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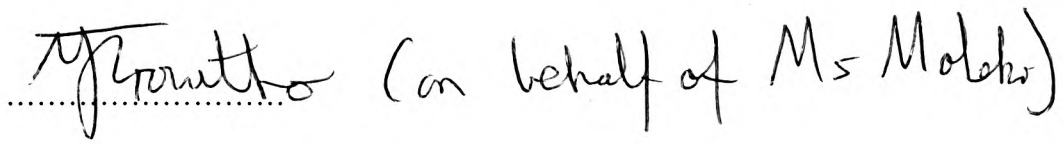
THE EFFECTS OF VARIOUS SOUTH AFRICAN MUTIS ON INSULIN SECRETION AND ACTIVITY

AN MSC MED DISSERTATION BY MAKHALA CONSTANCE MOLEKO

DECLARATION

I, Makhala Constance Moleko, declare that this dissertation is my own work, and has not been submitted or incorporated in another dissertation or thesis for any other degree.



ABSTRACT

Throughout the world, many traditional plant treatments for diabetes exist and therein lies a hidden wealth of potentially useful natural products for diabetes control. Despite this, few traditional antidiabetic plants have received scientific or medical scrutiny, and the World Health Organisation (1980) recommended accordingly that this area warrants further evaluation.

The present study was undertaken to examine the antidiabetic effects of the aqueous extract of coriander (*Coriandrum sativum*), Devil's claw (*Harpagophytum procumbens*), *Trichilia emetica* and *Ziziphus mucronata* on 3T3-L1 and MIN 6 cells. Insulin and plant extracts were included in the culture medium to test their effects on lipolysis in 3T3-L1 adipocytes. Following a 2 hr incubation, insulin, linalyl acetate aqueous extracts of coriander and Devil's claw were found to inhibit isoproterenol-stimulated lipolysis. The effect of insulin, linalyl acetate and plant extracts were concentration dependent. Insulin caused a significant ($p<0.05$) inhibition of lipolysis.

After a 2 hour incubation, aqueous extracts of coriander and Devil's claw produced dose-dependent, reductions in the isoproterenol-stimulated lipolysis. A component of coriander oil (linalyl acetate) caused 82.1% reduction in isoproterenol stimulated lipolysis and at a concentration as low as 4µl the effect of linalyl acetate was already evident. Aqueous extracts of Trichilia emetica and Ziziphus mucronata also lowered lipolysis.

Glucose at 40 mmol/l caused a statistically significant 2-fold increase in insulin secretion from MIN 6 cells. The coriander aqueous extract and linalyl acetate both caused dose-dependent increases in insulin secretion, but not to a statistically significant level.

The results of this *in vitro* study indicate that coriander and Devil's claw possess anti-diabetic activity and one of the possible active principles in coriander may be linalyl acetate. The active component of Devil's claw remains to be fully elucidated.

Furthermore, plants that are not traditionally used for the treatment of diabetes possess some level of anti-lipolytic activity and suggest that such plants should also be tested more fully for anti-diabetic activity.

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I would like to acknowledge and appreciate all the assistance I received from Professor N.J. Crowther, my supervisor, who devoted a lot of his valuable time to guiding me through the different steps in the process of conducting this study; and I would also like to express thanks to those who offered suggestions, assistance and support to make this research report possible.
My final and greatest thanks, go to God Almighty for giving me the strength to finish this project.

It was a pleasure to deal with persons who made a little effort to be as courteous and as helpful as possible.

DEDICATION

I dedicate this thesis to my son Tshepo Moleko

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADP	adinosine diphosphate
AMP	adinosine monophosphate
ATP	adenosine triphosphate
BSA	Bovine serum albumin
C	Celsius
CaCl	calcium chloride
cAMP	Cyclic adenosine-5- monophosphate
Cl	chlorine
C- peptide	Connecting peptide
Cori	Coriander
D/claw	Devil's claw
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's medium
ELISA	enzyme-linked immunosorbant assay
ERK-1	extracellular signal regulated kinase
FFA	free fatty acid
g	gram
Glut-2	glucose transporter
GDP	guanine diphosphate
GTP	guanine triphosphate
h	hours
IBMX	3-isobutyl-1- methyl- xanthine
Ins	Insulin
IR	Insulin receptor
IRS	Insulin receptor substrate
Iso	Isoproterenol
KCl	potassium chloride
Kg	kilogram
KH_2PO_4	potassium dihydrogen orthophosphate
KRB	Krebs Ringer Buffer
m	meter
M	moles per litre
MAP	mitogen- activated protein
ml	milliliter
mM	millimoles per litre
mm	millimetre
mRNA	Messenger RNA
mw	molecular weight
µl	microlitre
µM	micro moles per litre

MAP	mitogen-activated protein
NA	nicotinic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
Na_2HPO_4	disodium hydrogen orthophosphate
NaOH	sodium hydroxide
NIDDM	Non-insulin dependent diabetes mellitus
PIA	phenylisopropyladenosine
P1-3 K	Phosphatidyllinositol –3- kinase
PC 1	prohormone convertase
RNA	Ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SH2	*src* homology 2
STH	Somatotropin
Tri/emetica	Trichilia emetica
Tyr	Tyrosine
TZD	Thiazolidinedione
WHO	World Health Organization
Zi/mucronata	Ziziphus mucronata

CHAPTER 1

1.1 INTRODUCTION

The development of new dietary adjuncts and novel antidiabetic agents, which reinstate a normal metabolic environment, thereby reducing the long-term complications associated with diabetes is required. Such agents would both ideally stimulate the secretion and improve the action of insulin (Bailey and Flatt, 1995). Throughout the world, many traditional plant treatments for diabetes exist and therein lies a hidden wealth of potentially useful natural products for diabetes control (Bailey and Day, 1989, Gray and Flatt, 1997, Swanston-Flatt *et al.*, 1991). Despite this, few traditional antidiabetic plants have received scientific or medical scrutiny, and the World Health Organisation (1980) recommended accordingly that this area warrants further evaluation.

The World Health Organisation estimates that there are 150 million people with diabetes world wide, a figure that is expected to double by the year 2025 (WHO, 1995). Until recently diabetes was considered to be rare in Sub-Saharan Africa, but as a result of demographic and lifestyle changes, as well as increasing recognition, it is being identified as a major health problem (Gill *et al.*, 1997). In urbanised black populations in South Africa, recent surveys estimate the prevalence of diabetes at around 8% in those over 30 yrs of age (Omar *et al.*, 1993) and it is a major cause of mortality and morbidity (Kalk *et al.*, 1997).

A scientific investigation of traditional herbal remedies for diabetes may provide valuable leads for the development of alternative drugs and therapeutic strategies. Alternatives are clearly needed because of the inability of current therapies to control all of the pathological aspects of diabetes, and the high cost and poor availability of current therapies for many rural populations, particularly in developing countries (Kalk *et al.*, 1997). The enormous costs of modern treatment indicate that alternate strategies for the prevention and treatment of diabetes must be developed. Since almost 90% of the people in rural areas of developing countries still rely on traditional medicines for their primary health care, and scientific investigation of traditional medicines have led to the discovery of at least 88 drugs now in professional use world-wide (Soejarto and Farnsworth 1989), a synthesis of

local traditional and modern knowledge and techniques for the management of diabetes should be feasible.

Recent studies showed that aqueous extracts of agrimony (*Agrimony eupatoria*), Lucerne (*Medicago sativa*), Coriander (*Coriandrum sativum*), eucalyptus (*Eucalyptus globulus*), edible mushroom (Agaricus campestris) and mistletoe (Viscum album) enhanced insulin secretion and mimicked the effect of insulin on glucose metabolism *in vitro* (Gray and Flatt 1997, Gray and Flatt 1998). Such dual pancreatic and extrapancreatic actions would prove to be an important advance on existing therapies used to treat and control diabetes, such as oral hypoglycaemic drugs (which act either by enhancing insulin secretion or by improving the action of insulin). These combined findings illustrate the enormous potential of plants for use as possible dietary adjuncts and the discovery of natural products for diabetes therapy.

1.2 AIMS AND OBJECTIVES

The purpose of this study was to determine the effects of some South African plants and a known antidiabetic European plant, i.e coriander on insulin action and secretion. This was accomplished by:

- Studying the effect of coriander and South African plant extracts on lipolysis in cultured murine 3T3-L1 preadipocytes.
- Studying the effect of above plant extracts on insulin secretion from the rodent β-cell line, MIN6

1.2.1. Coriander

Coriandrum sativum (coriander) has been reported to have a number of possible medicinal attributes including antispasmodic, carminitive and stomachic properties (Lust, 1986). Additionally, coriander has been advocated as an anti-diabetic remedy (Farnsworth and Segelman, 1971; Lewis and Elvin-Lewis, 1977). More recent studies have confirmed the antihyperglycaemic effect of coriander in streptozotocin-diabetic mice (Swanston-Flatt *et al.*, 1990)

1.2.2. Devil's claw

Devil's claw is native to South and Central Africa. Its medicinal value lies in its fleshy roots, which are sliced and chopped while fresh. Researchers have found that the herb stimulates production of digestive juices (health.yahoo.com/health/alternative-medicine/herbal-remedies). Devil's claw (*Harpagophytum procumbens*) has been recommended for treating a wide variety of conditions : diabetes, cholecystitis, cholelithiasis, gout ,obesity, osteoarthritis, rheumatoid arthritis; dyspepsia; hypercholesterolemia; hyperlipidemia. It is a remedy from the Kalahari desert in Namibia with a well-deserved reputation as an effective rheumatic remedy. A group of glycosides called harpagosides found in the root show a marked anti-inflammatory effect.

In the west, Devil's claw has been recommended for treating a wild variety of conditions including diseases of the liver, kidneys, and bladder, as well as allergies, arteriosclerosis, lumbago, headache, heartburn, nicotine poisoning, and above all, rheumatism and arthritis. Diabetes, hepatitis, kidney and bladder deficiency, nervous malaise and respiratory ailments are all treated with Devil's claw preparations (http://www.herbnet.com/Herb%2OUses-CDE.htm).

1.3. LITERATURE REVIEW

1.4. INSULIN

Insulin is commonly felt to be the primary regulator of the blood glucose level. Indeed, an absence of insulin results in an elevated blood glucose concentration, whereas an excess of the hormone results in hypoglycemia. Insulin levels increase with food ingestion and fall with food deprivation, thereby providing coordination for the metabolic changes that accompany feeding and fasting. The hormone exerts its effects on muscle, fat, and the liver, tissues primarily concerned with the regulation of fuel metabolism.

1.4.1. INSULIN BIOSYNTHESIS

Insulin biosynthesis by the islets of Langerhans of the adult mammalian pancreas is influenced markedly by glucose concentrations over the physiological range of 2 to 8 mM and is therefore unique when compared to the synthesis of proteins by other mammalian tissues (Permutt and Kipnis, 1972). Some of the mechanisms proposed to account for the unique effect of glucose on insulin synthesis include: (a) coupling between insulin secretion and synthesis, (b) glucose functioning as a non-specific energy source for islet protein synthesis and (c) stimulation of RNA synthesis (Permutt and Kipnis, 1972).

The genetic information which will initiate biosynthesis is, of course, present in the β-cell deoxyribonucleic acid (DNA). It is here that the amino acid sequence of the protein is encoded. In a process requiring ribonucleic acid (RNA) polymerase, a messenger (m) RNA is transcribed from the gene. Insulin mRNA has a half-life of approximately 2 hours (Permutt and Kipnis, 1972). The initial translation product has been termed preproinsulin (Chan et al., 1976).

Preproinsulin, a protein of about 100 amino acids (MW 12000), is formed by ribosomes in the rough endoplasmic reticulum of the pancreatic β-cells. The "pre" portion of the molecule has a half-life of about 1 minute (Patzelt *et al.,* 1978), may be cleaved before the synthesis of the protein is completed, and probably serves to facilitate the association of ribosomal-bound messenger RNA with the membrane of the endoplasmic reticulum (Blobel and Dobberstein, 1975). Preproinsulin is not detectable in the circulation under normal conditions because it is rapidly converted by cleaving enzymes to proinsulin (MW 9000), an 86- amino-acid polypeptide. This is stored in secretory granules in the Golgi complex of the β-cells, where proteolytic cleavage to insulin and connecting peptide (C- peptide) occurs. It is here that insulin granules are first recognizable, although these initially appear immature and less dense than the fully developed granules. The Golgi apparatus is responsible for the synthesis of the membranous sac surrounding the developing granule (Greider *et al.,*1969). By facilitating fusion with the cell membrane, this sac may play a vital role in the process

of granule extrusion from the cell (Blaschko *et al.*, 1967). In the developing granules, several important cellular processes occur. First, the proinsulin is cleaved to insulin and connecting C-peptide, a process involving proteolytic cleavage (Kemmler *et al.*, 1970, Grant *et al.*, 1970). Second, zinc is taken up and together with insulin, forms what appear to be microcrystals of zinc insulin, the probable storage form of the hormone (Greider *et al.*, 1969). Because these conversion processes occur in the membranous sac of the developing granule, C-peptide and zinc are sequestered here to be released with insulin upon appropriate β-cell stimulation (Rubenstein *et al.*, 1969). In parallel with changes in the pH within the secretory granules, insulin is deposited in microcrystalline form in the lumen of these granules. At the cell membrane, insulin and C peptide are released into the portal circulation in equimolar amounts (Howell, 1991). Under normal conditions, 95% of the hormone product is secreted as insulin and less than 5% as unconverted proinsulin. The biosynthesis of proinsulin is stimulated by nutrient secretagogues to a greater relative extent than that of other islet proteins. The structural conformations of proinsulin and insulin are very similar, and a major function of the C peptide is to align the disulphide bridges that link the A and B chains so that the molecule is correctly folded for cleavage (Howell, 1991, Malaisse, 1996).

The stimulation of proinsulin biosynthesis by nutrient secretagogues occurs first at the transcriptional level. The cellular content of the preproinsulin mRNA may be affected by both transcription of the insulin gene and degradation of messenger RNA. The threshold value for stimulation of proinsulin synthesis at increasing concentrations of D-glucose (2-4mM) is lower than the minimal concentration of the hexose required to stimulate insulin release. This difference may prevent depletion of the hormonal stores whenever insulin release is stimulated by agents failing to affect the biosynthesis of the hormone. If the biosynthetic rate exceeds that of insulin release, excess insulin may undergo lysosomal degradation in a process of granulolysis termed crinophagy.

The stimulation of proinsulin biosynthesis by glucose and other nutrient secretagogus is closely connected with the capacity of these nutrients to augment the rate of ATP generation in islet cells. The precise nature of the coupling between metabolic and biosynthetic events remains unknown. An increase in the intracellular concentration of K^+, secondary to decreased efflux of this cation, may be involved in such a coupling process.

1.4.2. Factors stimulating insulin biosynthesis.

Compounds which are metabolised by the β-cells are best able to augment biosynthesis. Consequently, glucose, mannose, glyceraldehyde, and N-acetylglucosamine, compounds avidly utilised by the β-cell, promote this effect, while poorly metabolised hexoses such as fructose, are ineffective. Several metabolised non-sugars also augment biosynthesis. Thus, leucine, α-ketoisocaproate, and several ribonucleosides, stimulate the synthetic process. The biosynthetic effect can easily be dissociated from secretion since several compounds, most notably sulfonylureas, stimulate the secretion, but not the biosynthesis, of insulin.

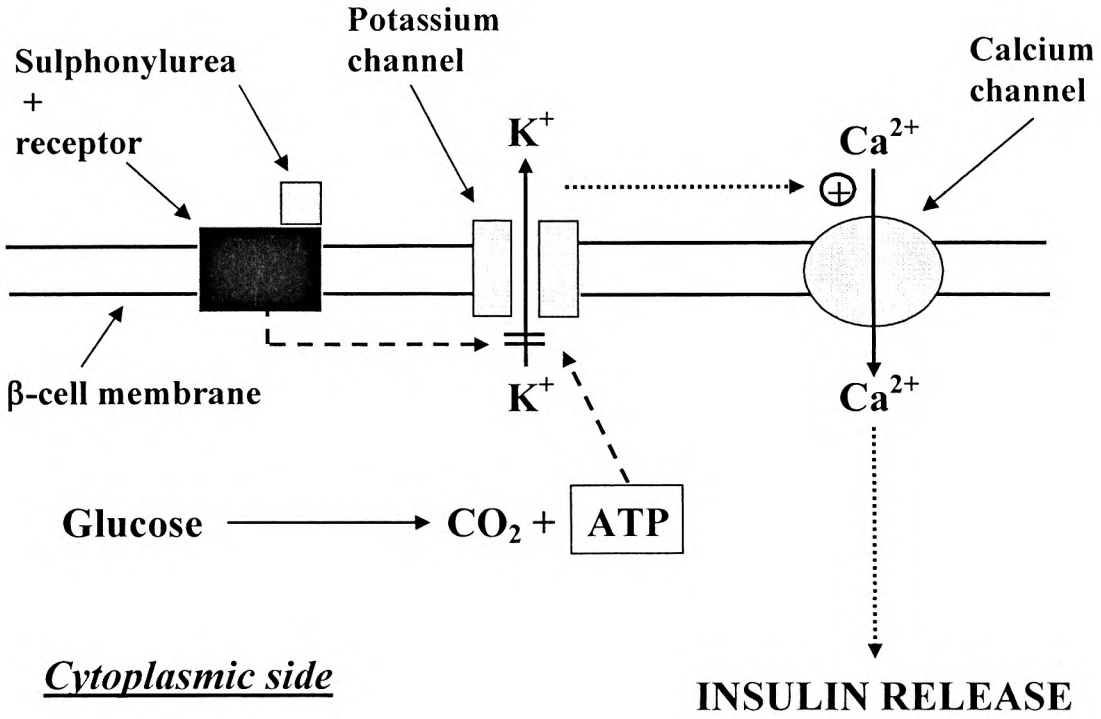

Figure 1.1. Insulin release from the pancreatic β-cell and its relationship with glucose metabolism and ion channel activity.

ATP and sulphonylureas both block the ATP-sensitive potassium channel leading to membrane depolarization and activation of the voltage-dependent calcium channel. This allows calcium into the β-cell leading to stimulation of calcium-dependent signalling events required for insulin secretion.

1.5. INSULIN RELEASE

In normal β-cells, insulin is released by the process of exocytosis, in which the granules first move close to the cell membrane and the granule membrane and cell membrane then fuse together, releasing the granule contents. Obviously, the continuity of both membranes must be breached, releasing insulin into the extracellular space for subsequent transport to distant tissues (Malaisse, 1996). Following the incorporation of the granule membrane, the newly expanded plasma membrane is partly reabsorbed into the cell by endocytosis and recycled back to the Golgi complex for re-use; the irregularities left in the plasma membrane after exocytosis are called microvilli. It seems likely that the transport of granules to the membrane is regulated independently from the final secretion process, so allowing a reservoir of granules to accumulate close to the internal surface of the plasma membrane. Release of this readily available pool might account for the rapid first-phase release of insulin that is seen in response to glucose stimulation (Malaisse, 1996).

