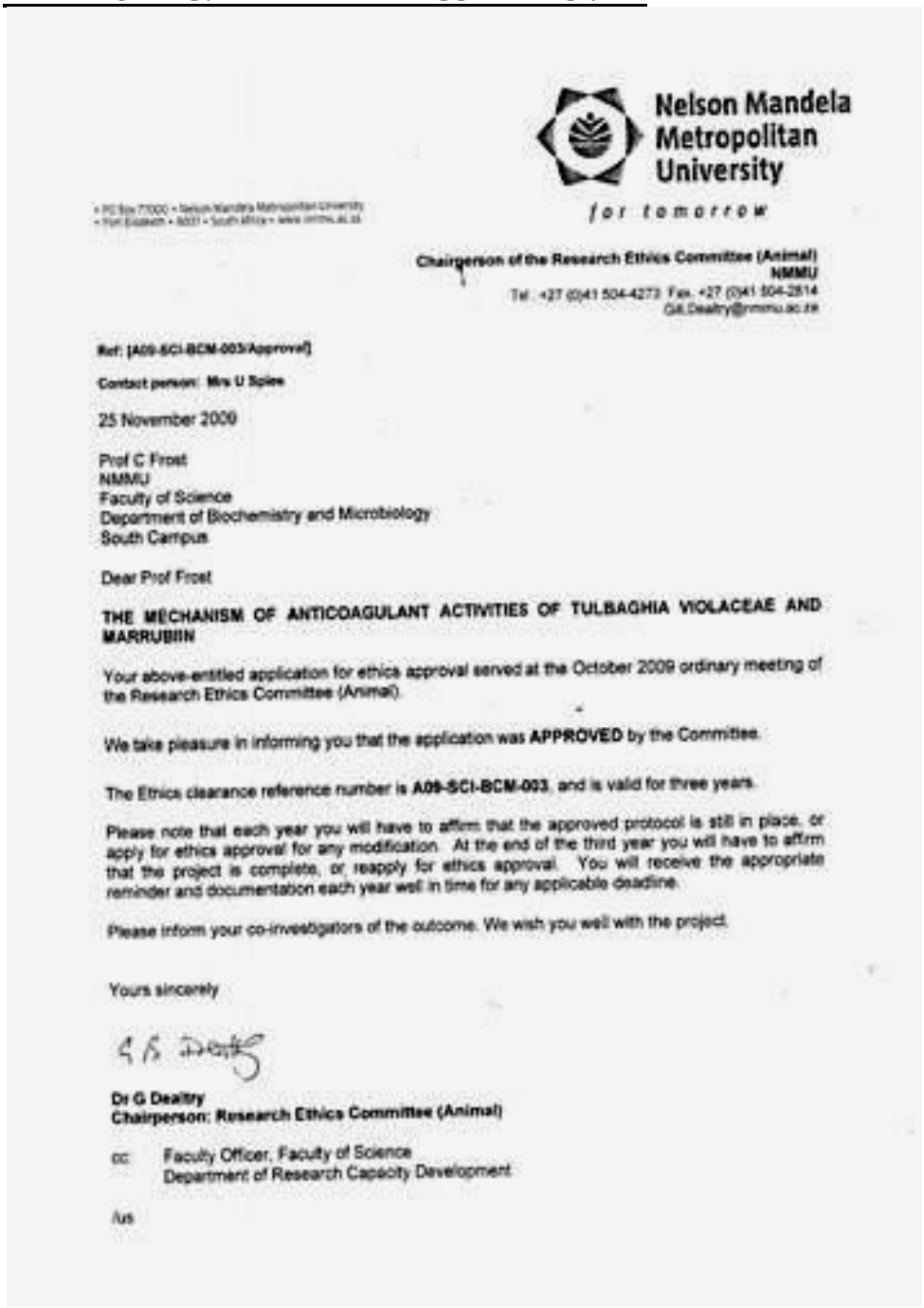
Please inform your co-investigators of the outcome. We wish you well with the project

Yours sincerely

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

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

ANNEXURE C: ANIMAL ETHICS APPROVAL



Nelson Mandela Metropolitan University

for tomorrow

Chairperson of the Research Ethics Committee (Animal)
NMMU
Tel: +27 (0)41 504-4273 Fax: +27 (0)41 504-2514
Gil.Dealtry@nmmu.ac.za

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 MARRUBIIN

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