Antidiabetic and Toxicological properties of *Dianthus thunbergii* (Caryophyllaceae) roots and *Hypoxis argentea* (Hypoxidaceae) corms

University of Fort Hare

Together in Excellence

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201514802

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Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Biochemistry in the Faculty of Science and Agriculture, University of Fort Hare, Alice, South Africa

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CO-SUPERVISOR: Prof. Anthony J. Afolayan

Dedication

This work is dedicated to God Almighty, the fountain of all knowledge and wellspring of all wisdom; to my wife who was there throughout the whole process and to the little one, Akinbambo Lewis who arrived at the nick of time.

Declaration

I, Akinleye Stephen Akinrinde (Student number: 201514802), hereby declare that the work contained in this thesis submitted for the degree of Doctor of Philosophy in Biochemistry in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture, is my original work and has never been submitted for a degree in this University or any other university.

Signature…… Date…….

I am aware of the University of Fort Hare policy on plagiarism and I have taken every necessary precaution to comply with the regulations of the University, while ensuring that all sources of materials used for this work have been duly acknowledged.

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I further declare that I am aware of the policy of the University of Fort Hare on research ethics and that there was no need for ethical clearance for the studies contained in this thesis after duly appraising all ethical considerations.

Signature…….. Date…….

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$^1O_2$	Singlet oxygen
4-HNE	4-hydroxy nonenal
ABTS	2, 2'- azino-bis (3-ethyl benzothiazoline-6-sulphonic acid
ADP	Adenosine diphosphate
AGE	Advanced glycation end-products
AMPK	Adenosine monophosphate protein kinase
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyl toluene
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic adenosine monophosphate
cAMP	cyclic guanosine monophosphate
CBB	Coomasie Brilliant blue
CDK	Cyclin-dependent kinase
CRI-G1	Cambridge rat insulinomas-G1 cell line
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1, 1' diphenyl-2-picryl hydrazyl
DPP-IV	Dipeptidyl peptidase IV
EDTA	Ethylene diamine tetra acetic acid
EGCG	Epigallocatechin gallate
EMEM	Eagle's modified essential medium
ESI	Electrospray ionization
FAB	Fast atomic bombardment
FBS	Feta bovine serum
FFA	Free fatty acid
FRAP	Ferric reducing antioxidant power
FTIR	Fourier transform infra-red

GABA	Gamma amino butyric acid
GC-MS	Gas-chromatography-Mass spectrometry
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
H_2O_2	Hydrogen peroxide
IC_{50}	Median inhibitory concentration
IL-6	Interleukin 6
INS-1	Insulinoma cell line
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LC-MS	Liquid chromatography-Mass spectrometry
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption ionization
MDA	Malondialdehyde
MS/MS	Tandem mass spectrometry.
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-ĸB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\cdot -}$	Superoxide radical
OH·	Hydroxyl radical
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate-buffered saline
PDK-1	Phosphoinositide-dependent kinase 1
PI3-K	Phosphatidyl inositol 3-kinase
PIP3	Phosphatidyl inositol 3, 4, 5-triphosphate
PKB	Protein kinase B

PNP	p-nitrophenol
PNP-GLUC	p-nitrophenyl-α-D-glucopyranoside
PPAR	Peroxisome proliferator activated receptor
RAGE	Receptor of advanced glycation end-products
RIN	Rat insulinomas cell line
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SH2	Src homology 2
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SUR-1	Sulfonylurea receptor 1
TLR4	Toll-like receptor 4
TNF-α	Tumor Necrosis factor alpha
TOF	Time-of-flight
TPTZ	Tripyridyl triazine
VLDL	Very low density lipoprotein
XTT	2, 3 – bis (2-methoxy-4-nitrro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt

Abstract

Diabetes mellitus is a chronic metabolic disorder primarily characterized by elevated blood glucose levels. Its rapidly increasing prevalence as a major non-communicable disease is now a growing concern for both developed and developing countries. The development of safe, cost-effective and pharmacologically-effective medications continues to a major challenge of antidiabetic management. Since most conventional antidiabetic drugs are costly and present with numerous adverse effects, alternatives are increasingly being sought from natural sources, most notably medicinal plants, as viable approaches to tackling the scourge of this disease. In the Eastern Cape Province of South Africa, the roots of *Dianthus thunbergii* and corms of *Hypoxis argentea* are frequently used traditionally for the treatment of diabetes mellitus, although no scientific evidence exists to validate their effectiveness for diabetes management. The studies conducted in the resent research were, therefore, aimed at investigating the antidiabetic and toxicological properties of these plants, in an attempt to providing data towards validating their use in traditional management of diabetes mellitus.

Aqueous and ethanol extracts of the underground parts of *D. thunbergii* and *H. argentea* were initially subjected to analysis of their phytochemical composition, relative to standard compounds, and the nature of their *in-vitro* antioxidant activities using standard spectrophotometric methods. The potentials of these extracts for cytotoxicity and/or cell proliferation were evaluated using MTT assay in HepG2 cells and Crystal violet assay in INS-1 cells. These activities were further examined in INS-1 cells using live cell fluorescence imaging techniques. To evaluate the antidiabetic properties of the extracts, they were screened for their inhibitory effects on the activities of different enzymes including α-amylase, α-glucosidase, porcine pancreatic lipase, Dipeptidyl peptidase IV (DPP-IV), collagenase and the drug

metabolizing enzyme, CYP3A4, while also assessing their effects on protein glycation using *in-vitro* visible and fluorescence spectrophotometric approaches.

Cell culture procedures were carried out to evaluate the effects of the extracts on glucose utilization in HepG2 cells and L6 myotubes; nitric oxide production in RAW 264.7 macrophages; glucose metabolism in INS-1 cells, as well as triglyceride accumulation in 3T3-L1 pre-adipocytes. Furthermore, identification of compounds present in the aqueous and ethanol extracts was carried out by Liquid chromatography- Mass spectrometry (LC-MS), while volatile oils extracted from fresh and dried parts of the two plants by hydrodistillation were also analyzed by Gas chromatography-Mass spectrometry (GC-MS).

The ethanol extracts of both *D. thunbergii* and *H. argentea* contained higher amounts of total phenols, flavonoids, tannins, proanthocyanidins and alkaloids, when compared with the aqueous extracts. This finding was in direct correlation with the antioxidant activities of the extracts, with the ethanol extracts of both plants demonstrating stronger scavenging activities against hydrogen peroxide, nitric oxide, ABTS and DPPH radicals, while also exhibiting higher ferric reducing antioxidant potentials, when compared with the aqueous extracts, and in some cases, the standard antioxidants, Vitamin C, butylated hydroxytoluene and rutin. The aqueous extracts of *D. thunbergii* exhibited the highest toxicity in HepG2 cells with IC_{50} < 50 µg/ml, while also producing a concentration-dependent reduction in the viability of INS-1 cells up to 41.81% at 50 µg/ml. Both extracts of *H. argentea,* however, did not produce any significant toxicity in these cells. Fluorescence imaging of live INS-1 cells using Hoechst and propidium iodide staining revealed stimulation of cell proliferation by *H. argentea,* while the cytotoxicity of *D. thunbergii* was further confirmed.