1.5.1. Regulation of insulin secretion

The major physical determinant of insulin secretion in mammals is the blood glucose concentration. Pancreatic and gastrointestinal hormones (e.g., glucagon, gastrin, secretin, pancreozymin, gastrointestinal polypeptide), endocrine, neural and pharmacological agents (e.g., sulfonylureas and β-adrenergic agonists) stimulate insulin secretion (Malaisse, 1996). These agents can be divided into two groups : *initiators* or primary stimuli, i.e. agents capable of provoking insulin release alone, such as glucose and certain amino acids; and *potentiators*, i.e. agents which are ineffective alone but will increase the insulin released in response to glucose or amino acids, such as glucagon and phoshodiesterase inhibitors .

Insulin release is inhibited by hypoglycemia, somatostatin , and various drugs (e.g. α-adrenergic agonists, β-adrenergic blockers, diazoxide, phenytoin, phenothiazines, and nicotinic acid). In healthy individuals, insulin is secreted in a pulsatile fashion, with glucose and insulin the main signals in the feedback loop. Glucose elicits the release of insulin from the pancreas in two phases. The first phase begins 1 to 2 min after intravenous injection of glucose and ends within 10 min. This phase represents the rapid release of insulin stored in the secreting granules close to the plasma

membrane. The second phase, beginning at the point where the first phase ends, depends on continuing insulin synthesis and transport to the plasma membrane and lasts until normoglycemia has been restored, usually within 60 to 120 min. (Malaisse, 1996).

1.5.1 (i) Factors stimulating insulin release directly are:

a. Glucose

b. Leucine, arginine, histidine, phenylalanine

c. Sulfonylureas

d. ACTH, glucagon, somatotropin

e. α_2-adrenergic blockade

f. β_2-adrenergic stimulation

g. Vagal stimulation

h. Gut hormones : glucagon, secretin, cholecytokinin

(ii) **Factors inhibiting insulin release directly are:**

a. . α_2-adrenergic stimulation

b. β_2-adrenergic blockade

c. Thiazide diuretics

d. Diphenylhydantoin

e. Diazoxide

(iii) **Factors decreasing the tissue response to insulin are:**

a. Growth hormone, placental lactogen, glucocorticoids, estrogens, progestins, oral contraceptive agents

b. Obesity

c. Inactivity

d. Poor physical condition

e. Low carbohydrate diet (Best and Malaisse, 1984).

1.5.2. Glucose-induced insulin release

Among circulating nutrients, D-glucose plays a key role in the regulation of insulin release. This hexose represents the sole substrate able, at its normal concentration, to stimulate insulin release even when tested *in vitro* in the absence of any other exogenous nutrients. Under steady-state conditions, the rate of insulin release is linked to the extracellular concentration of D-glucose by a sigmoidal relationship, with a threshold concentration close to 5.0 mM, a half-maximum response at about 10.0 mM and a maximal insulin output at approximately 20.0 mM D-glucose (Malaisse, 1996).

The immediate stimulation of insulin release by D-glucose represents the outcome of a sequence of metabolic, ionic and mechanical events in pancreatic islet β-cells (Malaisse, 1996). The transport of D-glucose across the β-cell plasma membrane is mediated by GLUT-2, allowing for an immediate equilibration of the extracellular and intracellular concentrations of the hexose. The phosphorylation of D-glucose which allows the hexose to enter the glycolytic pathway is catalysed in insulin-producing cells not only by a low-Km hexokinase (Km close to 0.05 mM), but also by a high Km glucokinase. This allows the rate of phosphorylation of the hexose to increase in the physiological range of glycaemia. Glucose causes a preferential stimulation of oxidative mitochondrial events, a phenomenon attributable to a Ca^{2+} - dependent activation of key mitochondrial dehydrogenases (Malaisse, 1992). The net result of the events is an increase in mitochondrial production of ATP.

The factors that couple islet β-cell catabolism of D-glucose to more distal events in the secretory sequence are not yet fully elucidated (Malaisse *et al.*, 1984). A current concept ascribes a key role to raised intracellular ATP levels resulting from the oxidative metabolism of glucose, this leads to ATP- induced closing of K^+ channels located at the plasma membrane (Fig 1.1). This results in depolarization of the cell membrane and subsequent gating of voltage-dependent Ca^{2+} channels. The increased influx of the latter divalent cations then leads to an increase in cytosolic Ca^{2+} concentration and the activation by Ca^{2+} of the microtubular-microfilamentous system responsible

for the oriented intracellular translocation of secretory granules and their release by exocytosis at the cell boundary (Malaisse et al., 1981).

Other factors also participate in the coupling of D-glucose metabolism to insulin release in the islet β-cells. They are thought to ensure further enhancement of insulin output at increasing concentrations of the hexose. For instance, the islet content of cyclic AMP is increased by D-glucose. This seems attributable to the activation, by calcium-calmodulin, of adenylate cyclase (Vaverde *et al.,* 1979). It may favour the release of insulin at any given concentration of cytosolic Ca^{2+}. Likewise, phospholipase C is apparently activated in islets exposed to high concentrations of D-glucose, resulting in the production of both diacylglycerol, an activator of protein kinase C, and inositol triphosphate, which mobilizes Ca^{2+} from intracellular sequestering sites (Best and Malaisse, 1984).

Table 1.1. Major cellular events involved in glucose-induced insulin release

1. **Metabolic events**

- GLUT-2 mediated equilibration of D-glucose concentration across the β-cell plasma membrane
- Resulting increase in D-glucose phosphorylation by glucokinase
- Increase in glycolytic flux
- Preferential stimulation of mitochondrial oxidative events

2. **Ionic events**

- ATP- induced closing of K^+ channels
- Subsequent depolarization of the plasma membrane
- Resulting gating of voltage-sensitive Ca^{2+} channels
- Ca^{2+} influx and increase in cytosolic Ca^{2+} concentration

3. **Mechanical events**

- Back-and- forth translocation of secretory granules along oriented microtubular pathways
- Access of secretory granules to exocytotic sites under control of the contractile microfilamentous cell web
- Fusion and fission of membranes at the exocytotic site
- Exocytosis-endocytosis coupling (recirculation of membranes)

(Physiology, pathology and pharmacology of insulin secretion, Acta Clinica Belgica 2000, W.J. Malaisse, Vol 55 [3]: 141).

1.6. INSULIN ACTION

1.6.1. The three levels of insulin action

The first level of action of insulin is composed of the initial events related to receptor tyrosine kinase activity. This includes the insulin receptor itself, the insulin receptor substrate (IRS), and the molecules that interact with this substrate. The second level is a cascade of serine phosphorylation and dephosphorylation reactions centred around an enzyme called MAP (mitogen-activated protein/microtubule-associated protein) kinase. The third level is final biological effectors of the insulin cascade. This includes the glucose transport molecules themselves, which reside in an intracellular pool and are translocated to the plasma membrane following insulin stimulation, the enzymes for glycogen and lipid synthesis, and the proteins involved in insulin action on gene expression and cell growth (Kahn, 1994).

1.6.2 Insulin action within the cell

Circulating insulin rapidly reaches the target tissue, where it interacts with its cognate receptor. The insulin receptor (IR), which is widely expressed, is a transmembrane tyrosine (Tyr) kinase that is expressed as a tetramer in an $\alpha 2\beta 2$ configuration (Ebina *et al.,* 1985, Ullrich *et al.,* 1985). Insulin binding to specific regions of the α-subunit leads to a rapid configuration change in the receptor that eventuates in autophosphorylation of specific Tyr residues of the intracellular region of the β - subunits through a transphosphorylation mechanism.

Autophosphorylation results in activation of the Tyr kinase activity of the receptor (Lee *et al.,* 1997). In the inactive state, the catalytic site of the Tyr kinase is occluded by the "activation-loop", preventing access of ATP and various substrates (Hubbard *et al.,* 1994). The activated IR kinase phosphorylates substrate proteins on Tyr residues, and these phosphorylated Tyr residues serve as docking sites for downstream effectors. Molecules such as Shc and IR substrate (IRS) (Feinstein *et al.,* 1993), engage the IR directly and provide a docking interface with downstream substrates.

Insulin-receptor signalling involves two major kinase pathways : the mitogen-activated protein (MAP) kinase and the phosphatidylinositol - 3- kinase (PI-3-K). Although these pathways act in a linear fashion, each pathway could, under certain circumstances, activate the other. The MAP kinase pathway is activated by binding of Grb2 to Tyr-phosphorylated Shc or IRS via its SH2 domain. Grb2 is prebound to mammalian Son of sevenless (mSOS), a nucleotide exchange protein that catalyzes the exchange of GDP for GTP on Ras (a small GTPase protein); this results in activation of Ras. The prenylated form of Ras binds the inner leaflet of the plasma membrane, and on activation, it binds the NH_2-terminal region of Raf, recruiting Raf to the plasma membrane (Roy *et al.,* 1998).

Raf-1 activates dual-specificity kinase, MEK1, by phosphorylation of two regulatory Ser residues. In turn, MEK1 activates extracellular signal-regulated kinase (ERK)-1 and ERK2 by phosphorylating regulatory Tyr and Thr residues (Marshall, 1995). Activated ERKs mediate the growth-promoting effects of insulin by phosphorylating transcription factors such as ELK-1, leading to the induction of genes.

TABLE 1.2 Metabolic responses of Tissues to Insulin

Tissue	Fasting; Low Insulin State (<10 mU/L serum)	Fed; High Insulin State (>10 mU/L serum)
Adipose	Failure to inhibit cAMP-induced lipolysis stimulated by glucagon, ACTH, STH, epinephrine Increased free fatty acid synthesis	Activation of lipoprotein lipase clears serum of endogenous and exogenous lipoproteins; increased glucose uptake and triglyceride synthesis
Muscle	Release of amino acids; leucine, valine, isoleucine; alanine, glycine, threonine, serine (glycogenic)	Amino acid uptake and protein synthesis; glucose uptake and glycogen synthesis
Hepatic	Gluconeogenesis from amino acids with energy From free fatty acids	Activation of glycogen Synthetase; glycogenesis

ACTH= Adrenocorticotropic hormone, corticotropin; cAMP= cyclic adenosine 5'- monophosphate; STH= somatotropin, growth hormone.

(Applied biochemistry of clinical disorders, 2nd ed, Allan G. Gornall (1986, p.385).

1.7. Insulin resistance

1.7.1. β-cell dysfunction

β-cell dysfunction is initially characterized by an impairment in the first phase of insulin secretion during glucose stimulation and may antedate the onset of glucose intolerance in type 2 diabetes (Marshall, 1995). The delay in the first phase of insulin secretion, although of some diagnostic importance, does not appear to act independently in the pathogenesis of type 2 diabetes. In some early-onset patients with type 2 diabetes (perhaps as many as 20%) (Ward *et al.,* 1986), there may be a deficiency in insulin secretion that may or may not be due to autoimmune destruction of the β-cell, and is not due to a deficiency in the glucokinase gene. In the great majority of patients with type 2 diabetes (± 80%), the delay in immediate insulin response is accompanied by a secondary hypersecretory phase of insulin release as a result of either an inherited or acquired defect within the β- cell or a compensatory response to peripheral insulin resistance. Over a prolonged period of time, perhaps years, insulin secretion gradually declines, possibly as a result of intra-islet accumulation of glucose intermediary metabolites (Kruszynska and Olefsky, 1996).

1.7.2. Insulin resistance

Insulin resistance is found in the majority of patients with type 2 diabetes, and it is present in the early pre-diabetic stage of impaired glucose tolerance. Insulin resistance is strongly linked to obesity, which is growing in prevalence within the developed world.

Insulin resistance in fat cells leads to increased lipolysis with release of fatty acids and a variety of sequelae, including dyslipidemia and vascular abnormalities caused by excessive amounts of circulating free fatty acids (Ginsberg, 2000). High free fatty acid concentrations also contribute to resistance to the action of insulin by enhancing glucose output from the liver and reducing glucose disposal in skeletal muscle (Boden, 1999).

In type 2 diabetes, a number of insulin's actions are impaired, which results in elevated levels of glucose and FFA. A reduction in insulin's ability to suppress gluconeogenesis and glycogenolysis in the liver may lead to a rise in hepatic glucose production, and the overall outcome will be elevated

basal glucose levels (hyperglycaemia). A reduction in skeletal muscle glucose uptake may also contribute to elevated blood glucose levels. This implies that in type 2 diabetes both glycogen synthesis and glucose oxidation are impaired and both contribute to postprandial hyperglycaemia. The loss of insulin's antilipolytic action as seen in Type 2 diabetes lead to an increase in adipose tissue lipolysis. This leads to elevated FFA and glycerol concentrations in the circulation. High circulating FFA levels, lead to a reduction in hepatic insulin clearance, which may accentuate hyperinsulinemia. Chronically elevated glucose and FFA may impair β-cell function as opposed to stimulation, a condition found in type 2 diabetes (McGarry and Dobbins, 1999; Wilding and Williams, 1998). In type 2, diabetes insulin resistance is primarily a post-receptor event rather than a defect in the insulin receptor itself.

Insulin resistance leads to type 2 diabetes only if there is an associated inability of the β-cell to compensate for the insulin resistance with appropriate hyperinsulinemia. The majority of obese individuals are insulin resistant, but only a small fraction progress to type 2 diabetes. Type 2 diabetes is preceded by insulin resistance, hyperinsulinemia, the unique dyslipidemia, and obesity in 75 – 85% of the patients.

Table 1.3. Causes of insulin resistance

I.	Abnormal β- cell secretory product
	A. Abnormal insulin molecule
	B. Incomplete conversion of proinsulin to insulin
II.	Circulating insulin antagonists
	A. Elevated levels of counterregulatory hormones, e.g.,growth hormone, cortisol, glucagons, or catecholamines
	B. Anti- insulin antibodies
	C. Anti- insulin receptor antibodies
III.	Target tissue defects
	A. Insulin receptor defects
	B. Post- receptor defects

Olefsky J.M. Insulin resistance and insulin action, Diabetes vol 30, pg149, 1981.

1.7.3. Obesity

Obesity is known to cause insulin resistance (Kahn, 1996). The mechanism by which this occurs has been extensively studied and it is now thought that factors secreted by adipocytes such as TNF alpha, free fatty acids and adiponectin (Ahima and Flier, 2000) may influence insulin sensitivity. Furthermore, triglyceride deposition in the muscle, which is higher in obese subjects has also been shown to reduce insulin sensitivity (Perseghin *et al.,* 1999). A large body of recent research has begun to elucidate the underlying pathophysiologic mechanisms linking adipose tissue with insulin sensitivity as well as vascular endothelial dysfunction (Ahima and Flier, 2000). One of the most revolutionary concepts is that adipose tissue is not only a simple reservoir of energy stored as triglycerides, but also serves as an active secretory organ, releasing many peptides, complement factors, and cytokines into the circulation (Ahima and Flier, 2000). The secretion of these factors is altered by increasing overall adiposity as well as the distribution of the stored adipose tissue, in particular attributing adverse effects of obesity to visceral and intra-abdominal adiposity as opposed

to subcutaneous adipose tissue which appears to be less pathogenic (Ahima and Flier, 2000). Recent research has delineated specific mechanisms whereby excess adiposity can influence the normal cellular actions of insulin and contribute to insulin resistance. Among other factors released by adipose tissue, free fatty acids (FFA), which comprise the lipid side-chains of the triglyceride molecule, are elevated in persons with increased visceral adipose tissue (Boden, 2001). When FFA are elevated for a prolonged amount of time, they have a direct effect on insulin action in skeletal muscle tissue and liver, reducing the normal responses to insulin to promote glucose uptake and to suppress hepatic glucose output, respectively (Boden, 2001; Shulman, 2000). Other proteins secreted by adipose tissue, including the important inflammatory mediators interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα), have adverse effects on energy metabolism and insulin sensitivity in liver and muscle, and play key roles in the development of insulin resistance in obesity (Goldstein, 2003).

Table 1.4

Influences of Obesity on the pathogenesis of Type 2 diabetes and Cardiovascular disease

- Factors secreted from tissue with adverse effects ; eg. Free fatty acids, cytokines
  - Promote resistance to insulin effects in skeletal muscle and liver
  - Contribute to endothelial dysfunction
  - Chronic exposure may adversely affect pancreatic β-cell function
- Factors secreted from adipose tissue with salutary effects; eg, adiponectin
  - Enhances insulin sensitivity in skeletal muscle and liver
  - Protective effects on vascular function
  - Unknown effects on pancreatic β-cell
- Factors related to local accumulation of visceral fat and increased tissue acyl CoA derivatives
  - Specific defects in insulin signal transduction
  - Toxic effects on pancreatic β-cell insulin secretion and β-cell mass

(Adapted from Goldstein, 2003)

1.8. TYPE 2 DIABETES THERAPY

Therapy of type 2 diabetes involves modifications of lifestyle and diet, and use of oral hypoglycaemic agents or insulin. Dietary modifications include limiting total caloric intake, increasing the percentage of calories from complex carbohydrates and reducing the intake of fats and cholesterol (Ilarde and Tuck, 1994). To be effective, therapeutic interventions for type 2 diabetes must reduce hepatic glucose production either by improving islet dysfunction and raising plasma insulin levels, or improving the effectiveness of insulin on the liver, skeletal muscle and adipose tissue (Porte and Kahn, 1991). The use of oral hypoglycemic drugs may be effective in controlling blood glucose levels, but may not prevent all the complications of diabetes..

The fasting blood glucose (FBG) concentration is a reproducible and reliable indicator of overall glycaemic control in type 2 diabetes, whilst random glucose measurements are helpful as postprandial glycaemic excursions are variable. In most type 2 diabetic patients, treatment should aim to lower FBG to below 6 mmol/l; symptomatic control may be adequate in elderly patients (Shulman, 2000).

1.8.1 Sulfonylurea therapy

The sulfonylureas bind to specific membrane receptors on β-cells and cause membrane depolarisation by directly inhibiting K_{ATP} channels (Phillipson and Steiner, 1995), which permits intracellular build up of calcium. The resultant stimulus- secretion coupling leads to insulin secretion (Misler and Giebisch, 1992; Phillipson and Steiner, 1995). Some patients with type 2 diabetes do not respond to sulfonylurea therapy (primary failure), and others fail to do so after having responded for several years (secondary failure) (Groop,1992).

None of the currently available sulfonylureas completely normalise insulin secretion and action (Beck-Nielsen *et al.*, 1988). Failure to respond to sulfonylurea drugs may be primary (25% to 30% of initially treated patients) or secondary, occuring in 5% to 10% of patients per year (Ilared and Tuck, 1994). Combination therapy with insulin and a sulfonylurea agent only slightly improved glycemic control in type 2 diabetics - less exogenous insulin was needed but fasting serum insulin

levels showed no difference between treatment groups. This therapy did not produce near - normal blood glucose levels and so it is not recommended for poorly controlled type 2 diabetics receiving insulin (Peters and Davidson, 1991).

Hypoglycemia, weight gain, and hyperinsulinemia are notable side effects of sulfonylureas (Dagogo-Jack and Santiago, 1997).

1.8.2 Biguanide therapy

Another class of drugs, the biguanides, was also shown to be effective. Metformin is a less toxic biguanide which can be a useful adjunct in type 2 diabetic therapy, since it improves peripheral sensitivity to insulin through stimulating tissue glucose uptake (Sirtori and Pasik, 1994).

Metformin enhances peripheral glucose uptake and reduces hepatic glucose output; it lowers blood glucose concentrations in hyperglycaemic, but not in normoglycaemic, people. Its main side effect, lactic acidosis, is more common in renal impairment and may be precipitated by tissue anoxia or ethanol ingestion (Vaverde *et al.*, 1979). Its antidiabetic action seems to be targeted at the underlying insulin resistance (Bailey and Turner, 1996). Unlike other therapies for diabetes, metformin does not cause weight gain.