H. argentea caused stimulation of glucose uptake in HepG2 cells up to 119.58% at 100 µg/ml and as much as 116.96% in L6 myotubes at 50 µg/ml, without showing toxicity to these cells. *D. thunbergii* produced 18.39% increase in L6 glucose uptake above untreated control; although its effect on HepG2 glucose uptake was irrelevant as significant toxicity was produced in these cells. *H. argentea* produced a concentration-dependent reduction in nitric oxide production in RAW macrophages, although not as effectively as the positive control, aminoguanidine. Again, the toxicity of *D. thunbergii* to this cell line precludes the relevance of nitric oxide inhibition as an antidiabetic mechanism for this plant. *D. thunbergii* produced a concentration-dependent increase in 3T3-L1 triglyceride accumulation, as measured by Oil red O staining, compared to untreated cells, while *H. argentea* exerted no significant alterations in pre-adipocyte differentiation. Generally, the two plants produced weak inhibition of the activities of the various enzymes measured, suggesting that this mechanism may not play a major role in the activities of these plants as possible antidiabetic agents.

GC-MS analysis revealed major differences in the volatile oil composition between fresh and dried plant parts for both plants. Most notably, total terpenoid content of *D. thunbergii* oils reduced significantly from 77.17% in the fresh root oil to 47.58% in the dried root oil. Total terpenoid content was much lower in *H. argentea* oils, but similarly reduced from 10.58% in the fresh corm oil to 4.00% in the dried corm oil. LC-MS analysis enabled the tentative identification of compounds including phenolic glycosides, flavonoids, alkaloids, terpenoids saponins and sapogenins, many of which have been reported in literature to exert bioactivities relevant to the ones elucidated in the present study.

Overall, *H. argentea* exhibited antidiabetic properties that may be mediated by its stimulation of glucose uptake in HepG2 and L6 cells; stimulation of proliferation in INS-1 cells;

lack of stimulation of 3T3-L1 triglyceride accumulation and a tendency to reduce nitric oxide production in RAW macrophages. These activities suggest that *H. argentea* has promise for further investigations as an antidiabetic agent. On the contrary, *D. thunbergii* exhibited significant toxicity to HepG2 cells, INS-1 cells and RAW macrophages. Its cytotoxicity at the concentrations investigated in the present studies raises significant concerns about any potential antidiabetic applications for this plant.

Keywords: *D. thunbergii, H. argentea,* Hyperglycemia, antidiabetic, toxicity, HepG2, INS-1, L6 myotubes, 3T3-L1 pre-adipocytes, RAW macrophages.

CHAPTER 1

General Introduction and Literature Review

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GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Diabetes mellitus: Classification and prevalence

Diabetes mellitus is a chronic metabolic disorder characterized by abnormal increase in blood sugar levels (hyperglycemia), resulting from an absolute or relative deficiency of insulin secretion, with associated alterations in metabolism of carbohydrates, proteins and lipids (Genuth *et al.*, 2003; American Diabetes Association, 2009). The chronic hyperglycemic status in diabetics produces an increased risk of complications due to long-term damage and dysfunction of various organs such as the eyes, kidneys, nerves, heart and blood vessels. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, blurred vision and susceptibility to infections, while long term complications may include retinopathy with potential loss of vision, nephropathy progressing into renal failure and cardiovascular complications such as hypertension. Other complications include peripheral neuropathy with the associated risk of foot ulcers and autonomic neuropathy giving rise to gastrointestinal and genitourinary symptoms (American Diabetes Association, 2004).

Several specific types of diabetes have been recognized, but the vast majority of cases fall into two broad categories:

1. Type 1 diabetes (insulin-dependent diabetes or juvenile onset diabetes), caused by an absolute deficiency of insulin secretion usually due to a cell-mediated autoimmune destruction of β cells of the Islets of Langerhans of the pancreas, which may either have a genetic predisposition (Ozougwu *et al.*, 2013). This form of diabetes accounts for 5–10% of patients with diabetes.

2. Type 2 diabetes (non-insulin dependent diabetes or adult onset diabetes), which accounts for 90–95% of patients with diabetes and is associated with a reduced sensitivity to insulin, also called insulin resistance and/or impaired insulin secretion, rather than an absolute insulin deficiency. The pathogenesis of this form of diabetes is more variable, although auto-immune destruction of β cells does not occur (Cnop *et al.*, 2005).

Gestational diabetes, often regarded as a third type of diabetes, refers to any disease of glucose intolerance with onset or first recognition during pregnancy (Azevedo and Alla, 2008).

Diabetes mellitus is the third most significant, chronic, non-communicable disease now believed to be responsible for most deaths and disabilities in the world (WHO, 2007), superseded only by cancer and cardiovascular diseases. The disease is primarily associated with a modern lifestyle and had previously been thought to be uncommon in rural Africa. However, rapid uncontrolled urbanization and major lifestyle changes are believed to be driving its emergence as an important non-communicable disease in recent years in sub-Saharan Africa (Motala *et al.*, 2008; Levitt, 2008).

Among adults (20–79 years), a worldwide prevalence of about 8.3% was estimated for the year 2013, and this was predicted to rise to 10.1% in 2035. Interestingly about 80% of the people affected are those living in low and middle-income countries. Current regional prevalence figures for Africa stand at about 4.6% (International Diabetes Federation, 2013). A few published studies in sub-Saharan Africa estimate low prevalence for Type 1 diabetes with 0.33 per 1000 in Nigerians and about 0.94 per 1000 in the Sudanese population (Mbanya *et al.*, 2010). Type 2 diabetes, however, is the most common form of diabetes in sub-Saharan Africa, similar to other regions of the world. Frequencies of 3–10% for Type 2 diabetes have been noted in urban

and peri-urban populations in South Africa (Levitt *et al*., 1993; Omar *et al.*, 1993) and in Sudanese communities of African origin (Elbagir *et al*., 1998) comparable with rates in developed countries. According to the International Diabetes Federation, there were about 2.28 million cases of diabetes in South Africa in 2015 with prevalence in the same period placed at 7.0% among adults 20–79 years old. In the same period, 57,318 deaths in adults were reported due to diabetes, while average cost per person with diabetes amounted to about USD 918.9 (IDF, 2015)

1.2 Normal Glucose Homeostasis

Glucose is a general metabolic fuel for virtually all cell types, and when present in sufficient quantities, it is the preferred fuel for most tissues of the body. A continuous supply of this metabolic fuel is required for the proper functioning and survival of all organs. While hypoglycemia can result in cell death, chronic hyperglycemia can also produce organ damage. The concentration of glucose in plasma is determined by the rate of glucose entry into the circulation, balance by the rate of glucose removal from the circulation (Gerich, 1993).

Circulating glucose may be derived from:

1. Intestinal absorption during the fed state,
2. Breakdown of glycogen to release glucose, called glycogenolysis, and
3. Synthesis of glucose from non-carbohydrate precursors (lactate and amino acids), called gluconeogenesis, in the fasting state.

Historically, the regulation of glucose metabolism has been viewed from a bi-hormonal perspective involving insulin and glucagon as primary regulators. In the 1970s, several gut hormones and other contributors to the maintenance of glucose homeostasis were identified (Moore and Cooper, 1991). The array of gluco-regulatory hormones now include other

molecules including glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide-1 (GLP-1), epinephrine, cortisol, amylin, and the growth hormone. These molecules are all involved in maintaining circulating glucose concentrations in a relatively narrow range.

According to the common bi-hormonal model (Figure 1.1), secretion of insulin in the fed state by the pancreatic β cells, coupled with responsiveness to insulin by major glucose metabolic tissues such as muscle, liver and fat, causes the mobilization of dietary glucose from circulation, thereby controlling plasma glucose. Insulin promotes glucose uptake, glycogen synthesis in the liver and muscle, formation of storage lipids in the adipose tissue and protein synthesis in most cells (Shakur *et al*., 2001; Barthel and Schmoll, 2003). The major portion of post-prandial glucose is used by skeletal muscles, an effect of insulin-stimulated glucose uptake. Other tissues, most notably the brain, use glucose in a non-insulin dependent fashion.

In the fasting state, however, there is a decreased plasma insulin concentration with an increased secretion of glucagon, glucocorticoids and catecholamines, all of which contribute to an increased glucose output via glycogenolysis, gluconeogenesis and lipolysis, as well as decreased synthesis and/or increased degradation of proteins.

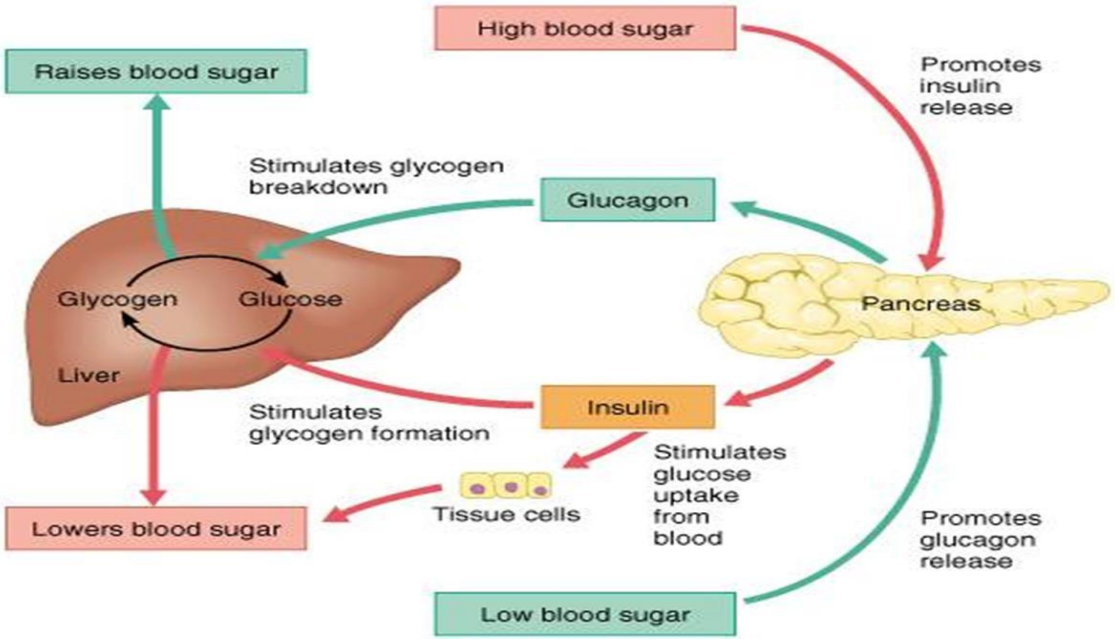

Fig.1.1: Simplified Regulation of glucose homeostasis (Marieb, 2001).

1.3. The pancreatic β-cell and insulin action

The islets of Langerhans, comprising 1–2% of the volume of the entire pancreas, consist of a population of 66–80% insulin-secreting beta (β) cells, and 15–20% glucagon-releasing alpha (α) cells. Other cell populations include somatostatin-producing delta (δ) cells (3–10%) and cells secreting the pancreatic polypeptide (1%) (Elayat *et al.*, 1995). β cells are the most studied of these cells and their primary function is to store and release insulin. The β cell couples nutrient (mainly glucose) metabolism with electrical activity to modulate the synthesis and release of insulin.

Pancreatic β cell mass is regulated by a combination of factors, including:

a. β cell replication
b. β cell size
c. β cell neogenesis, and

d. β cell apoptosis.

One of the features of Type 2 diabetes is a progressive decrease in β cell mass arising from marked increase in β cell apoptosis prevailing over β cell replication and neogenesis (Rhodes, 2005). A number of growth factors such as the epidermal growth factor, transforming growth factor α, growth hormone and insulin growth factor-1, are linked to β cell renewal and growth. Glucose is also able to stimulate β cell proliferation and destruction.

A number of cell lines that mimic primary β cell physiology have been developed. They represent extremely valuable tools for the study of molecular events connected to β cell function and dysfunction. B cell lines are usually derived insulinoma cells possessing the capability for unlimited growth in tissue culture. However, there are distinct differences in the responsiveness to glucose-induced insulin secretion, with most of them exhibiting marked hypersensitivity to glucose. The most widely used tumour cell lines are radiation- or virus-induced insulinomas. Examples of cell lines that have retained some characteristics of normal β cells include the rat insulinoma cell line (RIN), insulinoma cell line (INS-1) and the Cambridge rat insulinoma-G1 cell line (CRI-G1). In most cell lines hormone secretion is high at the beginning but decreased with time in culture (Ulrich *et al.*, 2002) and the capacity to respond to glucose is high at low or intermediate passages, but this property may be lost at higher passages.

1.3.1 Insulin: Structure and actions

The human biologically active form of insulin circulates in plasma as a monomer consisting of two chains, an A chain of 21 amino acids and a B chain of 30 amino acids (in man), linked by two disulfide bridges, A7–B7 and A20–B19 (DeMeyts, 2004). The A chain contains an intra-chain disulfide bridge between A6 and A11 (Fig. 1.2). At micro-molar concentrations, insulin dimerizes and, in the presence of zinc ions, further associates into hexamers, creating a

symmetrical structure. As the concentration of zinc ions is low in plasma, it is unlikely that it contains a substantial amount of the hexamer. The A chain has an N-terminal helix, A1–A8, linked to an antiparallel C-terminal helix, A12–A20. The B chain has a central helix B8–B19, extended by N- and C-terminal strands. This crystallographic conformation is referred to as the T conformation. An alternative conformation exists in which the B-chain helix extends all the way to the N-terminal, referred to as the R conformation (Fig. 1.2).