1.8.3 Insulin therapy

Other therapeutic options for NIDDM include the use of insulin - sparing antihyperglycemic agents such as α- glucosidase inhibitors, thiazolidinediones, chloroquine or hydroxychloroquine, or fibric acid derivatives such as clofibrate. Other experimental agents include fatty acid oxidation inhibitors and dichloroacetate. To prevent the complications arising from the spectrum of clinical and metabolic abnormalities which arise from insulin resistance, other specific agents may be used, including antihypertensives, lipid lowering agents and sorbitol inhibitors (Ilarde and Tuck, 1994). Insulin treatment of type 2 diabetes is indicated for (1) initial stabilization of patients with severe hyperglycemia, (2) established patients with hyperglycaemic crises, and (3) patients in whom oral hypoglycaemic therapy has failed (Dagogo-Jack and Santiago, 1997). Sufficient doses of exogenous

insulin reduce hyperglycemia and improve glucose toxicity in virtually all persons, but this treatment also causes hyperinsulinemia and weight gain (Ducimeteire *et al.*, 1980).

1.8.4 Thiazolidinedione therapy

There is a growing body of evidence that thiazolidinediones (TZDs) rejuvenate β-cells and improve their function. Studies in diabetes-prone (db/db) mice have shown rejuvenation of β-cells with rosiglitazone but not with glyburide or metformin (Lister *et al.*, 1999). After 28 days of treatment with rosiglitazone, metformin, or glyburide, only rosiglitazone-treated mice had significantly increased pancreatic insulin concentrations. In addition, in obese Zucker diabetic rats, rosiglitazone maintained β-cell proliferation, thus preventing loss of β-cell mass (Finegood *et al.*, 2001) Troglitazone is a member of antidiabetic drugs that improve insuin resistance. Although its exact mechanism of action is unknown, troglitazone appears to improve the action of insulin in liver, skeletal muscle, and adipose tissue directly (Wamoto *et al.*, 1991), and it reduces elevated plasma glucose and insulin concentrations in diabetic animals with hyperinsulinemia. The administration of troglitazone to patients with NIDDM improves both fasting and postprandial hyperglycemia and insulinemia. This reduction in hyperglycemia is associated with a near normalization of the rates of hepatic glucose production and a 40-to- 60- percent increase in insulin-mediated glucose disposal as measured by the glucose-clamp technique (Suter *et al.*, 1992).

Several clinical studies using the homeostasis model assessment method, which indirectly calculates insulin production, have shown an increase in endogenous insulin production with TZDs when used either as monotherapy or in combination with another antidiabetic agent. In type 2 diabetes, as pancreatic β- cells fail, more of the insulin precursor proinsulin is produced and the proinsulin- to – insulin ratio increases (Suter *et al.*, 1992). The proinsulin-to- insulin ratio has been shown to decrease (i.e. improve) significantly with rosiglitazone treatment, but not with sulfonylureas or placebo (Ward *et al.*, 1987). This indirectly indicates that rosiglitazone treatment improves β- cell function, whereas pancreatic β-cells continue to function at reduced levels despite sulfonylurea therapy.

1.8.5 Lipolysis inhibitors

Nicotinic acid (niacin), a vitamin of the B complex, has been used for almost 50 years as a lipid-lowering drug. The pharmacological effect of nicotinic acid requires doses that are much higher than those provided by a normal diet. Its primary action is to decrease lipolysis in adipose tissue by inhibiting hormone-sensitive triglyceride lipase. This antilipolytic effect of nicotinic acid involves the inhibition of cyclic adenosine monophosphate (cAMP) accumulation in adipose tissue through a G(i)- protein- mediated inhibition of adenylyl cyclase (Unaru *et al.*, 2003).

Acipimox (5-methylpyrazine carboxylic acid 4-oxide) is a potent and long acting antilipolytic drug derived from nicotinic acid. Acute acipimox administration to type 2 diabetics markedly (30%) improved both oxidative and nonoxidative glucose disposal rates during a high physiological insulin infusion (Unaru *et al.*, 2003). Furthermore, acute acipimox administration enhanced the suppressive effect of insulin on HGP in patients with type 2 diabetes (Vaag *et al.*, 1991) In contrast, prolonged (3 months) acipimox treatment (250mg, three times per 24h) had no beneficial effects in type 2 diabetics on fasting plasma glucose or glycohemoglobin (percentage) levels . This was apparently related to a rebound increase in NEFA levels (Saloranta *et al.*, 1991).

Both nicotinic acid (NA) and the adenosine receptor agonist phenylisopropyladenosine (PIA) are potent antilipolytic agents (Reaven *et al.*, 1988). Given the evidence that elevations in FFA concentration can impair the ability of insulin to both stimulate glucose uptake and suppress hepatic glucose output, it is surprising that uncertainty still exists as to the ability of antilipolytic agents to lower plasma glucose concentrations. This situation is almost certainly due to the fact that nicotinic acid (NA), an extremely potent antilipolytic agent, has been reported to both increase and decrease plasma glucose concentration (Ammon *et al.*, 1970, Dzedin *et al.*, 1970). PIA and NA seem to suppress lipolysis by interacting with specific, distinct, cell surface receptors that inhibit adenylate cyclase activity (Aktories and Jakobs, 1982). Indeed, measurements of the effect of PIA on glucose metabolism have indicated that glucose levels significantly increase after PIA injection into nondiabetic animals, possibly due to stimulation of glucogon secretion (Petrack *et al.*, 1981). However, those studies were carried out with relatively large amounts of PIA (1 μmol/kg). The fact

that the decrease in plasma FFA precedes the decrease in glucose concentration when either PIA or NA is given to rats with streptozotocin-induced diabetes does not necessarily mean that the changes in plasma glucose are caused by the decrease in plasma FFA. However, the common parallels between these two distinct drugs, which act at different receptors, suggest that their similar ability to inhibit lipolysis and suppress plasma concentration of FFA may be closely related to their ability to decrease plasma glucose in diabetic animals (Reaven *et al.*, 1988).

The antilipolytic effects of PIA and NA could enhance insulin-stimulated glucose uptake by lowering plasma FFA concntration. Furthermore, both PIA and NA were shown to increase glucose uptake in isolated adipocytes, and the ability of PIA to do this is consistent with previous observations (Taylor and Halperine, 1979). There is evidence that an increase in FFA flux to the liver can augment hepatic glucose production in perfused rat liver (Blumenthal, 1983; Ruderman *et al.*, 1969) and a direct relationship between hepatic glucose production and fasting and ambient plasma FFA concentrations has been documented in patients with diabetes.

1.9. ANTI-DIABETIC EFFECT OF PLANTS

Since the discovery of insulin, numerous substances, natural and synthetic, have been investigated for their blood sugar lowering effects. Although most of these drugs do not necessarily resemble insulin in their mechanism of action, some of them have certain beneficial effects on certain types of diabetes. Plants and plant products are also known to have certain antidiabetic effects. In this respect, Best and Scott in 1923 demonstrated an insulin - like activity in extracts prepared from potatoes, wheat, beetroot and celery and in 1938, Otto Hippokrates noticed that immature green bean pods contain a substance having an effect similar to insulin, which in experiments on animals lowered the blood sugar during fasting and weakened the hyperglycemia following the administration of dextrose and adrenaline (Sharaf *et al.*, 1963). He also noticed that a diet of bean pods reduced the glycosuria, acetonuria and actonemia of diabetic persons (Sharaf *et al.*, 1963).

1.9.1 South African antidiabetic plants

Various morphological parts of *Clausena anisata* (Wild hook) [family: Rutaceae], locally known as " isifudu" in the South African isizulu language have been reported to be useful as effective remedies against epilepsy or convulsions, arthritis, rheumatism and other inflammatory conditions, hypertension, heart failure and other heart ailments, schistosomiasis, taeniasis and other parasitic infections, constipation and abdominal pains, malaria and other febrile conditions, headaches, body pains, eye complaints, impotence and sterility. South African traditional healers have stated that adult diabetic patients who took *C. anisata* methanolic root preparation as a remedy for hypertension had their elevated blood glucose concentrations markedly reduced (Ojewole, 2002).

A study to evaluate the hypoglycaemic effect of *Sclerocarya birrea* [family: Anacardiacea], also known as 'Marula' in South Africa., in normal (normoglycemic) and in streptozotocin (STZ)-treated, diabetic Wistar rats has been carried out in South Africa. Following acute treament, relatively moderate to high doses of *S. birrea* stem- bark extract (SB, 100 – 800 mg/kg p.o.) produced dose-dependent, significant reductions in the blood glucose concentrations of both fasted normal and fasted diabetic rats. Administration of the single dose of *S. birrea* stem-bark aqueous extract (SB, 800 mg/kg p.o.) significantly reduced the blood glucose levels of both fasted normal (normoglycemic) and fasted STZ-treated, diabetic rats (Ojewole, 2003).

Hypoxis hemerocallidea (family: Hypoxidaceae) corm extract (locally known as African "Potato") at a dose of 800 mg/kg, caused 30.20% and 48.54% reductions in the blood glucose concentrations of fasted normal and STZ–treated diabetic rats, respectively. While it is likely that the hypoglycaemic effect of the plant extract is largely due to its phytosterols and/or sterolin content, the exact mechanism of its hypoglycaemic action is still obscure and will have to await further studies. The herb is used in the control and/or management of adult- onset, type 2 diabetes mellitus in some communities of South Africa (Mahomed and Ojewole, 2004).

In one exploratory study done at the university of Port Elizabeth , South Africa, fasting blood glucose levels of rats drinking *Sutherlandia frutescens* (kankerbos – a medicinal plant used for

diabetes) increased glucose uptake in the muscle, more so than in fat and liver tissue. *S. frutescens* seemed to cause weight gain and a decrease in metabolic rate (Chadwick *et al.*, 2002).

Traditional use of an infusion of *Medicago sativa* (alfalfa) leaves in South Africa may be associated with the high manganese content of this plant (Rubenstein *et al.*, 1962). Manganese chloride has been shown to exert a hypoglycemic action in type 1 diabetic patients, and manganese is now recognised as a necessary cofactor for ATP phosphorylation of the β - subunit of the insulin receptor (Rubenstein *et al.*, 1962; Reddy and King, 1987). An antidiabetic effect of alfalfa, which has been substantiated in streptozocin-induced diabetic mice, might also be associated with the plant's high concentration of vitamin K (Swanston-Flatt *et al.*,1989a). The synthetic analogue vitamin k_5 can acutely mimic certain actions of insulin (Kuo, 1969).

Catharanthus Roseus is used in South Africa as an antidiabetic. Water decoction of the leaves and/or the whole plant is used as household remedy for diabetes in several countries. Earlier reports indicate significant blood glucose lowering activity with hydroalcoholic or dichloromethane-methanol extracts of leaves of *C. roseus* in laboratory animals (Stolle *et al.*, 1967). Alloxan-treated animals receiving the leaf juice of C. roseus showed rapid normalization of blood glucose levels in comparison to control, and this could be due to the possibility that some β-cells are still surviving to act upon by *C. roseus* to exert its insulin releasing effect. Moreover, like sulphonylureas oral administration of *C. roseus* produced hypoglycaemia in normal animals (Nammi *et al.*, 2003).

1.9.2. Non-African antidiabetic plants

Viscum album (mistletoe) is a member of the Loranthacaea familly. It has been reported to have a number of medicinal properties, including the ability to lower blood pressure, slow the heart beat, stimulate the immune system, relax spasms and exert sedative, diuretic and anti- cancer effects. A tea prepared from the leaves of mistletoe is used traditionally to treat diabetes in the West Indies (Peters, 1957). This treatment has been shown also to relieve the diabetic symptoms of severely hyperglycemic streptozotocin - diabetic mice, including polydipsia, hyperphagia and body weight loss (Swanston-Flatt *et al.*, 1989b).

Raw onion bulbs (*Allium cepa*) and garlic cloves (*Allium sativum*) have long been used as dietary supplements for the traditional treatment of diabetes in Asia, Europe, and the MiddleEast (Day, 1984). Concentrated extracts from the plant organs exerted a weak hypoglycemic effect in healthy and alloxan- induced diabetic animals and healthy humans. In these studies, fasting glucose concentrations were lowered, and oral glucose tolerance was improved by 7- 18% within 1-2 h after oral administration of aqueous and ethanolic extracts of onion and garlic at doses of ~ 10g extract/kg body weight. This effect has been attributed to the volatile oils, allylpropyldisulfide and diallyldisulfide oxide, excessive amounts of which can have detrimental effects on hepatic metabolism (Augusti and Benaim, 1975, Augusti, 1974).

The root of *Panax ginseng*, or Asian ginseng, has been used clinicaly to treat type 2 diabetes (Bensky *et al.*, 1993; Huang, 1999) and has been used as a tonic, often taken for years without evidence of adverse effects or toxicity (Attele *et al.*, 1999). Results of *in vitro* and *in vivo* animal studies and clinical trials (Sotaniemi *et al.*, 1995; Vuksan *et al.*, 2000) support the claim that the root of panax ginseng and the root of other ginseng species (e.g. *Panax quinquefolius*, or American ginseng) possess antihyperglycemic activity. These previous studies have not investigated the mechanisms responsible for the antidiabetic effects of *Panax ginseng*, which are yet unknown. The active components of ginseng are considered to be ginsenosides, a group of steroidal saponins (Huang 1999; Attele *et al.*, 1999). Ginsenosides are distributed in many parts of the ginseng plant, including the root, leaf, and berry (Attele *et al.*, 1999).

The history of biguanides can be traced from the use of *Galega officinalis* (goat's rue or French lilac) as a treatment for diabetes in medieval Europe (Bailey and Day, 1989). Guanidine, the active component of galega, was used to synthesize several antidiabetic compounds in the 1920s, and metformin and phenformin, the two biguanides, were introduced in the late 1950s (Sterne, 1969; Beckman, 1971). In medieval times, a prescription of *Galega offinalis* was said to relieve the intense urination accompanying the disease that came to have the name of diabetes mellitus. *G. officinalis*, was also given during the plague epidemics to promote perspiration, and has been used as a galactogogue in cows. The active ingredient in the French lilac that produced the lowering of

blood glucose was shown to be galegine or isoamylene guanidine (Cusi and DeFronzo, 1998). Guanidine and some of its derivatives are too toxic for the treatment of diabetes mellitus, the biguanides (two linked guanidine rings) have proved useful (Cusi and DeFronzo, 1998). Metformin, a less lipophilic biguanide, proved safer and, after 20 years of use in Europe, was approved for use in the USA in 1995. It is now a mainstay of therapy in the treatment of type 2 diabetes and, as noted in the recently terminated Diabetes Prevention Program, is also an effective agent to decrease the risk of development of the disease (Cusi and DeFronzo, 1998; Bailey *et al.*, 1996; Wiernsperger *et al.*, 1999). Metformin not only lowers blood glucose but also inhibits adipose tissue lipolysis, reduces circulating free fatty acids, and diminishes VLDL production (Cusi and DeFronzo, 1998; Bailey 1996). In addition, metformin appears to suppress the gluconeogenic effects of glucagon and to increase the translocation of glucose transporters to the cell surface (Wiernsperger and Bailey, 1999).

Agaricus bisporus (common edible mushroom) is considered a useful dietary adjunct for diabetes in Europe, and a hypoglycemic effect has been shown in streptozocin-induced diabetic mice. *A. bisporus* consumption improved insulin sensitivity in diabetic mice, and a lectin from this mushroom stimulated insulin release by isolated islets of healthy rats (Swanston-Flatt *et al.*, 1989b; Ahmad *et al.*, 1984).

Consumption of several plant remedies reduced the rate of development and the extent of hyperglycemia in streptozocin-induced diabetic mice: leaves of *Agrimonia eupatoria* (agrimony) and *Eucalyptus globulus* (eucalyptus), seeds of *Coriandrum sativum* (coriander), and berries of *Juniperus communis* (juniper) (Swanston-Flatt *et al.*, 1989b).

Several traditionally recommended vegetables, notably leaves of *Brassica oleracea* (cabbage) and *Letuca sativa* (lettuce), tubers of *Solanum tuberosum* (potato), and roots of *Brassica rapa* (turnip) have been claimed to yield nonfibrous hypoglycemic extracts, some of which lowered glucose concentrations after parenteral administration (Peters, 1957; Sharaf *et al.*, 1963; Collip, 1923).

Sambucus nigra (elder) flowers contain water-soluble natural products which directly stimulate glucose metabolism by muscle and promote insulin secretion from clonal pancreatic β-cells. Studies to evaluate the possible mechanisms underlying the insulin-releasing action of elder indicated a similarity to the sulfonylurea class of drugs currently used for diabetes therapy (Bailey and Day 1989). Elder flowers contain a number of established natural products including lectin, the flavenoid rutin, choline tannin and lipophilic triterpenoid and sterol compounds such as lupeol and β-sitosterol.

In North America, an alkaloid extract from *Ruta graveolens* (rue) rutin has been recommended for diabetes, but its efficacy is unconfirmed (Duke, 1985). *R. graveolens* is added in trace amounts to foods and beverages as a flavouring, although large amounts are narcotic, irritating to skin, abortifacient, and contra-indicated in pregnancy. Rutin has been ascribed beneficial effects in patients with cardiovascular and thrombotic disoders in addition to diabetes, but its mode of action is uncertain (Duke, 1985).

Several plants that are traditionally used for the treatment of diabetes exerted little or no effect on glycemic control during recent studies in streptozocin-induced diabetic mice i.e. *Anacardium occidentale* [cashew], *Taraxacum officinale* [dandelion], *Sambucus nigra* [elder], *Humulus lupulus* [hops], *Salvia officinale* [sage], *Daucus carrota* [wild carrot] and *Convallaria majalis* [lily of the valley] (Swanston-Flatt *et al.*,1989b).

1.9.3 Diabetic complications and plant remedies

It is possible that some traditional treatments for diabetes may create a feeling of improved well-being without necessarily reducing hyperglycemia. No controlled studies of microvascular, macrovascular, or neuropathic complications have been undertaken with traditional therapies. A plant that is thirst quenching or increases sympathetic tone might gain a traditional reputation as efficacious without providing any long-term benefits to the underlying malady. Members of the allium family, particularly garlic, are traditionally considered to give strength and combat arterial vascular disease (Day, 1984). Garlic reduces polydipsia and weight loss in severely streptozocin-

induced diabetic mice without improving glycemic control (Swanston-Flatt *et al.*, 1989b). Other traditional antidiabetic plants have been anecdotally claimed to possess hypolipidemic properties.

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1. Plant Material

(i) Dried coriander seeds

(ii) Linalyl acetate

(iii) Devil's claw (*Harpagophytum procumbens*)

(iv) Natal mahogany (*Trichilia emetica*)

(v) Buffalo-thorn (*Ziziphus mucronata*)

The Devil's claw roots were supplied by Dr A.M. Viljoen of the Department of Pharmacy and Pharmacology, University of the Witwatersrand. The plant had previously been confirmed to be Devil's claw by Dr Viljoen, and was a commercial sample obtained from Namibia. The coriander seeds were bought from a commercial source in Johannesburg, South Africa. Linalyl acetate is one of the components of the oil obtained from coriander, and was obtained from Sigma. Natal mahogany and buffalo-thorn are not normally used for the treatment of diabetes and were therefore used to determine whether anti-diabetic properties are specific to plants used for treating the disease. The Natal mahogany was collected by Dr A. DeCastro in Swaziland, whilst the buffalo thorn was collected in Johannesburg by Dr A.M. Viljoen.