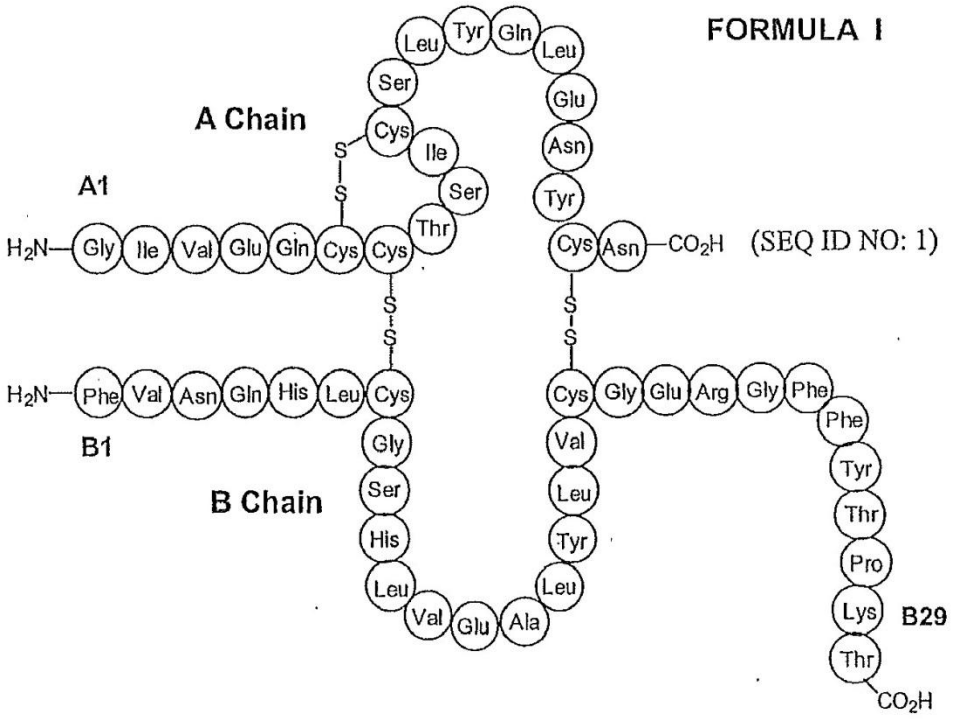

Figure 1.2: Structure of insulin

In terms of structure-activity relationships, the insulin monomer is the structure that binds to the insulin receptor and triggers signals through it, although it is not clear the extent of conformational change the insulin molecule undergoes upon binding to the receptor.

1.3.2. Cellular glucose metabolism of the β cell and insulin secretion

Glucose metabolism is the primary physiological event that stimulates insulin gene transcription and mRNA translation (Poitout *et al*., 2006). However, other factors such as nutrients (e.g., amino acids, fatty acids); small peptides (e.g., acetylcholine, pituitary adenylate cyclase-activating polypeptide (PACAP); incretins (e.g., glucose-dependent insulinotropic polypeptide (GIP) glucagon-like peptide-1 (GLP-1); and several other agonists also influence these processes (Bratanova-Tochkova *et al*., 2002).

β cell glucose metabolism has certain distinct characteristics that are intricately linked to insulin secretion:

1. The β cell expresses across its membranes, the high capacity, low affinity glucose transporter, GLUT-2, which mediates glucose transport by facilitated diffusion and allows rapid equilibration of glucose across the β cell membrane. GLUT-2 is the only glucose transporter expressed in β cells. It is also expressed in the liver, renal and intestinal absorptive cells, although these tissues express some other isoforms of glucose transporters. Unlike GLUT-4, which is primarily expressed in muscle and fat cells, mobilization of GLUT-2 to the plasma membrane is insulin-independent and the transporter protein shows a low substrate affinity, ensuring high glucose influx (Newsholme *et al.*, 2010).
2. Once glucose has entered the β cell, it is phosphorylated to glucose-6-phosphate by the high Km glucokinase (also called hexokinase IV). This step constitutes the flux-determining step for glycolysis, ensuring that glucose which enters the cell is committed to metabolism within the cell (MacDonald *et al*., 2005). Glucokinase is expressed only in

four types of mammalian cells: hepatic cells, pancreatic β cells, enterocytes and glucose-sensitive neurons.

Two important properties enable glucokinase to function as a glucose sensor in β cells:

a. It has relatively lower affinity for glucose than other hexokinases
b. It is not inhibited by its product, an otherwise common regulatory feature in the metabolism of substrates. Therefore, its activity continues even at high concentrations of glucose (Suckale and Solimena, 2008).

3. Once phosphorylated, glucose is metabolized by glycolysis to pyruvate, which is then oxidized through the TCA cycle by the mitochondria, to produce ATP. In both processes, NADH is also produced. β cells express very low levels of lactate dehydrogenase. This ensures that pyruvate cannot shunt away from the mitochondrial metabolic pathways (Zhao *et al.*, 2001).

Pyruvate oxidation through the TCA cycle is the major signal pathway coupled to the ATP-sensitive potassium (K_{ATP}) channel-dependent insulin release. Mitochondrial oxidative metabolism has been estimated to produce 98% of β cell ATP and this serves as the main effector of insulin secretion. Increased metabolism of glucose and pyruvate increases the ATP/ADP ratio in the β cell. Elevated ATP levels displace bound ADP on the K^+ channels, resulting in channel closure in normal β cells. The closure of these channels causes membrane depolarization and activation and opening of voltage dependent Ca^{2+} channels, with the resultant influx of Ca^{2+} into the cytosol. Elevated cytosolic Ca^{2+} concentration leads to the stimulation of exocytosis of insulin-containing granules (Fu *et al.*, 2013) (Fig. 1.3).

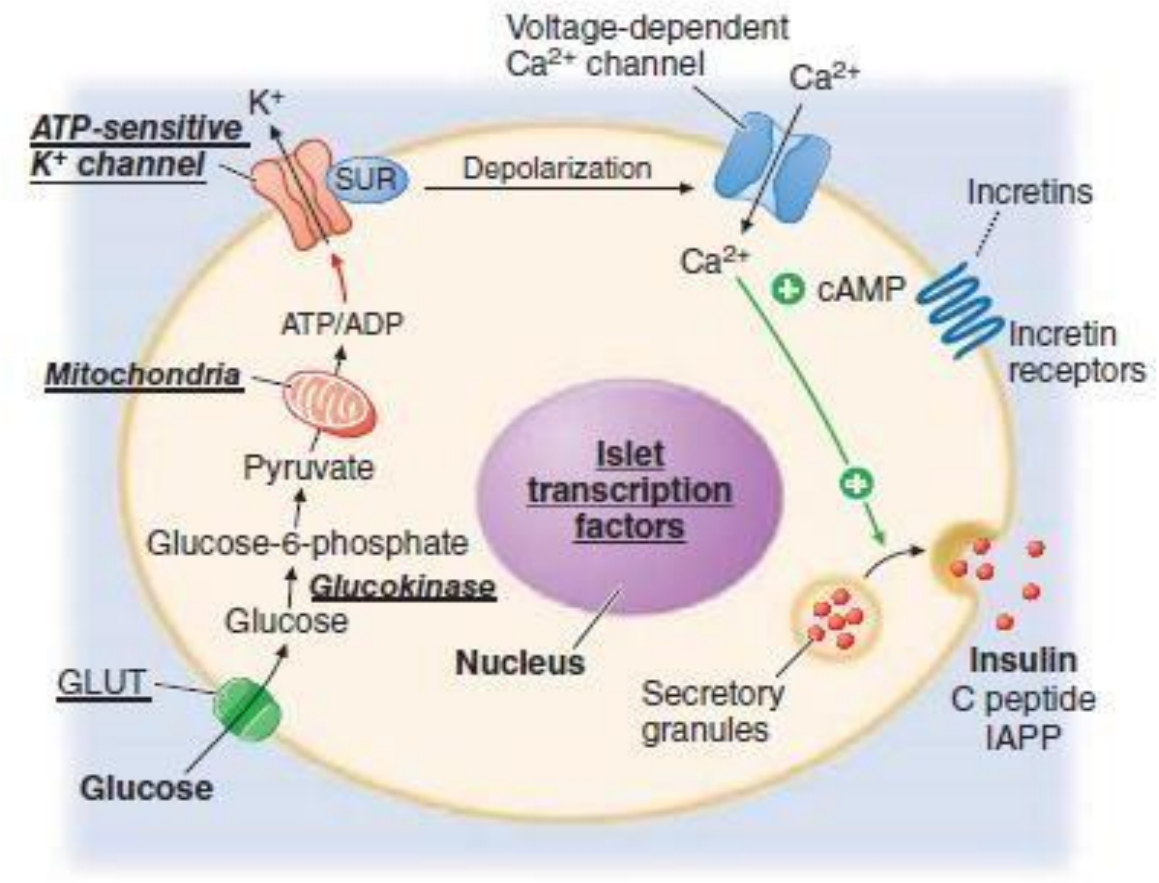

Fig. 1.3: Mechanism of glucose-induced Insulin secretion (GIIS) in the β cell (Source: Kasper *et al.*, 2015).

Exocytosis is highly regulated by the molecular machinery broadly known as soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Bratanova-Tochkova *et al.*, 2002). The SNARE complex is composed of two groups of SNARE proteins: those associated with the secretory vesicles and those associated with the plasma membrane. In the β cell, both plasma membrane and vesicle bound SNAREs are involved in docking the secretory vesicle to the plasma membrane (Pasyk *et al.*, 2004). Increased cytosolic Ca^{2+} activates a Ca^{2+}-sensitive protein in the SNARE complex called synaptotagmin (Gauthier and Wollheim, 2008), leading to SNARE-mediated fusion of the secretory vesicle with the plasma membrane, and thus insulin secretion from the β cell.

In effect, glucose induces insulin secretion by both triggering K^+ channel closure and also by amplifying K_{ATP} closure effects. It should be noted, however, that cell signals other than increased intracellular Ca^{2+} concentration are activated by glucose. These include cAMP, cGMP, inositol 1, 4, 5-triphosphate and diacylglycerol (Fu *et al.*, 2013), cAMP being the most important among these for potentiating insulin secretion.

1.3.3. Insulin signaling and Glucose utilization/uptake by other cells

As the glucose molecule is very hydrophilic, it cannot ordinarily diffuse through the lipid bilayer of cell membranes. Its entry into cells, therefore, is mediated by specific membrane transporters which are mainly of two different families: the sodium-dependent glucose transporters (SGLT) and the facilitative glucose transporters (GLUTs) (Scheepers *et al.*, 2004). SGLTs are primarily involved in glucose absorption across the intestinal and renal tubular epithelium in which glucose is co-transported with sodium ions.

GLUTs mediate the transport/uptake of glucose by facilitated diffusion. There are about 14 genes coding for individual proteins of the GLUT family (GLUT 1 – GLUT 14). The proteins are different in their tissue distribution, as well as their relative affinities for glucose (Scheepers *et al.*, 2004). In skeletal muscle and adipose tissue, three glucose transporters are expressed, including GLUT1, GLUT 3 AND GLUT 4. GLUT 4 is the most abundant and has the highest turnover number (Waddell *et al.*, 1992).

One of the primary effects of insulin upon its secretion is to regulate the disposal or storage of glucose by stimulating its uptake into insulin-responsive tissues such as muscle and adipose tissue. Glucose transport is the key step in insulin-regulated glucose metabolism and a dysfunction of this process in muscle or adipose tissue clearly represent an important defect in insulin action. Insulin mediates glucose transport by stimulating the translocation of GLUT 4

isoforms from the intracellular pools to the cell surface (Shepherd and Kahn, 1999). In the basal state (absence of insulin), these glucose transporters are normally sequestered intracellularly. It should be noted that glucose uptake into skeletal muscle tissues may also occur through an insulin-independent pathway activated by contractions through the AMP-protein kinase (AMPK) complex (Alvim *et al.*, 2015).

Insulin stimulation of glucose uptake involves a complex series of cellular and molecular signaling events. In insulin-responsive tissues, the action of insulin is initiated by its binding to the insulin receptor. The insulin receptor belongs to the family of receptors called receptor tyrosine kinases which plays critical roles in development, cell proliferation and differentiation, cell survival and metabolism (Blume-Jensen and Hunter, 2001). Activation of receptors in this family occurs by the binding of their cognate ligands leading to receptor dimerization (or oligomerization) and auto phosphorylation, as well as phosphorylation of other protein substrates, exclusively on their tyrosine residues (Ward *et al.*, 2007). The insulin receptor is a hetero-tetramer made up of two α-subunits located extracellularly, and responsible for ligand binding, and two transmembrane β subunits, which possess intrinsic tyrosine kinase activity (Draznin, 2006).

The binding of insulin leads to a structural change in the α-subunits, producing an auto-phosphorylation of the tyrosine kinase domain in the β subunits, and subsequent tyrosine phosphorylation of several downstream protein substrates, including insulin receptor substrates (IRS) 1, 2, 3 and 4. IRS-1, when phosphorylated, recognizes and binds to proteins with src-homology 2 (SH2) domains, especially phosphatidyl inositol 3- kinase (PI3- K). PI3K is a heterodimeric protein composed of a regulatory subunit (p85) and a catalytic subunit (p110) (Cavalheira *et al.*, 2002). PI3K becomes activated when phosphorylated IRS-1 binds to its SH2

domains, thereby activating its catalytic subunit, which then phosphorylates phosphatidyl inositol 4,5 biphosphate at position 3 on the inositol ring, to form phosphatidyl inositol 3,4,5 triphosphate (PIP3) (Van Dam *et al.*, 2005). PIP3 provides the phosphorylation of phosphoinositide-dependent kinase 1 (PDK 1), which in turn activates protein kinase B (PKB or Akt) and protein kinase C. PKB is thus recruited to the plasma membrane where it stimulates the translocation of GLUT 4 to the plasma membrane, thus facilitating the entry of glucose into the cell (Fig. 1.4).