2.2. Preparation of an aqueous extract

The coriander, Devil's claw, Natal mahogany and buffalo-thorn aqueous extracts were prepared as follows: The dried coriander seeds and Devil's claw, Natal mahogany and buffalo-thorn roots were crushed to a fine powder and stored at room temperature in screw-top jars. One gram of powdered material was placed in 40 ml cold water, brought to boil, then removed from the heat source and allowed to infuse for 15 minutes. This suspension was filtered (Whatman no.1) and each volume readjusted to 40 ml with distilled water. Five millilitre portions of extract were stored at -20°C until use.

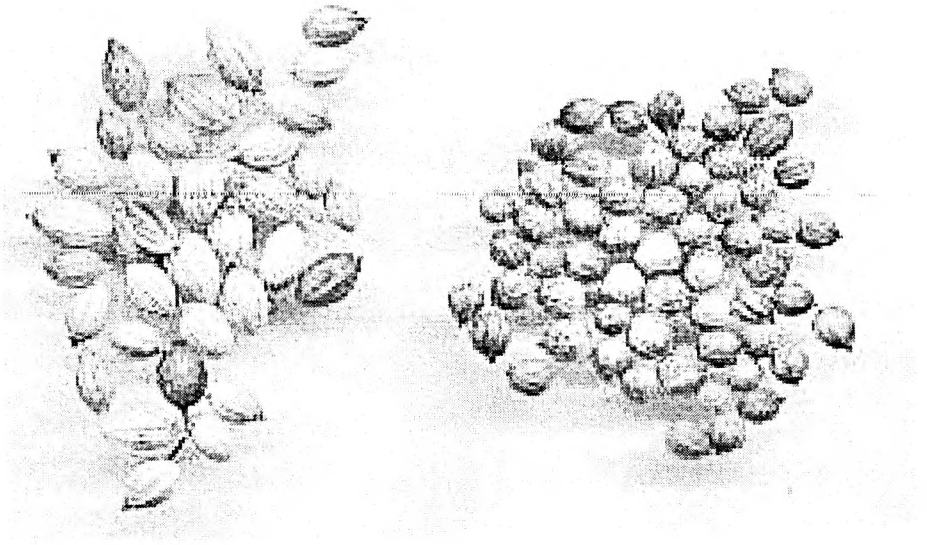

Fig 2.1. Coriander seeds

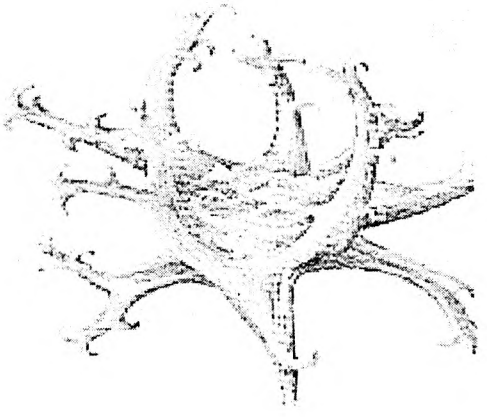

Fig 2.2. Devil's claw (*Harpagophytum procumbens)*

2.3. Experimental procedures

2.4. Cell culture

(i) 3T3-L1

The 3T3-L1 cells (preadipocytes) were grown to confluence in 4 ml of DMEM (Dulbecco's modified Eagle's medium, Invitrogen corporation) supplemented with 10% fetal calf serum (Gibco BRL life technologies Ltd) , and incubated at 37°C in 25cm^2 culture flasks. A day after confluence, the cells were fed with 4 ml DMEM containing 10% FCS, 220 nM dexamethasone, 0.5mM IBMX and 2 μM of insulin to induce the differentiation process. The culture medium was removed after 72 h and replaced with fresh DMEM containing 2 μM of Insulin. This culture medium was also removed after 72 h and replaced with fresh culture medium (DMEM) supplemented with 10% fetal calf serum only. After 8 days more than 90% of the cells differentiated into adipocytes, with large intra-cellular lipid droplets clearly visible. (see figure 3).

(ii) MIN 6

MIN 6 cells were grown in 1 ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen corporation) and incubated at 37°C in a 24-well plates. The medium was supplemented with 10% fetal calf serum. After 48-72 h of culture, the cells were confluent and ready to use.

2.5 Lipolysis assay

2.5.1. Isoproterenol test

The 3T3-L1 cells were incubated as described earlier and their lipolytic activity tested using the catecholamine derivative, isoproterenol.

Six (6) 25cm^2 tissue culture flasks containing the 3T3 cells were incubated for 2hrs with serum-free DMEM. After incubation the cells were then washed with Krebs Ringer –

Bovine Serum Albumin (KRB-BSA) [7.48 g/l NaCl, 0.35g/l KCl, 0.14g/l CaCl, 0.15g/l $MgSO_4$, 1.38g/l $NaH_2PO_4.H_2O$]. Three ml of KRB-BSA buffer was added to flask 1, and different concentrations of isoproterenol (10, 3, 1, 0.3, and 0.1 mM) in KRB-BSA buffer was added to the remaining five (5) flasks. Cells were incubated for 2 hrs. At 30 min, 1 hr and 2 hrs after incubation 300µl was removed from each flask and placed in Eppendorf tubes and stored at -20°C until assayed for FFA levels. FFA was measured by a spectrophotometric method. (Free fatty acid, Half- micro test kit, Roche Diagnostics GmbH).

2.6 Lipolysis inhibition assay

Six (6) 25 cm^2 tissue culture flasks containing the 3T3-L1 cells were incubated for 2 hrs with serum-free DMEM. After incubation the cells were then washed with KRB-BSA buffer. Three ml of KRB-BSA buffer was added to the first flask , 3 ml 1 mM isoproterenol was added to the remaining five (5) flasks with different volumes (300, 150, 50, 20 µl) of the plant aqueous solution or 60, 30, 10 and 4 µl of the coriander oil (lanolyl acetate) or 112, 56, 28 and 7 nM of insulin. Cells were incubated for 2 hrs. At 30 min, 1 hr and 2 hrs after incubation 300µl was removed from each flask and placed in Eppendorf tubes and stored at -20°C until assayed for FFA levels.

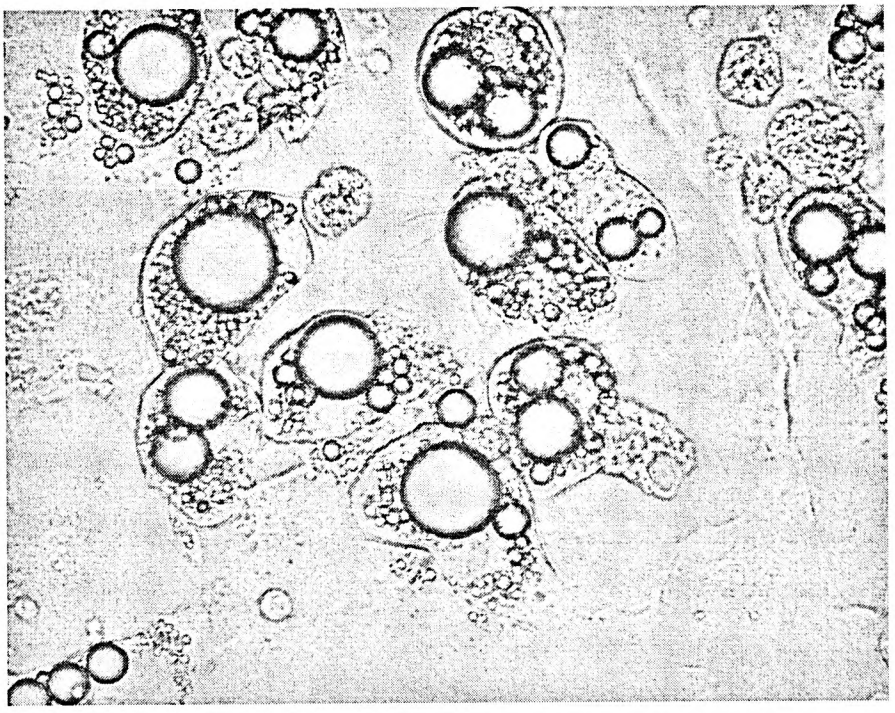

Figure 2.3 (a). Differentiated 3T3-L1 cells

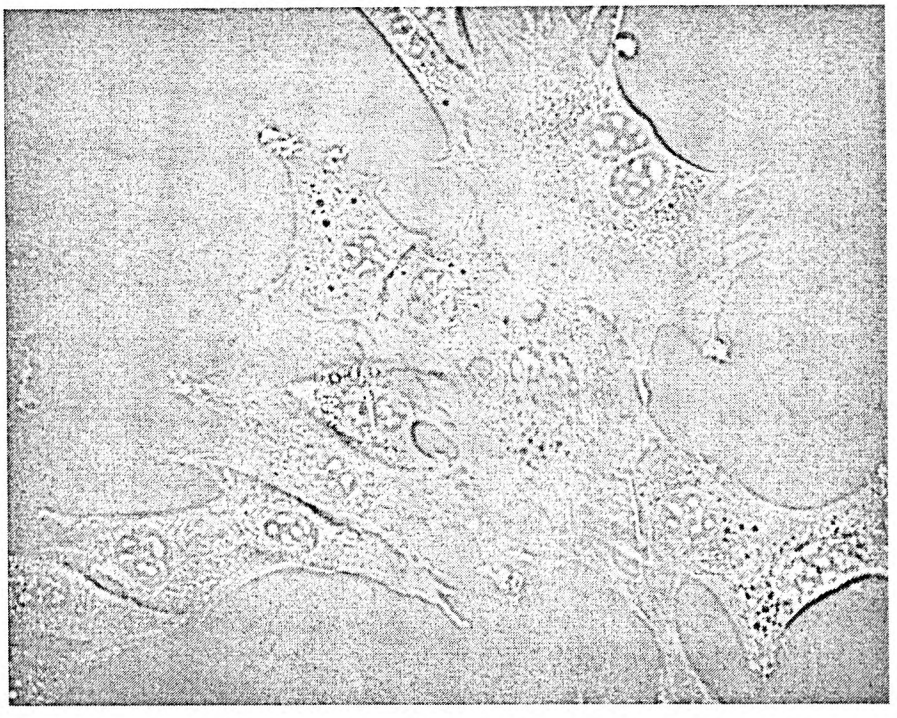

Figure 2.3(b). Unifferentiated 3T3-L1 cells

Table 2.1. The Free fatty acids, Half- micro test procedure

Add to wells	Blank	Standard	Sample
Reaction mixture A	200 µl	200 µl	200 µl
Sample	—	—	10 µl
Redist. water	10 µl	—	—
Mix, bring to 25°C for approx. 10 min			
2. Add N-ethyl-maleinimide (10µl) to each cuvette			
3. Mix and read absorbances of the solutions (at 540 nm).			
4. Start reaction with react Mixture B Mix, for 10 min and read absorbances of the solutions (540 nm)			

2.7. Palmitic acid (standard solution for FFA assay)

(i) Reagents

Triton X-100 (Roche Molecular Biochemicals)

Ethanol

Palmitic acid (Sigma)

(ii) Method

Solution 1: Six (6) grams of triton X-100 was added to 80 ml of redistilled water (30°-40°C), it was allowed to cool to 15 – 25°C and made up to 100 ml in a measuring cylinder.

Solution 2: Nine (9) mg of palmitic acid (molecular weight, 256.4) was dissolved in 6 ml of warm ethanol (about 35- 40 °C). the beaker was immediately sealed with Parafilm and allowed to cool to 15- 25°C. About 80 ml of solution 1 was added to solution 2, stirring slowly for 30 min using a

magnetic stirrer, the solution was made up to the mark in a 100 ml volumetric flask with solution 1 to give a final concentration of 0.38 mM.

2.8. Insulin secretion assay

MIN6 cells were grown in 1ml DMEM in 24-well plates as described previously. After confluence, the medium was removed and cells were washed once with HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mmol/l NaCl, 4.74 mmol/l KCl, 2.54 mmol/l $CaCl_2$, 1.19 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 10 mmol/l HEPES, pH 7.4) containing 0.5% bovine serum albumin (BSA) without glucose. Next, cells were preincubated for 0.5 h in HEPES-balanced Krebs-Ringer bicarbonate buffer with 0.5% BSA and 5 mmol/l glucose. After washing twice with HEPES-balanced Krebs-Ringer bicarbonate buffer, cells were incubated for 2 h in HEPES-balanced Krebs-Ringer bicarbonate buffer supplemented with 0.5% BSA and varying concentrations of glucose and a plant extract. The samples were removed for insulin assay using the Mercodia rat insulin ELISA kit. To each well 200 µl of 1 mol/l NaOH was added to solubilize cells for determination of cellular protein content using the Bradford method.

The insulin ELISA (see Table 2.2 for summary) used an anti-rat insulin monoclonal antibody immobilised in wells of a 96-well plate. Samples and standards (25 µl aliquots) were added to wells and incubated for 1 hour with 100 µl of an anti-rat insulin antibody-peroxidase conjugate at room temperature on a shaking platform. The plate was washed 6 times with wash solution and after the final aspiration, 200 µl of a solution containing the peroxidase substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added to each well for 15 minutes. The reaction was stopped by addition of 50 µl of 0.5 M H_2SO_4. The production of coloured-end product was assessed spectrophotometrically at 450 nm.

Table 2.2. Mercodia Rat Insulin ELISA

Add standards and samples	**25 µl**
Add Conjugate solution	**100 µl**
Incubate	**1 hour at 20- 25°C on a shaker**
Wash	**6 times**
Add substrate	**200µl**
Incubate	**15 minutes**
Add stop solution	**50 µl** **Shake for 5 sec to ensure mixing**
Measure A_{450}	

2.9. Bradford Method for Proteins

Reagents

100 mg Coomasie G-250

50 ml 95% Ethanol

100 ml 85% Phosphoric acid

850 ml distilled water

The reagents were mixed, stirred, filtered and stored in a dark bottle.

To prepare albumin standard

Stock 1 mg/ml in water

50 mg albumin in 50 ml water was prepared by dissolving and stored frozen in 1ml aliquots.

Table 2.3. Preparation of the Standard curve

Stock Albumin (µl)	H2O (µl)	Concentration (ug/ml)
0	1000	0 (blank)
20	980	20
40	960	40
60	940	60
80	920	80
100	900	100

2.10. Assay method

Five millilitre tubes were labelled in duplicate according to standards and samples, 300 µl standard or sample was added to each of the duplicate and 2.7 ml Coomasie reagent was added mixed and the absorbance was read at 595 nm on a spectrophotometer.

2.11 Statistical analyses and data presentation

The FFA release from the 3T3-L1 cells was used as a measure of lipolysis. This variable was expressed as the concentration, µmol/l of FFA present in the incubation medium. In the dose-response experiment with isoproterenol, FFA release was also expressed as a percentage of the FFA concentration in the absence of isoproterenol. In the experiments in which lipolysis was measured in the presence of insulin or plant extracts FFA release was expressed as a percentage of the FFA concentration observed in the presence of 3 mmol/l isoproterenol but absence of insulin or plant extract.

Insulin secretion from MIN6 cells was expressed as nmol insulin released per g of cell protein and was also expressed as a percentage of the insulin levels in the absence of glucose or the absence of plant extracts.

Mean levels of FFA release were compared with that in the presence of 3 mmol/l isoproterenol using the Students paired t-test. Differences in total (area-under-the-curve, AUC) FFA release, calculated using the trapezoid rule, were analysed across the different incubation time points using ANOVA. Insulin secretion was compared with that in the absence of any treatment using the Students paired t-test.

3.0 RESULTS

3.1 Effects of isoproterenol on lipolysis

Figure 3.1 shows that isoproterenol over the concentration range of 0.1 to 10 mmol/l increased the lipolytic activity of the 3T3-L1 cells, and that FFA release into the medium increased over time. A statistically significant ($p<0.05$ vs 0 mmol/l isoproterenol) activation of FFA release was demonstrated after 2 hours of incubation with 3mmol/l isoproterenol (see Fig 3.1A). When FFA release was expressed as a percentage of that observed in the absence of isoproterenol, statistically significant increases in lipolysis were observed in the presence of 3 and 10 mmol/l isoproterenol at 1 hour and with 3 mmol/l isoproterenol at 2 hours (Fig 3.1B).

Table 3.1 gives values for the area-under-the-curve (AUC) for the graphs in Fig 1. It can be seen that FFA release in to the medium did increase over time, but the trend was not significant (see Table 3.1A). However, this trend was significant for FFA release when expressed as a percentage (Table 3.1B). This may be due to the smaller SDs obtained for the percentage compared to the molar data.

These data demonstrate that 3 mmol/l isoproterenol causes a significant activation of lipolysis and therefore, this concentration of isoproterenol was used in all future experiments.

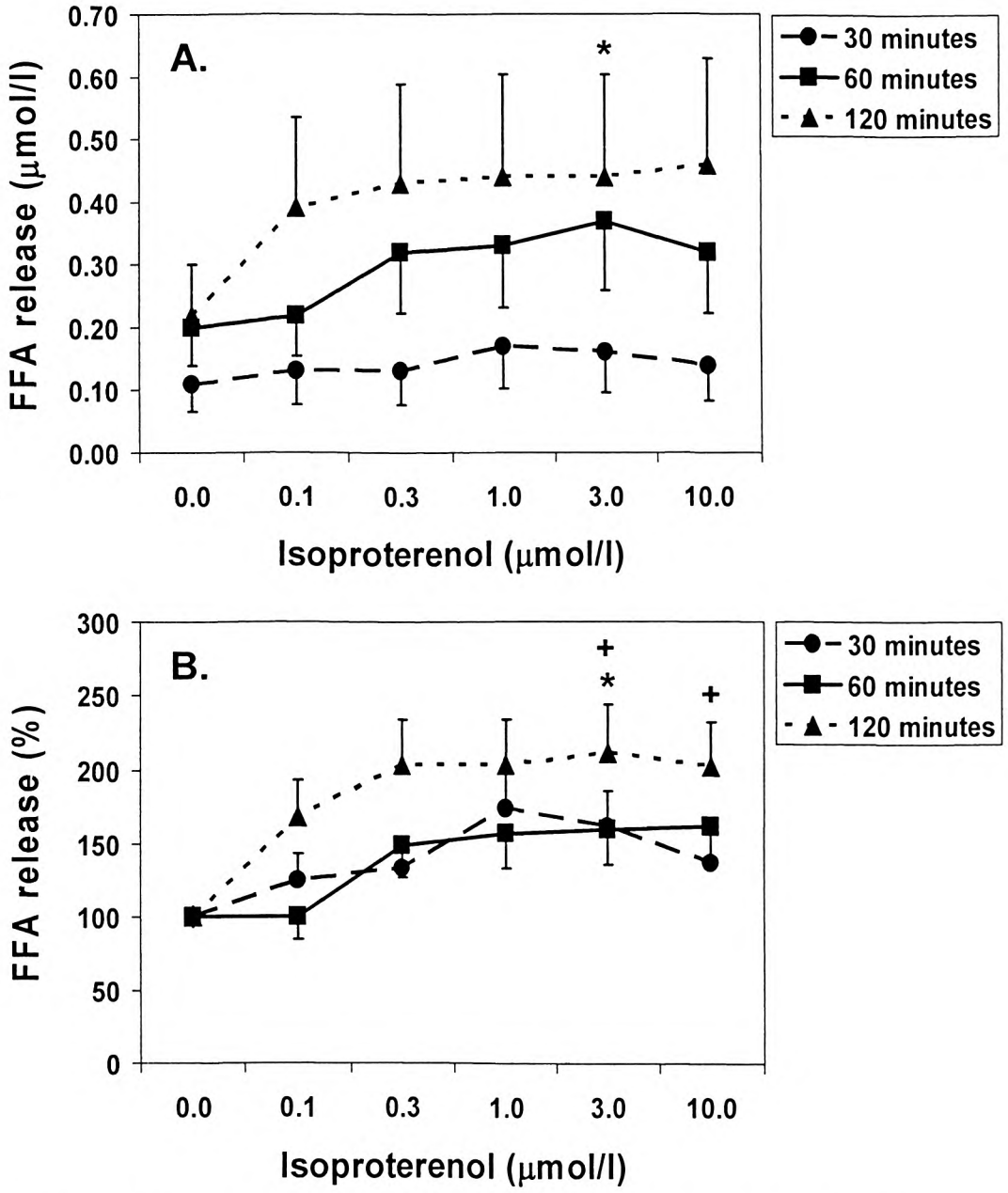

Fig 3.1 Effect of different isoproterenol concentrations over time on 3T3-L1 lipolysis expressed as A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *p<0.05, +p<0.05 vs. 0μM isoproterenol at 120 and 60 mins, respectively.