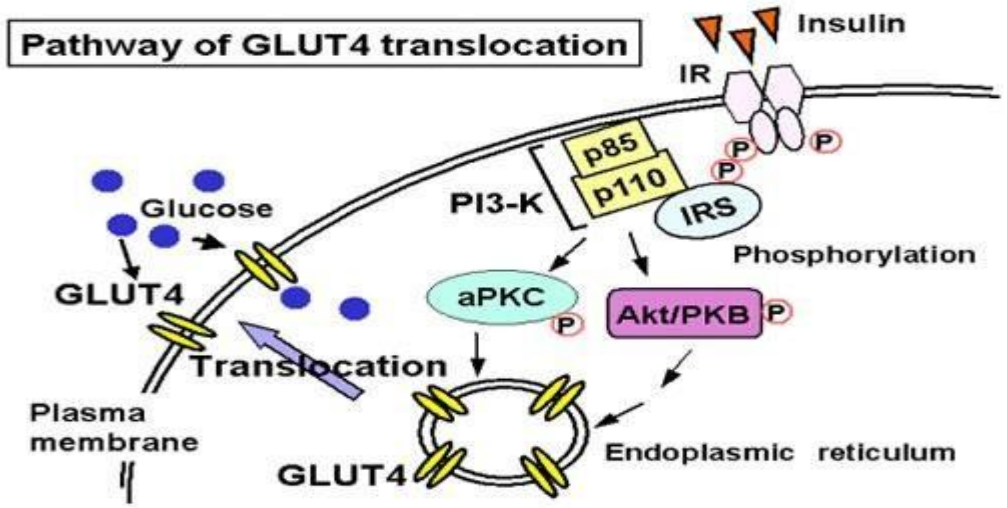

Fig. 1.4: Stimulation of glucose uptake by insulin (Jewwell *et al.*, 2010).

1.4 Insulin resistance

Insulin resistance has been defined as the inability of insulin to promote normal cellular glucose uptake at a given insulin concentration (Ducluzeau *et al.*, 2002). The molecular mechanisms of development of insulin resistance are still unclear. However, in most cases, insulin resistance is the result of alterations in insulin receptor functioning as well as post-receptor defects in insulin signaling. These may include receptor down-regulation, deficiencies

or genetic polymorphisms of tyrosine phosphorylation of the insulin receptor, IRS proteins or PI3-kinase, as well as abnormalities of GLUT-4 function (Wheatcroft *et al*., 2003).

Obesity, caused by excessive nutrition and sedentary lifestyles, is believed to be the leading cause of insulin resistance and therefore, a major risk factor of Type 2 diabetes mellitus. Obesity-associated exposure of tissues to elevated dietary nutrients results in excessive accumulation of lipids and toxic metabolic by-products (Finkelstein *et al.*, 2012). Insulin resistance is associated with increased lipolysis (producing elevated plasma free fatty acids, FFAs) with an impaired ability of adipocytes to store excess calories as triglycerides, leading to abnormal redistribution of lipids and their metabolites to other tissues that are not normally adapted to lipid storage such as the muscle and liver, a condition called lipotoxicity. Circulating FFAs contribute to the development of insulin resistance via several mechanisms.

Hyperlipidemia and hyperglycemia caused by excessive nutrients, lipolysis and gluconeogenesis induce mitochondrial dysfunction, endoplasmic reticulum stress and oxidative stress, which stimulate stress-responsive signaling molecules such as JNK (Abel *et al.*, 2012). Further, saturated FFAs bind to toll-like receptor 4 (TLR4), causing the activation of NF-KB and JNK, with its subsequent inflammation and insulin resistance (Shi *et al*., 2006). Inflammation in adipose tissue is mediated by a group of hormones and cytokines called adipokines which are produced by adipocytes and infiltrating pro-inflammatory immune cells such as macrophages (Muoio and Newgard, 2008). Pro-inflammatory adipokines include leptin, TNF-α, Interleukin-6, resistin, retinol-binding protein-4, and so on. Recent research efforts now target pro-inflammatory pathways as a novel therapeutic approach to prevent insulin resistance, particularly in obesity-induced insulin resistance (Goldfine *et al.*, 2010).

Insulin resistance in the liver and muscle tissue manifests as a consequence of metabolic overload. Accumulation of excess lipid in these tissues causes impairment of fatty acid oxidation, resulting in the redirection of long-chain fatty acyl chains into diacylglycerols, ceramides and triglycerides, the presence of which correlates negatively with insulin sensitivity (Abel *et al.*, 2001). Major hepatic manifestations of insulin resistance include increased hepatic glucose output by gluconeogenesis and altered lipoprotein metabolism. Increased FFA delivery and reduced catabolism of very low density lipoproteins by insulin resistant adipocytes increases hepatic triglyceride content and VLDL secretion. Studies have also shown impairment of muscle glycogen synthesis in insulin resistance, mediated by reduced intracellular glucose uptake (Hunter and Garvey, 1998).

Other factors proposed by Guyton and Hall (2006), to be involved in the development of insulin resistance are:

- Excessive glucocorticoids as in Cushing's syndrome and steroid therapy
- Excessive growth hormone as seen in acromegaly
- Pregnancy and gestational diabetes
- Polycystic ovarian disease
- Lipodystrophy associated with lipid accumulation in the liver
- Autoantibodies to the insulin receptor
- Mutations of the insulin receptor
- Mutations of the Peroxisome proliferator activated receptor γ
- Hemochromatosis – a hereditary disease that causes tissue iron accumulation.

1.5. Molecular pathophysiology of Type 1 and Type 2 diabetes

1.5.1. Type 1 diabetes

Type 1 diabetes is essentially a T-cell mediated auto-immune disease, characterized by the selective destruction of pancreatic β cells. Susceptibility to this disease is determined by a combination of genetic and environmental factors which may include viral infections, dietary factors in early infancy, vaccination, climatic influences, toxins and stress. The destruction of β cells leads to severe insulin depletion, with hyperglycemia resulting from overproduction of glucose from glycogenolysis and gluconeogenesis and decreased cellular uptake of glucose from circulation.

The onset of Type 1 diabetes usually coincides with the end stage of β cell destruction (β cell mass reduced by 70–80%). A variety of features characterizing the autoimmune nature of this disease have been identified in affected patients. These include the presence of islet cell-specific auto-antibodies and the presence of immune-competent and accessory cells in infiltrated pancreatic islets; response by patients to immunotherapy and alterations in T-cell mediated immune-regulation, especially the CD4+ cell compartment (Al Homsi and Lukic, 1992). Three types of autoantibodies are usually detected in Type 1 diabetes, including islet cell cytoplasmic antibodies, islet cell surface antibodies and specific antigenic targets of islet cells (Ozougwu *et al*., 2013).

Type 1 diabetes has strong associations with other endocrine auto-immunity disorders, e.g., Addison's disease. The auto-immune assault characterized by inflammatory reactions termed 'insulitis', features invasion of the islets by mononuclear cells leading to loss of β cells after prolonged periods of the disease. Beta cell death in insulitis may be caused by direct contact with activated macrophages and T cells and/or exposure to soluble mediators secreted by these

cells, including cytokines, nitric oxide and reactive oxygen species (Eizirik and Mandrup-Poulsen, 2010). Cytokines induce stress response genes that are either protective or deleterious for β cell survival.

Apoptosis is the main cause of β cell death at the onset of Type 1 diabetes, activated by extracellular signals, intracellular ATP levels, phosphorylation cascades and expression of pro- and anti-apoptotic proteins. β cell apoptosis can occur in response to several stimuli acting via three recognized pathways including *i.* Cytokine-induced cell death mediated by cell surface receptors such as TNF-α, *ii.* Mitochondrial disruption secondary to reactive oxygen species and release of cytochrome c, and *iii* Endoplasmic reticulum stress pathway (Lupi and Del Prato, 2008).