Table 3.1A, B. Area-under-the-curve data for effect of isoproterenol on lipolysis

A. FFA release expressed in μmol/l x μmol/l (data derived from Fig 3.1A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	1.536	1.008	.582
2	3	3.427	2.416	1.395
3	3	4.458	2.600	1.501

ANOVA: F=1.45, p=0.31

B. FFA release expressed in percent x μmol/l (data derived from Fig 3.1B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	1527.539	169.967	98.131
2	3	1585.288	107.642	62.147
3	3	2058.958	293.295	169.334

ANOVA: F=6.05, p=0.03

(1=30 mins; 2=60 mins; 3=120 mins)

3.2 Effects of insulin on isoproterenol-stimulated lipolysis

Figure 3.2 shows the effects of different concentrations of insulin on isoproterenol-stimulated lipolysis, over time. Isoproterenol increased FFA release producing statistically higher levels when compared to FFA release in absence of isoproterenol after 60 and 120 minutes. These differences were only observed when data was expressed as a percentage (see Fig 3.2B).

Insulin reduced FFA release, producing a statistically significant fall in FFA levels when compared to levels in absence of insulin, after 120 minutes incubation and at a concentration of 112 nmol/l (see Fig 3.2B). There was a trend for FFA levels to fall with increasing concentrations of insulin, and this is particularly clear at the 120 minute time point.

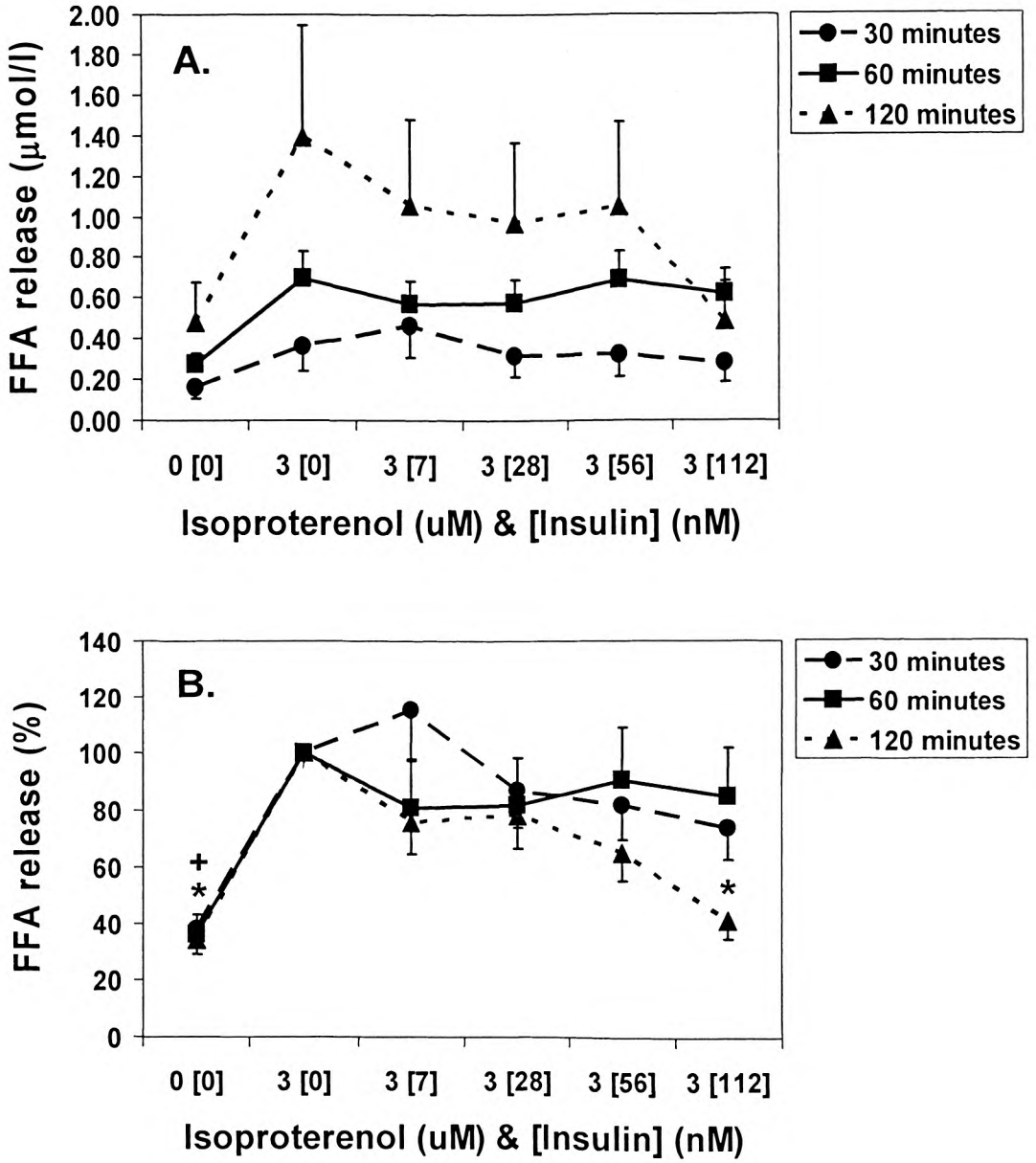

Fig 3.2 Effect of different insulin concentrations over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *$p<0.05$, +$p<0.05$ vs. 3μM isoproterenol, 0nM insulin, at 120 and 60 mins, respectively.

Table 3.2 A and B give the AUC data derived from Figures 3.2A and B, respectively. Table 3.2A shows that FFA release into the medium did increase over time but the trend was not significant. Table 3.2B shows that when the data is expressed as a percentage, no obvious trend over time is observed, although at 120 minutes the value is lower than at 30 or 60 minutes.

Table 3.2A, B. Area-under-the-curve data for effect of insulin on isoproterenol-stimulated lipolysis

A. FFA release expressed in μmol/l x nmol/l (data derived from Fig 3.2A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	36.813	20.250	11.691
2	3	70.748	43.144	24.909
3	3	100.998	73.446	42.404

ANOVA: F=1.21, p=0.36

B. FFA release expressed in percent x nmol/l (data derived from Fig 3.2B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	9663.750	2652.837	1531.616
2	3	9676.306	2927.039	1689.927
3	3	7216.818	494.220	285.338

ANOVA: F=1.14, p=0.38

(1=30 mins; 2=60 mins; 3=120 mins)

3.3 Effects of coriander extract on isoproterenol-stimulated lipolysis

The aqueous coriander extract inhibited lipolysis, as demonstrated in Fig 3.3A, B. Isoproterenol caused a stimulation of lipolysis at all time points when data was expressed in molar units (Fig 3.3A), but only caused a significant stimulation at 60 minutes when data was expressed as a percentage. The coriander extract inhibited FFA release in a dose-

dependent fashion with a significant inhibition of lipolysis occurring with 150 and 300 µl of extract at 120 minutes and with 300 µl of extract at 30 minutes (see Fig 3.3A). When the data was expressed as a percentage, 150 and 300 µl of the extract inhibited lipolysis at the 30 minute time point only (see Fig 3.3B).

When data from Fig 3.3 was expressed as AUC, it was observed that FFA release did increase with time and a statistically significant trend was noted (see Table 3.3A). When AUC data was expressed as a percentage, no such trend was observed (see Table 3.3B).

Table 3.3A, B. Area-under-the-curve data for effect of coriander extract on isoproterenol-stimulated lipolysis

A. FFA release expressed in µmol/l x µl (data derived from Fig 3.3A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	42.575	8.534	4.927
2	3	100.540	19.382	11.190
3	3	149.522	59.462	34.330

ANOVA: F=6.47, p=0.032

B. FFA release expressed in percent x µl (data derived from Fig 3.3B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	22534.684	3292.015	1900.646
2	3	24057.768	3017.727	1742.286
3	3	23262.426	5497.050	3173.723

ANOVA: F=0.10, p=0.90

(1=30 mins; 2=60 mins; 3=120 mins)

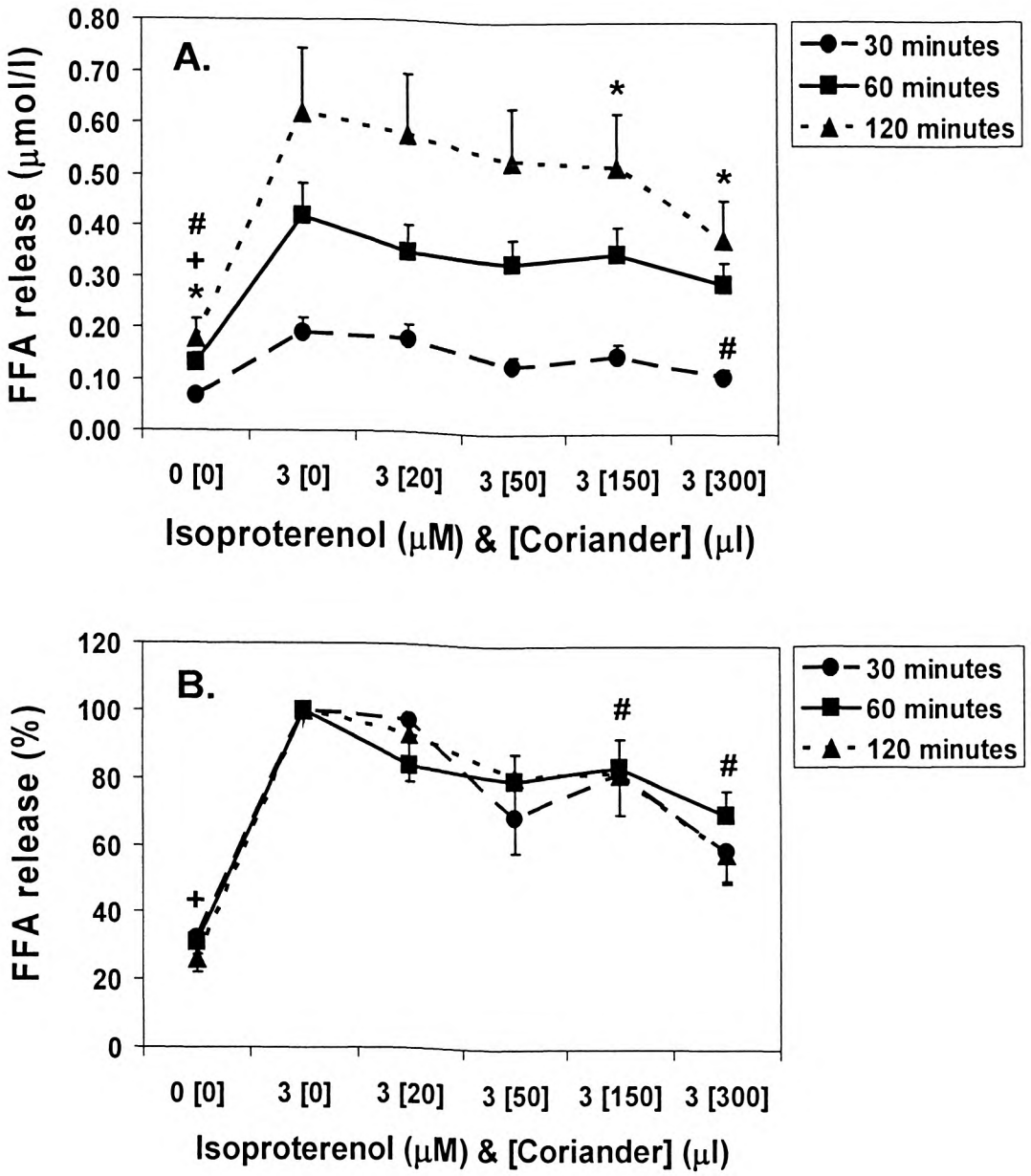

Fig 3.3 Effect of different coriander extract volumes over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *p<0.05, +p<0.05 and #p<0.05 vs. 3μM isoproterenol, 0μl coriander, at 120, 60 and 30 mins, respectively.

3.4 Effects of linalyl acetate on isoproterenol-stimulated lipolysis

When data was expressed in molar units (Fig 3.4A), isoproterenol did increase FFA release at all time points, but not to a statistically significant extent. Linalyl acetate at all concentrations, reduced FFA release but again, no statistically significant differences were noted. However, when data was expressed as percentages, the SD values were reduced and significant trends were observed (see Fig 3.4B). Thus, at 120 minutes, isoproterenol increased lipolysis to a statistically significant extent. Furthermore, linalyl acetate at all concentrations used significantly reduced FFA release at the 30 and 120 minute time points, and showed statistically significant FFA reductions at 60 minutes when used at concentrations of 6.2 and 94.0 mmol/l.

When the data from Figs 3.4 A and B were expressed as AUC, FFA levels did increase over time but the trend was not statistically significant (see Table 3.4A). When the AUC data was expressed in percentage terms, there was no significant trend over time (see Table 3.4B).

Table 3.4A, B. Area-under-the-curve data for effect of linalyl acetate on isoproterenol-stimulated lipolysis

A. FFA release expressed in μmol/l x mmol/l (data derived from Fig 3.5A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	.842	.577	.333
2	3	1.931	1.361	.786
3	3	3.111	2.190	1.264

ANOVA: F=1.66, p=0.27

B. FFA release expressed in percent x mmol/l (data derived from Fig 3.5B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	1716.114	903.725	521.766
2	3	2207.849	1430.269	825.766
3	3	2037.260	1076.698	621.632

ANOVA: F=0.14, p=0.87 (1=30 mins; 2=60 mins; 3=120 mins)

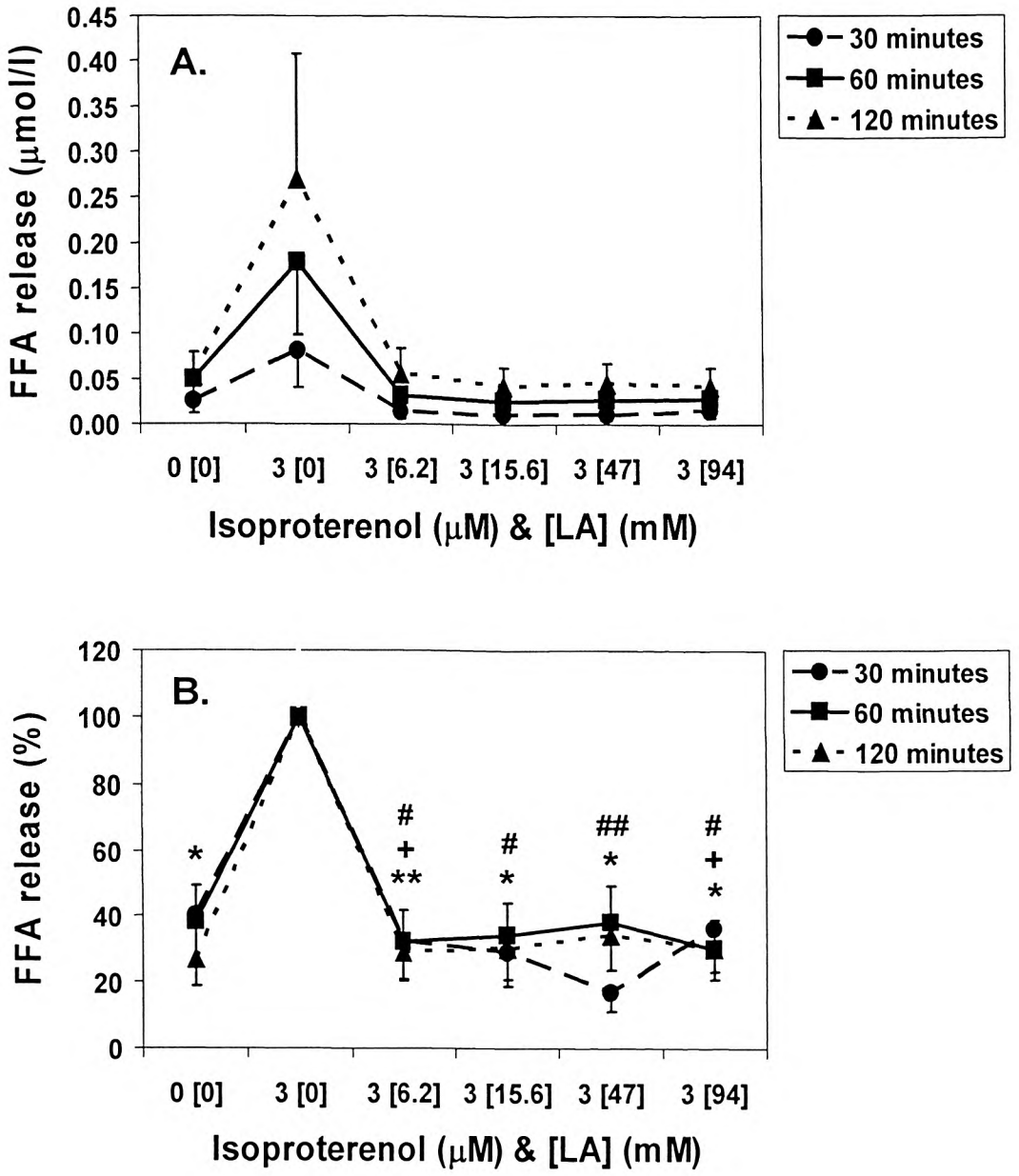

Fig 3.4 Effect of various linalyl acetate (LA) concentrations over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *p<0.05, **p<0.01; +p<0.05; #p<0.05, ##p<0.01 vs. 3μM isoproterenol, 0mM LA, at 120, 60 and 30 mins, respectively.

3.5 Effects of Devil's claw extract on isoproterenol-stimulated lipolysis

Isoproterenol increased lipolysis at all time points to a significant degree (see Fig 3.5A). The Devil's claw (*H. procumbens*) extract inhibited FFA release at the 60 and 120 minute time points when used at volumes of 150 and 300 µl (Fig 3.5A). When FFA release was expressed as a percentage very similar trends were observed to those seen when data was expressed in molar units (see Fig 3.5B).

When data was expressed as AUC, FFA release did increase over time but the trend was not statistically significant (see Table 3.5A). When the AUC data for FFA release was expressed as a percentage, no trends over time were observed (Table 3.5B).

Table 3.5A, B. Area-under-the-curve data for effect of Devil's claw on isoproterenol-stimulated lipolysis

A. FFA release expressed in µmol/l x µl (data derived from Fig 3.5A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	29.895	15.863	9.159
2	3	44.588	21.152	12.212
3	3	74.277	41.101	23.730

ANOVA: F=1.93, p=0.23

B. FFA release expressed in percent x µl (data derived from Fig 3.5B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	13161.664	7532.680	4348.995
2	3	10362.869	3810.085	2199.754
3	3	11994.387	4257.387	2458.003

ANOVA: F=0.20, p=0.82

(1=30 mins; 2=60 mins; 3=120 mins)

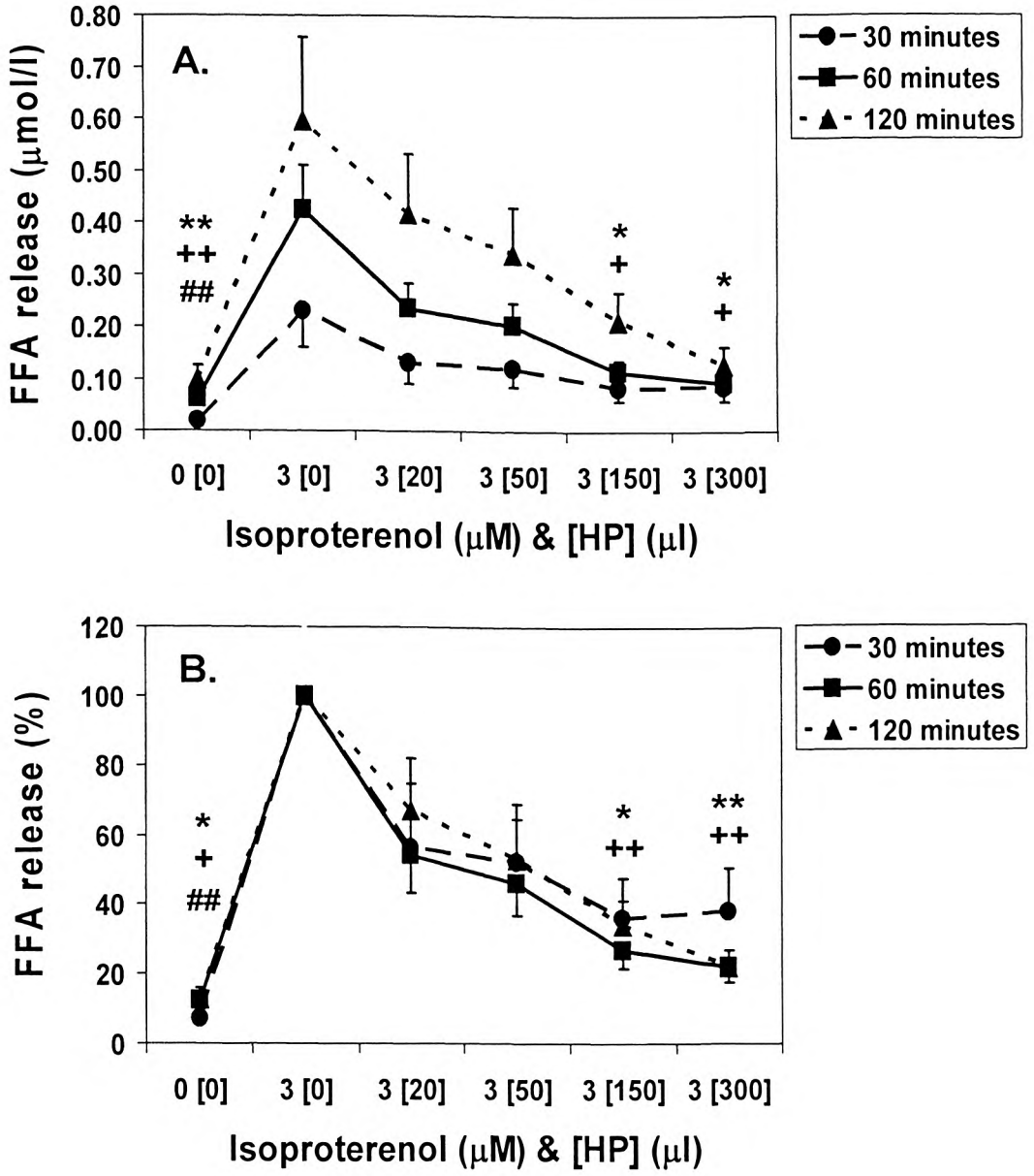

Fig 3.5 Effect of various levels of *H. procumbens* (HP) extract over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *p<0.05, **p<0.01; +p<0.05, ++p<0.01; ##p<0.01 vs. 3μM isoproterenol, 0μl HP, at 120, 60 and 30 mins, respectively.

3.6 Effects of *T. emetica* extract on isoproterenol-stimulated lipolysis

Isoproterenol increased lipolysis but produced statistically significant increases at all time points only when data was expressed as percentage values (see Fig 3.6B). *T. emetica* extract did not show any effect on FFA release when data was expressed as molar values (see Fig 3.6A) but when data was expressed as percentages, 150 µl of extract significantly inhibited lipolysis at the 120 minute time point (see Fig 3.6B).

The AUC data for *T. emetica* shows that FFA release does increase with time of incubation, but the trend is not statistically significant when data is expressed in molar terms (see Table 3.6A). When data is expressed as a percentage, FFA release tends to fall over time but the trend is not significant (see Table 3.6B).

Table 3.6A, B. Area-under-the-curve data for effect of *T. emetica* extract on isoproterenol-stimulated lipolysis

A. FFA release expressed in µmol/l x µl (data derived from Fig 3.6A)

	Count	Mean	Std. Dev.	Std. Err.
1	3	6.977	3.909	2.257
2	3	10.725	4.850	2.800
3	3	14.560	6.646	3.837

ANOVA: F=1.56, p=0.28

B. FFA release expressed in percent x µl (data derived from Fig 3.6B)

	Count	Mean	Std. Dev.	Std. Err.
1	3	28312.739	14166.493	8179.029
2	3	24344.797	9053.326	5226.940
3	3	20247.520	3488.740	2014.225

ANOVA: F=0.50, p=0.63

(1=30 mins; 2=60 mins; 3=120 mins)

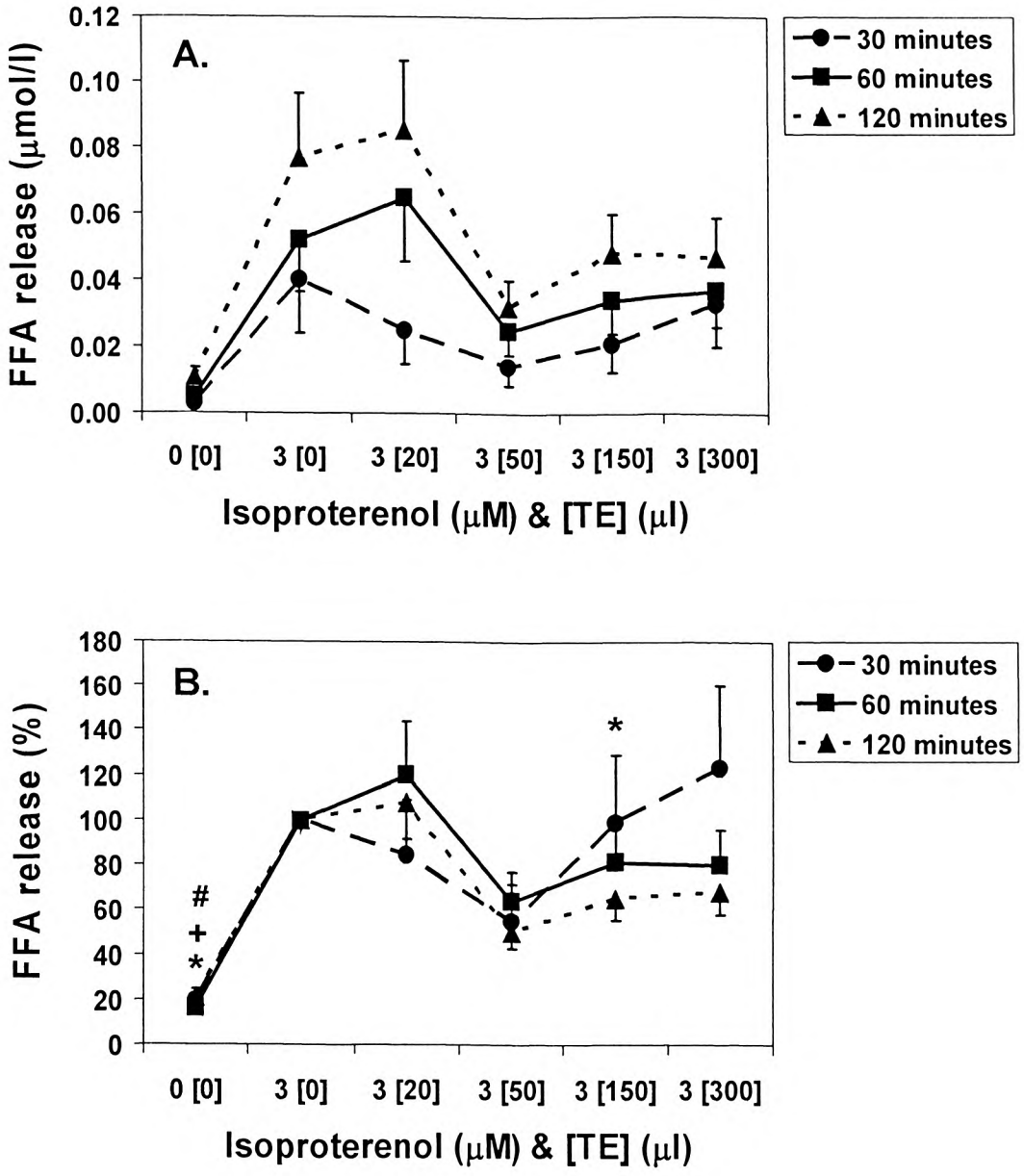

Fig 3.6 Effect of various volumes of *T. emetica* (TE) extract over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 3 experiments; *p<0.05, +p<0.05 and #p<0.05 vs. 3μM isoproterenol, 0μl TE, at 120, 60 and 30 mins, respectively.

3.7 Effects of *Z. mucronata* extract on isoproterenol-stimulated lipolysis

Isoproterenol treatment of the 3T3-L1 cells again increased lipolysis and statistically significant increases in FFA release were observed at all time points and for data expressed as molar release (see Fig 3.7A) and as a percentage (see Fig 3.7B). No significant inhibition of lipolysis was seen when data was expressed in molar terms, but when expressed as a percentage, 50 µl of *Z. mucronata* extract statistically inhibited FFA release at the 120 minute time point (see Fig 3.7B).

When FFA release was expressed as AUC, a statistically significant increase in FFA release with time was observed when data was expressed in moles (see Table 3.7A). When AUC data was expressed as a percentage, no significant trends were observed (see Table 3.7B).

Table 3.7A, B. Area-under-the-curve data for effect of *Z. mucronata* extract on isoproterenol-stimulated lipolysis

A. FFA release expressed in µmol/l x µl (data derived from Fig 3.7A)

	Count	Mean	Std. Dev.	Std. Err.
1	4	9.293	5.101	2.551
2	4	23.071	10.631	5.316
3	4	31.828	12.295	6.147

ANOVA: F=5.33, p=0.03

B. FFA release expressed in percent x µl (data derived from Fig 3.7B)

	Count	Mean	Std. Dev.	Std. Err.
1	4	23657.716	13201.905	6600.952
2	4	20264.353	14230.335	7115.167
3	4	22718.261	7454.945	3727.473

ANOVA: F=0.08, p=0.92

(1=30 mins; 2=60 mins; 3=120 mins)

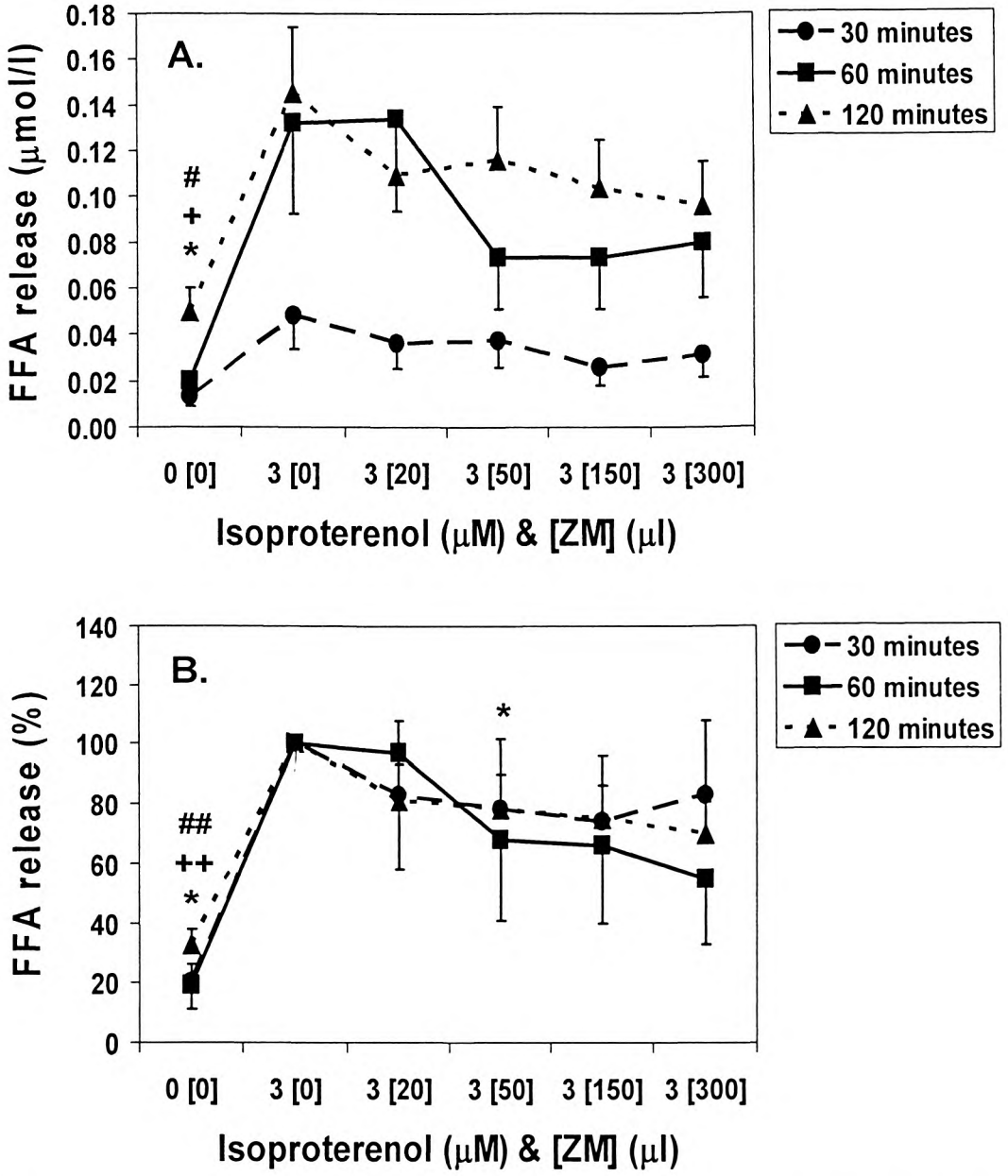

Fig 3.7 Effect of various volumes of Z. *mucronata* (ZM) extract over time on isoproterenol-stimulated lipolysis expressed as: A. Molar FFA concentration; B. Percentage FFA concentration.

Means ± SEM from 4 experiments; *p<0.05; +p<0.05, ++p<0.01; #p<0.05, ##p<0.01 vs. 3μM isoproterenol, 0μl ZM, at 120, 60 and 30 mins, respectively.

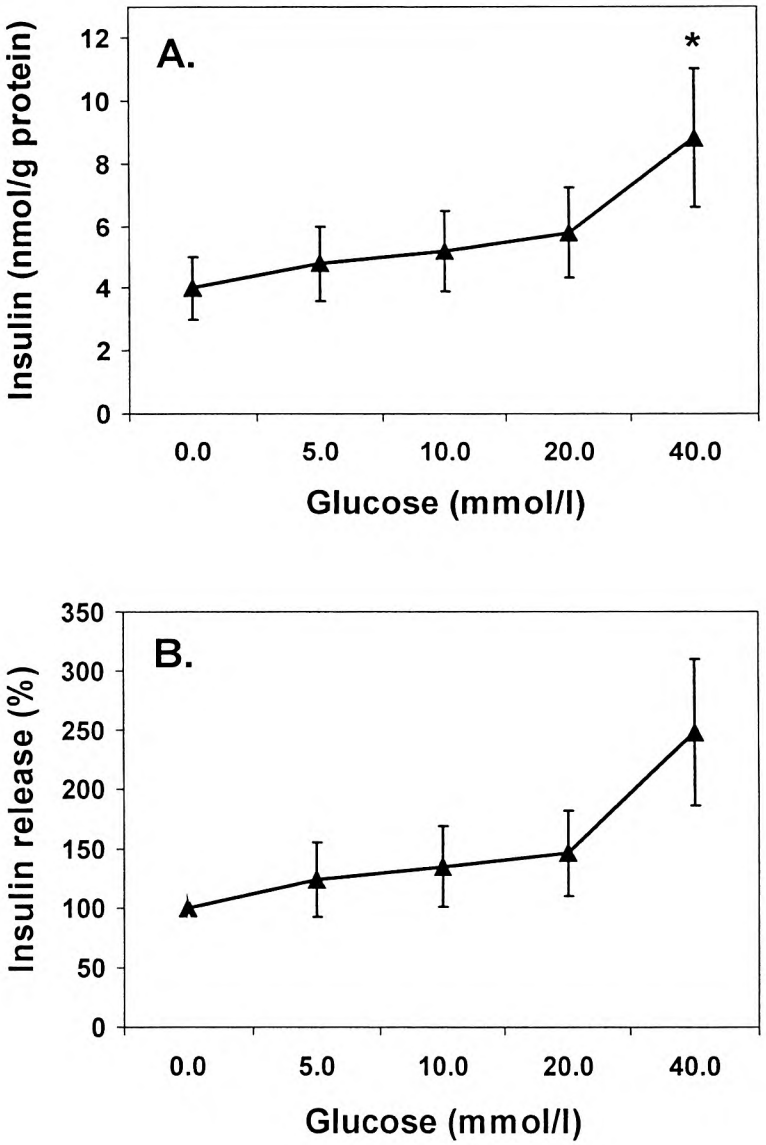

Fig 3.8 Effect of different glucose concentrations on MIN6 insulin release expressed as A. Molar insulin concentration; B. Percentage insulin levels.

Means ± SEM from 4 experiments; *p<0.05 vs 0 mmol/l glucose.