1.5.2 Type 2 diabetes

Two main pathological defects characterize the pathogenesis of Type 2 diabetes: impaired insulin secretion through a dysfunction of the pancreatic β cell and impaired insulin action due to insulin resistance (Holt, 2004). It is well known that insulin resistance in Type 2 diabetes occurs mainly as a result of defective post-receptor insulin signaling, causing alterations in the metabolic actions of insulin.

Several mechanisms such as tyrosine dephosphorylation, imbalance of serine/threonine phosphorylation, or insulin receptor internalization impair insulin signaling (Zick, 2004). A number of molecules associated with insulin resistance affect these mechanisms, and most of them are related to the adipose tissue. Obesity and lipodystrophy lead to insulin resistance in the skeletal muscles (Abel *et al*., 2001). Recent data suggest that molecules released from adipocytes, e.g., free fatty acids, tumor necrosis factor alpha (TNFα) and Interleukin 6 (IL-6)

inhibit insulin signaling and induce insulin resistance by activating serine/threonine kinases that phosphorylate IRS proteins, thus inhibiting their function (Schiner *et al*., 2005).

The mechanisms of lipotoxicity by free fatty acids in the β cells is largely uncertain, although it has been suggested that free fatty acids activate cellular kinases, including protein kinase C isoforms with the consequent elevation of diacylglycerol concentrations, which can inhibit the insulin-dependent tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase (Dresner *et al*., 1999). The IL-6 concentration is elevated in insulin resistance states. This cytokine decreases tyrosine phosphorylation of IRS-1 and decreases the association of the p85 subunit of PI3K with IRS-1 in response to physiologic insulin levels (Senn *et al*., 2002). TNFα participates in the pathogenesis of Type 2 diabetes via various mechanisms, including the stimulation of lipolysis, resulting in elevated levels of free fatty acids as well as the down-regulation of genes required for normal insulin action e.g. GLUT 4, as well as direct effects on insulin signaling and effects on the peroxisome proliferator activated receptor gamma (PPARγ).

PPARs are nuclear receptors that heterodimrize with the retinoid X receptor (RXR) and regulate transcription of a number of genes. Activation of PPARγ has been found to be one of the molecular mechanisms of anti-diabetic agents with insulin-sensitizing agents, such as seen in thiazolidinedione drugs (Saltiel, 2001).

Hyperglycemia itself decreases insulin secretion, and is implicated in the damage to β cells (Robertson *et al*., 2003). Chronic hyperglycemia impairs insulin gene expression of some major β cell transcription factors (Poitout and Robertson, 2002). The molecular mechanisms of glucotoxicity have been proposed to involve the generation of reactive oxygen species and oxidative stress (Robertson *et al*., 2003). In addition, hyperglycemia-induced mitochondrial superoxide production activates uncoupling protein 2, which decreases the ATP/ADP ratio and

reduces the insulin-secretory response (Brownlee, 2003). Reactive oxygen species are also known to enhance nuclear factor-kappa B activity which potentially induces β cell life and death.

1.6. Reactive oxygen species and common complications of diabetes mellitus

Diabetes mellitus is associated with a variety of complicating conditions that are largely responsible for the high morbidity and mortality caused by the disease. Regardless of the underlying causes of diabetes, there is a decrease in the uptake of glucose into muscle and adipose tissue producing chronic hyperglycemia that ultimately results in damage to various organs (Resnick and Howard, 2002; Luscher *et al*., 2003). Common long-term complications occurring in diabetes include eye damage (diabetic retinopathy and cataract formation), kidneys (diabetic nephropathy), nerves (diabetic neuropathy) heart (myocardial infarction) and blood vessels (atherosclerosis, hypertension) (ADA, 2010; Tesfaye and Gill, 2011; Chintan *et al*., 2011). Diabetic foot complications, (e.g., foot ulcerations), also result from complex interactions between peripheral neuropathy (with loss of pain sensation), microangiopathy and poor foot hygiene.

One major underlying phenomenon that has been proposed to explain hyperglycemia-induced onset of diabetic complications is the production of reactive oxygen species (ROS) (Sheetz and King, 2002; Creager *et al*., 2003; Niedowicz and Daleke, 2005). ROS include reactive metabolites of oxygen called free radicals, such as superoxide ($O_2^{\bullet -}$), hydroxyl ($OH^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, as well as non-radicals such as hydrogen peroxide, singlet oxygen, hypochlorous acid, and so on. Free radicals are chemical species capable of independent existence and possess a lone electron in an orbital, rendering the radical highly

unstable and very reactive towards nuleophilic sites in macromolecules (lipids, proteins and DNA) (Paravicini and Touyz, 2008).

In aerobic organisms, one major production site of ROS is the mitochondria, where transport of electrons through a series of protein complexes (I-IV) via oxidative phosphorylation, results in the reduction of molecular oxygen to water. Incomplete reduction of oxygen, via uncoupling of electron transport with the reduction of oxygen (due to uncoupling proteins) yields the superoxide radical. In these situations, the key sites for superoxide radical generation are complex I and the ubiquinone-complex III (Niedowicz and Daleke, 2005). In diabetes, alterations in both the electron transport activity and in some instances, uncoupling protein activity or expression have been proposed as responsible for an increase in superoxide radical production.

In addition, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are a major source of glucose-induced superoxide radical production in the blood vessels and kidneys (Inoguchi *et al*., 2003; Li and Shah, 2003). NAD (P) H oxidase is a multi-subunit enzyme that catalyzes $O_2^{\bullet -}$ production by the 1-electron reduction of O_2, using NADPH or NADH as the electron donor. Other potential sources include Nitric oxide synthase (NOS) (Ceriello, 2003), Xanthine oxidase and Cytochrome P450 monooxygenases (Desco *et al*., 2002). NOS can also transfer electrons directly to O_2 to form $O_2^{\bullet -}$ and it is also well known that nitric oxide produced by NOS can react with O_2 to form peroxynitrite, a highly reactive oxidant associated with diabetes and other disease states such as sepsis, inflammation and atherosclerosis. Xanthine oxidase, which catalyzes the oxidation of hypoxanthine and xanthine to form $O_2^{\bullet -}$

ROS generation by various mechanisms in diabetes can cause oxidation of lipids, leading to the production of highly reactive aldehyde end-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These products react with protein and DNA, altering their function and potentially inducing genetic mutations. In long-standing diabetes, protein oxidation and glycation results in the formation of advanced glycation end-products (AGEs), a process that alters protein structure and activity.

ROS, when produced, cause depletion of antioxidant defenses including enzymatic antioxidants such as glutathione peroxidase, catalase, superoxide dismutase and glucose 6-phosphate dehydrogenase, as well as, non-enzymatic antioxidants such as Vitamins A, C and E, glutathione, α-lipoic acid, and so on. Generally excessive production of ROS can overwhelm antioxidant defenses beyond their capacity to neutralize them. This creates an unbalanced state in favor of oxidants, called oxidative stress. Oxidative stress produces damage to cellular components that results in the various complications observed in long-standing hyperglycemia (Brownlee and Cerami, 1981; Giugliano *et al,* 1995).