3.8 Effect of glucose on insulin secretion from MIN6 cells

The data in Fig. 3.8A demonstrates that increasing glucose concentrations led to an increase in insulin secretion in the MIN6 cells, with 40 mmol/l glucose ($p<0.05$ vs insulin release in presence of 0 mmol/l glucose) producing the highest effect. When data was expressed as percentage insulin release (see Fig. 3.8B) a very similar trend was observed but this time no statistically significant difference was seen between 40 mmol/l glucose and 0 mmol/l glucose.

3.9 Effect of an aqueous coriander extract on insulin secretion from MIN6 cells

Insulin secretion was increased by incubating MIN6 cells with an aqueous coriander extract but the effect observed was not statistically significant at any of the different coriander extract volumes. This was true for insulin secretion data expressed as nmol/g protein or as a percentage (see Figs 3.9A and B, respectively).

3.10 Effect of linalyl acetate on insulin secretion from MIN6 cells

Linalyl acetate increased insulin release from the MIN6 cells, particularly at its highest (94 mmol/l) concentration. However, no statistically significant difference was noted between insulin release in absence and insulin release in the presence of linalyl acetate at any concentration used. This trend was the same for data expressed as nmol/g protein (see Fig. 3.10A) or as a percentage (see Fig. 3.10B). However, the p values were close to significant for the 6.2 ($p=0.097$), 15.6 ($p=0.083$) and 94 ($p=0.089$) mmol/l linalyl acetate concentrations, when insulin release was expressed in molar terms. When insulin release was expressed as a percentage, linalyl acetate at 94 mmol/l also demonstrated a close to significant effect ($p=0.072$).

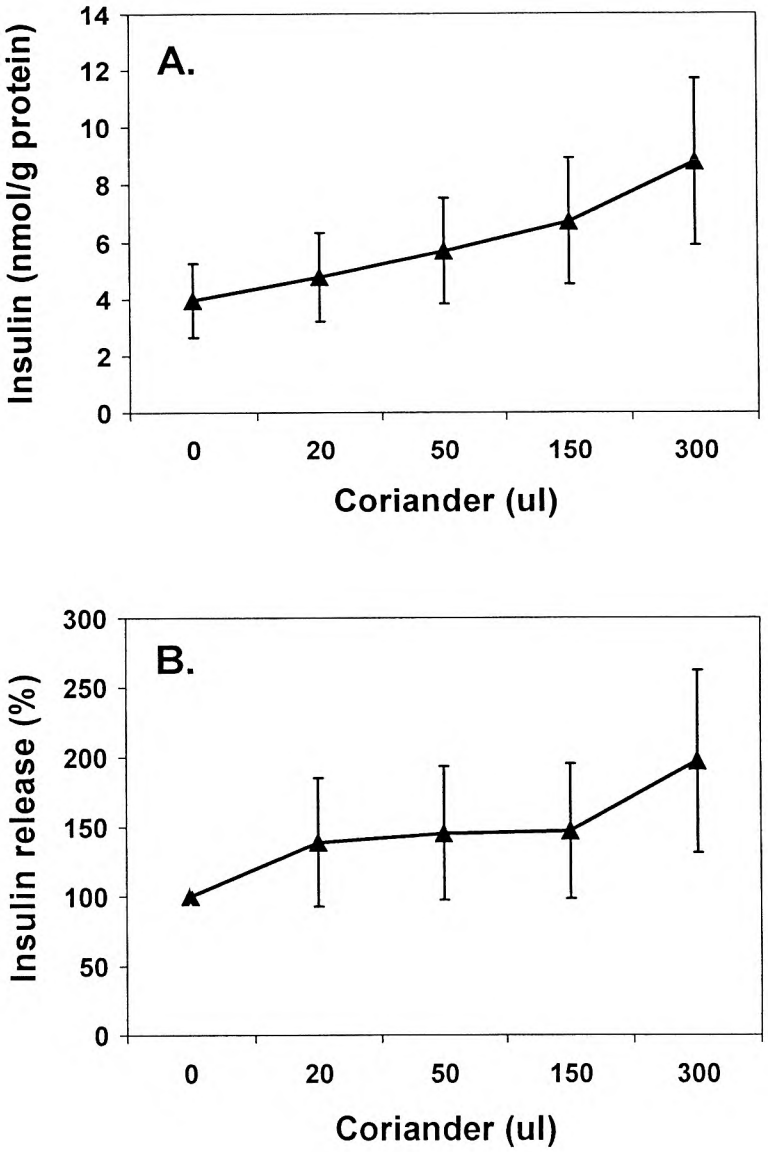

Fig 3.9 Effect of different volumes of aqueous coriander extract on MIN6 insulin release expressed as A. Molar insulin concentration; B. Percentage insulin levels.

Means ± SEM from 4 experiments.

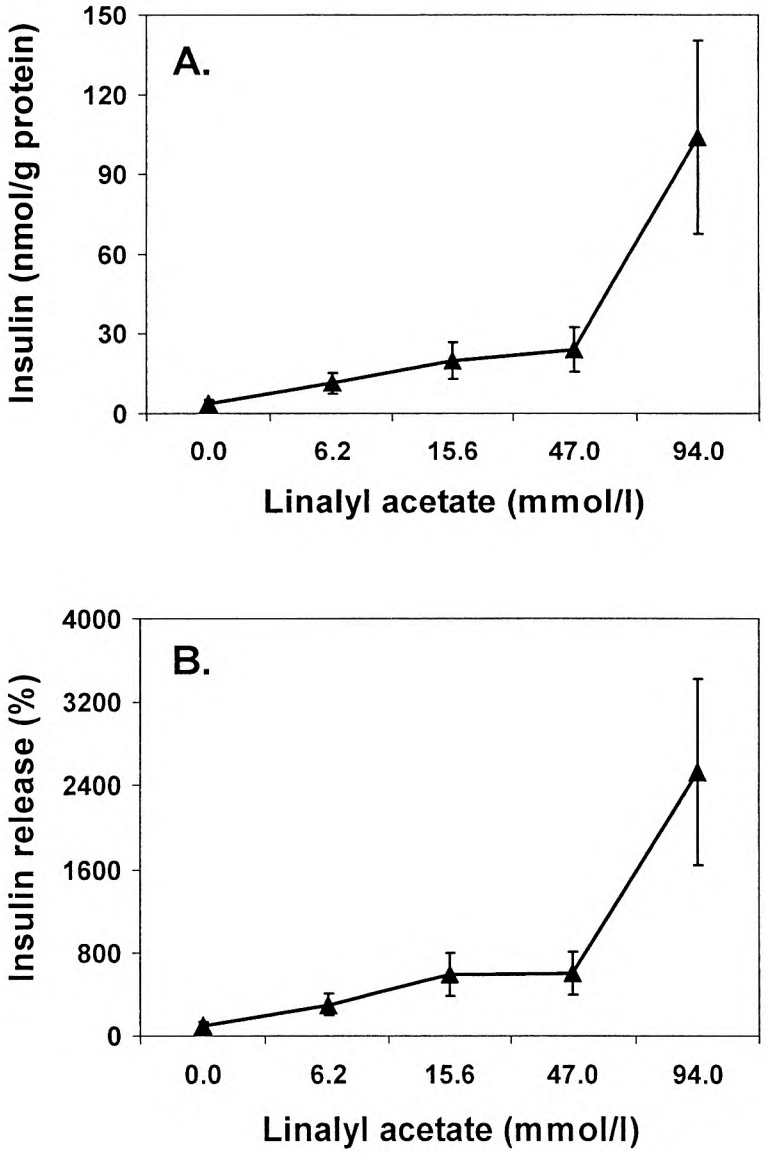

Fig 3.10 Effect of different linalyl acetate concentrations on MIN6 insulin release expressed as A. Molar insulin concentration; B. Percentage insulin levels.

Means ± SEM from 4 experiments.

3.11 Effect of an aqueous Devil's claw extract on insulin secretion from MIN6 cells

The effect of an aqueous Devil's claw (*H. procumbens*) extract on insulin release was minimal. Thus, a 20 µl volume of the extract had the maximal effect but this was only a 50% increase in insulin release when compared to the level observed in the absence of the extract (see Fig. 3.11B). No statistically significant increases in insulin release, whether expressed in molar (see Fig. 3.11A) or percentage (see Fig. 3.11B) terms, were observed for any of the extract volumes.

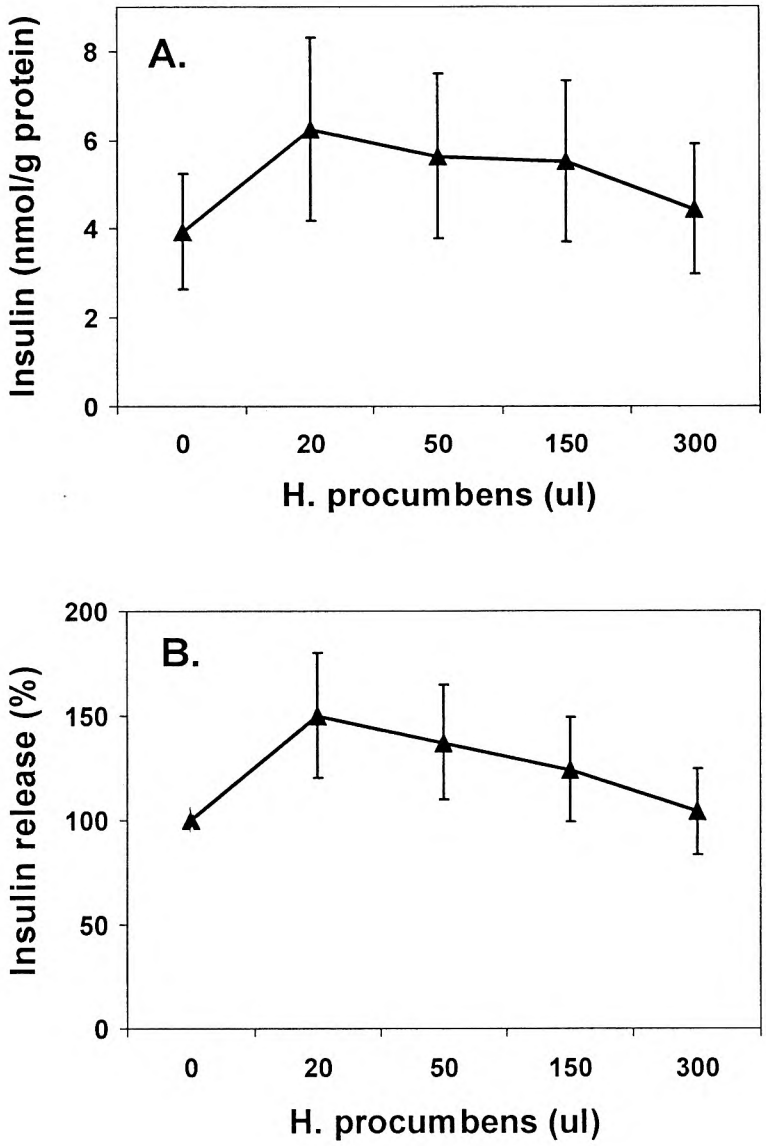

Fig 3.11 Effect of different volumes of *H. procumbens* aqueous extract on MIN6 insulin release expressed as A. Molar insulin concentration; B. Percentage insulin levels.

Means ± SEM from 4 experiments.

4.0 DISCUSSION, CONCLUSIONS AND LIMITATIONS OF STUDY

4.1 Discussion

Diabetes mellitus is possibly the world's largest growing metabolic disease, and as the knowledge on the heterogeneity of the disorder is advanced, the need for more appropriate therapy increases (Nammi *et al.*, 2003). It has been recognized as a clinical syndrome since ancient times, and it remains a crippling global health problem today. Despite the introduction of oral hypoglycaemic agents, diabetes and related complications continue to be a major medical problem. Traditional plant remedies are used throughout the world for the treatment of diabetes.

4.1.1 Experiments with 3T3-L1 cells

The present study used murine, preadipocyte 3T3-L1 cells to determine the effect of different plant extracts and related compounds on isoproterenol-stimulated lipolysis. This cell line was chosen because it has been used by many investigators to study adipocyte metabolic pathways, and is, therefore, a well characterized cell line with many similarities to non-immortalised adipocytes (Ntambi and Kim, 2000).

Incubation of the 3T3-L1 cells with isoproterenol concentrations ranging from 0.1 to 10 μmol/l caused a dose-related increase in lipolysis, demonstrating that a functional lipolytic response was present within this immortalized cell line. Data from the present study agrees with that from previous reports demonstrating that in 3T3-L1 cells, isoproterenol concentrations between 1 and 10 μmol/l cause maximal stimulation of lipolysis (Olansky and Pohl, 1984; Fong *et al.*, 1991; Adler-Wailes *et al.*, 2005). Furthermore, insulin over a concentration range of 7 to 112nmol/l caused a dose-related inhibition of isoproterenol-stimulated lipolysis, demonstrating that the 3T3-L1s were responsive to insulin. This agrees with previous studies showing that insulin from 1nmol/l to 1μmol/l can significantly inhibit catecholamine-induced lipolysis in 3T3-L1 cells (Olansky and Pohl, 1984; Elks and Manganiello, 1985; Chernick *et al.*, 1986).

Aqueous extracts of both coriander and Devil's claw (*H. procumbens*) caused a dose-dependent inhibition of lipolysis in the 3T3-L1 cells. This is the first study to demonstrate that aqueous extracts from these plants can inhibit lipolysis. Devil's claw caused a maximum inhibition of lipolysis of 80% compared to 40% for the coriander extract. This compares to a maximum inhibition of lipolysis of 60% for insulin. However, it should be noted that these maximum levels of inhibition were caused by the highest concentrations of each of these 3 different treatments. Therefore, it is possible that even greater levels of inhibition may have been produced by higher concentrations of coriander and Devil's claw extracts and insulin. The present study is not the only one to demonstrate that plant extracts can inhibit lipolysis. One other study has shown that aqueous extracts of Radix ginseng, *Radix rehmanniae* and *Radix puerariae* can inhibit isoproterenol-stimulated lipolysis in 3T3-L1 cells (Hong *et al.*, 2002). These plants are commonly used in Chinese medicines, and have been shown to have positive effects on glucose tolerance in animal experiments (Kimura and Suzuki, 1981; Attele *et al.*, 2002; Hsu *et al.*, 2003). Also, ethanol extracts of peanut (*Arachis hypogaea*) shell have been shown to inhibit lipolysis in 3T3-L1 cells (Moreno *et al.*, 2005).

Linalyl acetate is a monoterpene ester and is present in a number of aromatic plant oils, including lavender, and is also found in coriander seeds (Peana *et al.*, 2002). The present study shows that it is able to strongly inhibit lipolysis at all concentrations used. Thus, it is possible that the inhibitory effect of coriander on lipolysis is due to linalyl acetate. This is the first study to show that linalyl acetate inhibits lipolysis. Furthermore, no other studies have demonstrated a negative effect of terpenes on lipolysis.

The effects of aqueous extracts of two plants that are not used for the treatment of diabetes were also tested. This was done to determine whether anti-lipolytic activity is specific to plants that supposedly have anti-diabetic activity. The aqueous extract of *T. emetica* (Natal mahogany) did not have a dose dependent effect on lipolysis but did inhibit lipolytic activity by up to 50%. Natal mahogany is commonly used for stomach and intestinal complaints, kidney problems and parasites (van Wyk *et al.*, 1997). The

extract from *Z. mucronata* (buffalo-thorn) did inhibit lipolysis in a dose dependent fashion and caused a maximum inhibition of 50%. Buffalo-thorn is used for cough and chest complaints, diarrhoea and dysentery (van Wyk *et al.*, 1997). Thus, anti-lipolytic activity does not seem to be an effect that is specific to plants that are normally used for the treatment of diabetes.

The area-under-the-curve (AUC) data for the lipolysis experiments show that free fatty acid (FFA) release into the incubation medium increases over time. However, significant trends were noted only for isoproterenol, coriander and *Z. mucronata*. This data suggests that for isoproterenol, the percentage stimulation of lipolysis is greater after a 2-hour incubation. In the case of coriander and *Z. mucronata,* this data suggests that these two plants' extracts are less effective at suppressing lipolysis over time compared to the other plant extracts. The AUC data for percentage lipolysis demonstrates that for each plant extract, the effects on lipolysis do not change with time, i.e., the pattern of inhibition at 30 minutes is very similar to the pattern at 2 hours. The present study also demonstrates that the ability of some of these plant extracts to inhibit lipolysis are visible very quickly, i.e., at the 30 minute time point, and still visible after 2 hours.

The mechanism by which these various plant extracts may inhibit lipolysis is not known. Hormone sensitive lipase (HSL) is the major enzyme involved in lipolysis within adipocytes, and it is known to be inhibited by insulin. Insulin reduces intracellular cAMP levels by activating cAMP phosphodiesterase (Elks and Manganiello, 1985). This, in turn, reduces activity of cAMP-dependent protein kinase, the enzyme responsible for phosphorylation, and hence activation, of HSL (Belfrage *et al.*, 1981). Thus, inhibition of HSL activity could be produced by activating cAMP-phosphodiesterase or inhibiting cAMP-dependent protein kinase. Additionally, HSL can be inhibited by activation of protein phosphatases type 1, 2A or 2C. These enzymes can dephosphorylate the lipase (Belfrage *et al.*, 1985). Increased intracellular calcium levels also inhibit HSL activity by increasing cAMP- phosphodiesterase activity (Xue *et al.*, 2001). It is not known whether any of the plant extracts affect the activity of HSL via the mechanisms described above.

However, one study has shown that extracts from the leaf of *Toona sinensis* can activate lipolysis by increasing protein kinase C activity in 3T3-L1 cells (Hsu *et al.*, 2003). Thus, plant extracts are able to modulate the cell signaling machinery in adipocytes, resulting in changes in lipolytic rate.

Drugs that inhibit lipolysis have been shown to have positive effects on glucose tolerance. It is thought that elevated serum FFA levels cause insulin resistance by reducing glucose uptake and metabolism in insulin sensitive tissues (the Randle hypothesis) (Randle *et al.*, 1988). Therefore, it is possible that plant extracts that have been shown to improve glucose tolerance *in vivo* may act via blocking adipocyte lipolytic activity. Data from the present study shows that extracts taken from plants that are used for the treatment of diabetes do indeed inhibit lipolysis in 3T3-L1 cells.

4.1.2 Experiments with MIN6 cells

The MIN6 cell line was used to test the ability of plant extracts to promote insulin secretion. This cell line was derived from a transgenic mouse and was chosen for investigation because it secretes insulin in response to glucose in a fashion very similar to that seen in normal islet β-cells (Ishihara *et al.*, 1993).

The incubation of MIN6 cells with glucose produced a dose-dependent increase in insulin secretion, with increasing glucose concentration. This demonstrates that the MIN6 cells are responsive to glucose, as demonstrated in a previous study (Ishihara *et al.*, 1993). However, the results from the study by Ishihara *et al.*, (1993) showed an S-shaped relationship between glucose concentration and insulin secretion, with insulin secretion reaching a plateau at 20-25 mmol/l glucose. The present study demonstrated a much flatter insulin response with insulin secretion not being significantly stimulated until glucose levels reach 40 mmol/l. The difference between the 2 studies is possibly a result of the higher passage number for the present MIN6 cells. It is well known that cell lines do tend to change functionally with increasing passage number and is one of the disadvantages of using cell lines. However, the cells did respond to glucose, suggesting

that they do retain glucose responsiveness. Data from forthcoming experiments with plant extracts must, therefore, be interpreted with caution, particularly for those extracts that do not show an insulin response.

Incubation of the MIN6 cells with aqueous coriander extract produced a dose-dependent increase in insulin secretion, but this response was not statistically significant. The maximum response to glucose occurred at 40 mmol/l and gave a 2.5-fold increase in insulin secretion compared to insulin secretion in the absence of glucose. The coriander extract when used at 300µl, gave the maximal insulin response with a 2-fold increase in insulin secretion. A previous study has shown that an aqueous coriander extract induces insulin secretion from the rodent β-cell line, BRIN-BD11, and reduces hyperglycaemia in streptozotocin-diabetic rats (Gray and Flatt, 1999). Although the insulin response to coriander extract in the present study was not statistically significant there was a trend for the secretion level to increase with increasing coriander extract concentration. Thus, a higher concentration of coriander extract may be necessary, and also the cell line used in this study, and the study by Gray and Flatt (1999) were different.

Treatment of the MIN6 cell line with the monoterpene ester linalyl acetate also produced a dose-dependent increase in insulin secretion. The maximum response occurred at the highest linalyl acetate concentration (94 mmol/l), which produced a greater than 24-fold increase in insulin output above the baseline value. However, this was not a statistically significant difference with the p value just failing to reach significance ($p=0.072$ for percentage secretion). Statistical significance was not reached due to the high SEM values that resulted from large inter-experiment variation (see raw data in Tables H and I in the appendix). This variation may be due to the passage number of the cells. Extracts of leaves of the plant *Stevia rebaudiana* Bertoni have been used for the treatment of diabetes in South America. These extracts contain the diterpene glycosides, stevioside and rebaudioside A, and both compounds have been shown to induce insulin secretion in *in vitro* studies (Jeppesen *et al.*, 2000; Abudula *et al.*, 2004). Thus, terpene-related compounds isolated from different plant species are able to stimulate insulin secretion.

An aqueous extract of *H. procumbens,* when added to MIN6 cells, did not produce a dose-dependent increase in insulin secretion. This probably suggests either that a higher quantity of the plant extract is required to stimulate insulin secretion, or that *H. procumbens* does not contain an insulinotropic agent. It is also possible that the extract does contain an insulinotropic agent, or that the MIN6 cells are not responsive to it. A study using streptozotocin-diabetic rats has shown that treatment of these animals with an aqueous extract of *H. procumbens* does lead to a fall in blood glucose levels (Mahomed and Ojewole, 2004). However, it is not certain whether the hypoglycaemic effect of this plant extract is due to an insulinotropic effect, an insulin-sensitising effect or both.

The mechanism by which some of these insulinotropic plant extracts induce insulin secretion has been investigated. Thus an aqueous extract of coriander seeds increases insulin secretion from the BRIN-BD11 cell line by acting upon the ATP-sensitive potassium channel, a mechanism also used by the sulphonylurea class of anti-diabetic drugs (Gray and Flatt, 1999). This method of stimulating insulin secretion is also used by quinolizidine alkaloids isolated from Lupinus species (Garcia Lopez *et al.*, 2004). An ethanol extract of *Anemarrhena asphodeloides* increases insulin secretion in isolated rat islets but this effect is inhibited by pertussis toxin. This suggests that the insulinotropic component of this plant acts via pertussis toxin-sensitive Gi- (or Ge-) proteins (Hoa *et al.*, 2004). Thus, plant extracts are capable of stimulating insulin release by acting on different components of the intracellular signaling pathways that control insulin secretion in islet β-cells.

4.2 Conclusions

The present study demonstrates that linalyl acetate and aqueous extracts of coriander and *H. procumbens* are potent inhibitors of lipolysis in the 3T3-L1 cell line. This property was also observed in aqueous extracts taken from 2 plants that are not normally used for the treatment of type 2 diabetes. This suggests that insulin-like activity, i.e., inhibition of lipolysis, is not only restricted to plants that are traditionally used for the treatment of

diabetes. Studies with the insulin secreting cell line, MIN6, demonstrated that linalyl acetate and an aqueous extract of coriander caused a dose-dependent increase in insulin secretion, but not to a level that was statistically significant. Therefore, this data must be interpreted with caution. However, one other study using the rodent β-cell line, BRIN-BD11, has shown that an aqueous extract of coriander does cause a significant stimulation of insulin secretion (Gray and Flatt, 1999).

4.3 Limitations of this study and future investigations

There are a number of limitations to the present study, and these are as follows:

1. Data was obtained from experiments conducted on cell lines. Although the cell lines used have similarities to the primary cell types, i.e., adipocytes and β-cells, there are functional and biochemical differences. Data obtained therefore, must be interpreted with some caution, and results confirmed by *in vivo* studies in rodents. Furthermore, the functionality of cell lines changes with passage number, and this was particularly true for the MIN6 cell line. This may explain the large inter-experiment variation in results obtained with these cells.

2. The active chemical components of the plant extracts were not investigated and, therefore, we are not certain whether the effects are due to a single or multiple chemical components of the plant extracts. This must be further investigated by using chromatographic and other analytical techniques to isolate different components and testing their activities.

3. Experiments were carried out using only aqueous extracts of the different plants. This was done because these plants are administered to patients in the form of water-based extracts. However, because we have investigated only water-soluble components of the plants, it may be possible that we have overlooked other active components that are sparingly soluble in water or are not water soluble. Further studies must be performed on organic extracts of these plants, for instance using methanol.

4. The large inter-experiment variation in the data obtained from the MIN6 cells meant that any effects of the plant extracts on insulin secretion did not reach statistically significant levels. Each extract was tested in 4 separate experiments using the MIN6 cells and it is possible that significant effects may have been observed if more experiments had been performed. It is also possible that higher concentrations of the plant extracts should have been used.

5. It is known that seasonal and geographic variations do occur in plant biochemistry and, therefore, it is possible that the active components present in the plant extracts were at lower levels at the time or location of harvesting. Thus, experiments should be performed on plants isolated from different geographic locations and at different times of the year.

6. The toxicity of the plant extracts has not been investigated. Therefore, LD_{50} values of the plant extracts should be determined in rodents.

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APPENDIX

DATA FROM 3T3-L1 AND MIN6 EXPERIMENTS

Table A. Data from 3T3-L1 experiments with isoproterenol

Expt No	*Time*	*Iso0*	*Iso0.1*	*Iso0.3*	*Iso1*	*Iso3*	*Iso10*	*Iso AUC*	*Iso0 %*	*Iso0.1 %*	*Iso0.3 %*	*Iso1 %*	*Iso3 %*	*Iso10 %*	*Iso AUC%*
1	30	.058	.055	.072	.074	.099	.088	.90	100	95	124	128	171	152	1546
2	30	.200	.245	.224	.265	.278	.269	2.70	100	122	112	132	139	134	1349
3	30	.060	.095	.097	.157	.106	.075	1.01	100	158	161	261	176	125	1687
1	60	.115	.081	.200	.136	.147	.259	1.86	100	70	174	118	128	225	1617
2	60	.371	.406	.557	.555	.723	.536	6.21	100	109	150	149	195	144	1674
3	60	.151	.182	.188	.305	.236	.177	2.21	100	120	124	202	156	117	1465
1	120	.105	.191	.280	.174	.276	.249	2.51	100	182	266	165	263	237	2389
2	120	.378	.755	.756	.619	.691	.855	7.41	100	199	200	164	183	226	1960
3	120	.189	.229	.269	.530	.362	.270	3.45	100	121	142	280	191	143	1828

Isoproterenol (Iso) concentrations are in μmol/l.

Data is expressed as μmol/l of free fatty acid, except where %.

Table B. Data from 3T3-L1 experiments with insulin

Expt No	*Time*	*Ins0 Iso0*	*Ins0 Iso3*	*Ins7 Iso3*	*Ins28 Iso3*	*Ins56 Iso3*	*Ins112 Iso3*	*Ins AUC*	*Ins0 Iso0%*	*Ins0 Iso3%*	*Ins7 Iso3%*	*Ins28 Iso3%*	*Ins56 Iso3%*	*Ins112 Iso3%*	*Ins AUC%*
1	30	.044	.223	.141	.201	.122	.132	16.5	19.7	100	63	90	55	59	7399
2	30	.033	.410	.364	.319	.308	.345	36.9	8.0	100	89	78	75	84	9010
3	30	.396	.453	.881	.428	.534	.363	57.0	87.4	100	194	94	118	80	12582
1	60	.100	.461	.331	.402	.289	.310	36.9	21.7	100	72	87	63	67	8007
2	60	.197	.703	.627	.428	.454	.543	56.0	28.0	100	89	61	65	77	7965
3	60	.526	.914	.748	.887	1.325	1.010	119.3	57.5	100	82	97	145	111	13056
1	120	.121	.750	.558	.590	.450	.472	57.0	16.1	100	74	79	60	63	7601
2	120	.455	.904	.709	.904	.395	.299	60.2	50.3	100	78	100	44	33	6659
3	120	.874	2.514	1.899	1.424	2.295	.683	185.8	34.7	100	76	57	91	27	7390

Insulin (Ins) concentrations are in nmol/l and isoproterenol (Iso) concentrations are in μmol/l.

Data is expressed as μmol/l of free fatty acid, except where %.

Table C. Data from 3T3-L1 experiments with aqueous coriander extract

Expt No	*Time*	*Co0 Iso0*	*Co0 Iso3*	*Co20 Iso3*	*Co50 Iso3*	*Co150 Iso3*	*Co300 Iso3*	*Co AUC*	*Co0 Iso0%*	*Co0 Iso3%*	*Co20 Iso3%*	*Co50 Iso3%*	*Co150 Iso3%*	*Co300 Iso3%*	*Co AUC%*
1	30	.002	.171	.137	.071	.137	.082	33.0	1.2	100	80	42	80	48	19313
2	30	.083	.191	.230	.163	.171	.131	49.5	43.5	100	120	85	90	69	25893
3	30	.115	.202	.182	.160	.153	.122	45.2	56.9	100	90	79	76	60	22399
1	60	.001	.376	.287	.194	.323	.190	78.2	.27	100	76	52	86	51	20790
2	60	.187	.420	.363	.477	.312	.387	112.3	44.5	100	86	114	74	92	26739
3	60	.218	.451	.401	.334	.418	.302	111.1	48.3	100	89	74	93	67	24644
1	120	.002	.474	.438	.192	.339	.138	80.9	.42	100	92	41	72	29	17066
2	120	.111	.674	.672	.775	.607	.479	185.7	16.5	100	100	115	90	71	27554
3	120	.434	.723	.639	.630	.622	.534	182.0	60.0	100	88	87	86	74	25167

Coriander (Co) concentrations are in µl and isoproterenol (Iso) concentrations are in µmol/l.

Data is expressed as µmol/l of free fatty acid, except where %.

Table D. Data from 3T3-L1 experiments with linalyl acetate

Expt No	*Time*	*LA0 Iso0*	*LA0 Iso3*	*LA6.2 Iso3*	*LA15.6 Iso3*	*LA47 Iso3*	*LA94 Iso3*	*LA AUC*	*LA0 Iso0%*	*LA0 Iso3%*	*LA6.2 Iso3%*	*LA15.6 Iso3%*	*LA47 Iso3%*	*LA94 Iso3%*	*LA AUC%*
1	30	.056	.191	.019	.014	.018	.024	1.5	29	100	10	7	9	13	769
2	30	.009	.040	.013	.013	.008	.014	.7	23	100	33	33	20	35	1810
3	30	.009	.013	.007	.006	.003	.008	.3	69	100	54	46	23	62	2569
1	60	.126	.442	.064	.039	.040	.045	3.4	29	100	14	9	9	10	766
2	60	.012	.077	.023	.023	.028	.030	1.7	16	100	30	30	36	39	2231
3	60	.013	.019	.010	.012	.013	.008	.7	68	100	53	63	68	42	3626
1	120	.127	.644	.121	.077	.059	.062	5.3	20	100	19	12	9	10	823
2	120	.016	.129	.035	.032	.060	.051	3.1	12	100	27	25	47	40	2414
3	120	.016	.032	.013	.017	.015	.013	.9	50	100	41	53	47	41	2875

Linalyl acetate (LA) concentrations are in mmol/l and isoproterenol (Iso) concentrations are in μmol/l.

Data is expressed as μmol/l of free fatty acid, except where %.

Table E. Data from 3T3-L1 experiments with aqueous extract of *H. procumbens* (Devil's claw)

Expt No	*Time*	*HP0 Iso0*	*HP0 Iso3*	*HP20 Iso3*	*HP50 Iso3*	*HP150 Iso3*	*HP300 Iso3*	*HP AUC*	*HP0 Iso0%*	*HP0 Iso3%*	*HP20 Iso3%*	*HP50 Iso3%*	*HP150 Iso3%*	*HP300 Iso3%*	*HP AUC%*
1	30	.003	.222	.046	.040	.035	.046	13.8	1.4	100	21	18	16	21	6214
2	30	.049	.251	.187	.163	.069	.053	30.4	19.5	100	75	65	27	21	12104
3	30	.003	.215	.157	.159	.141	.153	45.5	1.4	100	73	74	66	71	21167
1	60	.006	.423	.132	.084	.063	.076	26.6	1.4	100	31	20	15	18	6280
2	60	.171	.491	.403	.381	.168	.095	67.9	34.8	100	82	78	34	19	13824
3	60	.006	.358	.171	.148	.110	.108	39.3	1.7	100	48	41	31	30	10985
1	120	.007	.540	.214	.176	.084	.111	41.0	1.3	100	40	33	16	21	7595
2	120	.278	.747	.687	.607	.353	.160	120.2	37.2	100	92	81	47	21	16094
3	120	.009	.501	.350	.232	.193	.115	61.6	1.8	100	70	46	39	23	12293

H. procumbens (HP) concentrations are in µl and isoproterenol (Iso) concentrations are in µmol/l.

Data is expressed as µmol/l of free fatty acid, except where %.

Table F. Data from 3T3-L1 experiments with aqueous extract of *T. emetica* (Natal mahogany)

Expt No	*Time*	*TE0 Iso0*	*TE0 Iso3*	*TE20 Iso3*	*TE50 Iso3*	*TE150 Iso3*	*TE300 Iso3*	*TE AUC*	*TE0 Iso0%*	*TE0 Iso3%*	*TE20 Iso3%*	*TE50 Iso3%*	*TE150 Iso3%*	*TE300 Iso3%*	*TE AUC%*
1	30	.003	.094	.050	.024	.027	.058	11.5	3.2	100	53	26	29	62	12207
2	30	.004	.013	.013	.013	.015	.025	5.1	30.8	100	100	100	115	192	38846
3	30	.003	.013	.013	.005	.020	.015	4.4	23.1	100	100	38	154	115	33885
1	60	.004	.091	.128	.027	.043	.066	16.2	4.4	100	141	30	47	73	17791
2	60	.007	.020	.025	.019	.024	.025	6.9	35.0	100	125	95	120	125	34675
3	60	.004	.044	.042	.029	.034	.019	9.1	9.1	100	95	66	77	43	20568
1	120	.011	.124	.159	.030	.072	.073	21.6	8.9	100	128	24	58	59	17452
2	120	.014	.035	.042	.022	.024	.035	8.5	40.0	100	120	63	69	100	24157
3	120	.009	.071	.054	.045	.049	.033	13.6	12.7	100	76	63	69	46	19134

T. emetica (TE) concentrations are in µl and isoproterenol (Iso) concentrations are in µmol/l.

Data is expressed as µmol/l of free fatty acid, except where %.

Table G. Data from 3T3-L1 experiments with aqueous extract of *Z. mucronata* (buffalo-thorn)

Expt No	*Time*	*ZM0 Iso0*	*ZM0 Iso3*	*ZM20 Iso3*	*ZM50 Iso3*	*ZM150 Iso3*	*ZM300 Iso3*	*ZM AUC*	*ZM0 Iso0%*	*ZM0 Iso3%*	*ZM20 Iso3%*	*ZM50 Iso3%*	*ZM150 Iso3%*	*ZM300 Iso3%*	*ZM AUC%*
1	30	.0240	.086	.062	.030	.030	.049	11.8	27.9	100	72	35	35	57	13703
2	30	.0001	.013	.017	.009	.021	.022	5.4	.8	100	131	69	162	169	41654
3	30	.0040	.034	.018	.024	.008	.018	4.7	11.8	100	53	71	24	53	13824
4	30	.0250	.060	.047	.083	.045	.033	15.3	41.7	100	78	138	75	55	25450
1	60	.0320	.091	.107	.098	.141	.121	36.7	35.2	100	118	108	155	133	40280
2	60	.0030	.213	.256	.017	.045	.042	18.4	1.4	100	120	8	21	20	8643
3	60	.0130	.100	.060	.066	.022	.030	11.8	13.0	100	60	66	22	30	11790
4	60	.0330	.125	.112	.112	.084	.048	25.4	26.4	100	90	90	67	38	20344
1	120	.0900	.124	.169	.078	.110	.145	35.2	72.6	100	136	63	89	117	28355
2	120	.0030	.096	.087	.079	.102	.068	26.1	3.1	100	91	82	106	71	27208
3	120	.0170	.156	.060	.116	.034	.052	18.8	10.9	100	38	74	22	33	12019
4	120	.0900	.203	.120	.190	.169	.117	47.3	44.3	100	59	94	83	58	23291

Z .mucronata (ZM) concentrations are in µl and isoproterenol (Iso) concentrations are in µmol/l.

Data is expressed as µmol/l of free fatty acid, except where %.

Table H. Data from MIN6 experiments with insulin release expressed as nmol per g cell protein

Expt	*G0*	*G5*	*G10*	*G20*	*G40*	*Co20*	*Co50*	*Co150*	*Co300*	*LA6.2*	*LA15.6*	*LA47*	*LA94*	*HP20*	*HP50*	*HP150*	*HP300*
1	5.78	4.67	6.60	10.35	12.88	2.80	5.94	8.66	18.56	19.58	18.85	20.18	130.7	9.11	9.10	11.53	4.80
2	2.41	1.58	1.27	2.30	9.23	3.42	2.04	.45	.15	13.00	33.09	20.20	82.8	3.26	2.60	1.60	1.17
3	2.88	5.35	7.31	6.70	7.57	6.88	5.75	2.98	8.15	2.24	3.50	2.03	4.79	2.95	3.73	3.04	2.58
4	4.68	7.73	5.50	3.63	5.67	5.95	8.99	14.75	8.30	10.77	25.20	53.55	199.7	9.65	7.11	5.91	9.22

Glucose (G) and linalyl acetate (LA) concentrations are both in mmol/l.

Coriander (Co) and H. procumbens (HP) concentrations are both in µl.

Table I. Data from MIN6 experiments with insulin release expressed as a percentage

Expt	*G0*	*G5*	*G10*	*G20*	*G40*	*Co20*	*Co50*	*Co150*	*Co300*	*LA6.2*	*LA15.6*	*LA47*	*LA94*	*HP20*	*HP50*	*HP150*	*HP300*
1	100	81	114	179	223	48	103	150	321	339	326	349	2260	158	157	199	83
2	100	66	53	95	383	142	85	19	6	540	1375	840	3442	136	108	67	49
3	100	186	254	233	263	239	200	103	283	78	122	71	167	103	130	106	90
4	100	165	117	78	121	127	192	315	177	230	538	1144	4265	206	152	126	197

Glucose (G) and linalyl acetate (LA) concentrations are both in mmol/l.

Coriander (Co) and H. procumbens (HP) concentrations are both in µl.