1.7 Glycation and advanced glycation end-products (AGEs)

Glycation is a term used to describe the non-enzymatic glycosylation reaction between reducing sugars and free amino groups of proteins, lipids and nucleic acids. Protein glycation differs from the enzymatic glycosylation of proteins that occurs as a post-translational modification to proteins in the endoplasmic reticulum and Golgi apparatus, resulting in the formation of glycoproteins (Negre-Salvayre *et al*., 2009). In glycation, the initial reaction is followed by a cascade of reactions resulting in the formation of a Schiff base, which spontaneously rearranges itself to form an Amadori and Maillard products (Fig. 1.5). The

reactions eventually lead to the formation of a variety of derivatives known as advanced glycation end-products (AGEs).

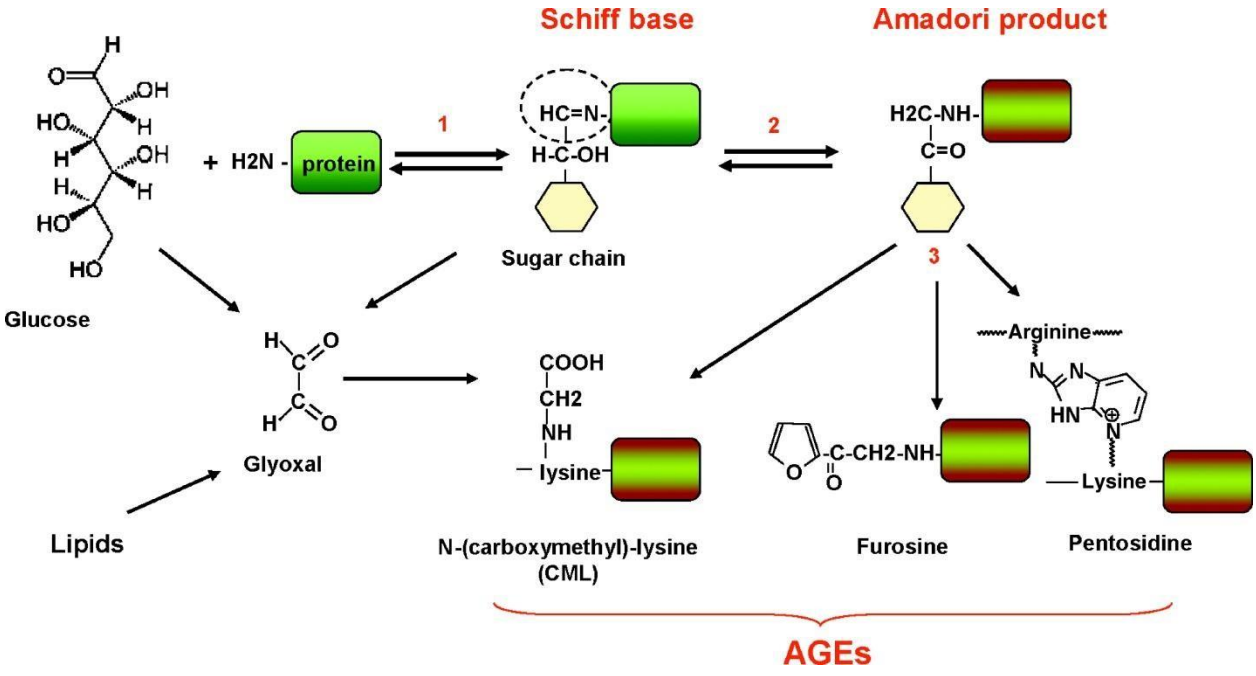

Fig. 1.5: Formation of AGEs during protein glycation (Source: Negre-Salvayre *et al*., 2009).

Protein glycation usually affects circulating proteins, e.g., serum albumin, lipoproteins, insulin and hemoglobin. AGEs characteristically form covalent cross-links with proteins, especially long-lived extracellular matrix proteins e.g. collagen. They can also react with a variety of cell surface AGE-binding receptors, referred to as Receptors of advanced glycation end-products (RAGEs). Interaction with RAGEs may lead to the endocytosis of AGEs and their subsequent degradation, or to the activation of pro-oxidant and pro-inflammatory events implicated in endothelium dysfunction and micro vascular complications of diabetes (Peppa *et al*., 2003).

AGEs participate in the progression of diabetic complications via different mechanisms in different tissues. For example, accumulation of AGEs in the glomerular basement membrane

of the nephron and their interaction with messangial cells, endothelial cells and podocytes triggers oxidative stress, inflammatory signaling and apoptosis which characterize diabetic nephropathy (Fukami *et al.*, 2008). Furthermore, RAGEs and AGEs are involved in oxidative stress and sustained NF-KB activation with induction of pro-inflammatory genes, resulting in neurologic dysfunction and loss of pain sensation which is a feature of diabetic neuropathy. Increased hemoglobin glycation causing decreased oxygen delivery to tissues, peripheral neuropathy and altered innate immunity have all been implicated in the impairment of wound healing, a frequent complication of diabetes. AGEs and RAGEs contribute to this process by decreasing phagocytosis and chemotaxis of phagocytes to wound sites, together with the increased secretion of pro-inflammatory cytokines, (e.g., IL-6 and TNF-α), matrix metallo-proteases and reduced synthesis of collagen (Goova *et al.*, 2001).

1.8. Conventional therapeutic management of diabetes

Current approaches employed in the management of diabetes can be broadly classified into two categories as:

a. Non-pharmacological interventions, e.g., modification of diet, increased physical exercise, acupuncture, and so on.
b. Pharmacological interventions, including oral and injectable anti-diabetic drugs.

The main treatment for Type 1 diabetes, aimed at achieving normal glycemia levels with minimal side effects, is insulin treatment. Severe forms of Type 2 diabetes may also require insulin treatment (ADA, 2002). Injectable insulin can be prepared from the pancreas of different compatible species (e.g. porcine or bovine pancreas), each having different pharmacological properties. Insulin injections are commercially available as rapid-, short- and long-acting types. Insulin extracted from other animals is made chemically identical to human insulin by

recombinant DNA technology or other forms of chemical modification. Insulin analogs have also been developed by modifying the amino acid sequence of insulin.

Currently available oral hypoglycemic drugs are classified into different categories, based on their mechanisms of action. The list includes, although is not limited to, Sulfonylureas, Meglitinides, Biguanides, Thiazolidinediones, Alpha-glucosidase inhibitors and DPP-IV inhibitors. These drugs are most commonly applied in the treatment of Type 2 diabetes.

1.8.1 Mechanisms of action of anti-diabetic medications

1. **Sulfonylureas,** e.g., glibenclamide, glicazide, glipizide, glimepiride.

These drugs act by stimulating insulin secretion by pancreatic β cells. They are effective in reducing blood glucose levels in the short term, although they have not been proved to offer much benefit against long-term diabetic complications. Stimulation of insulin secretion occurs by different mechanisms including the enhancement of the sensitivity of the β cells to glucose; binding to the trans-membrane sulfonylurea receptor (SUR-1) in the β cell to mediate the closing of the ATP-sensitive potassium channels (Aguilar-Bryan *et al*., 1995).

2 **Biguanides,** e.g., Metformin

These are thought to improve insulin sensitivity in peripheral tissues via modification of post-receptor signaling in the insulin signaling pathway. Their effects on the hepatic tissue produce a reduction in the hepatic production of glucose via the reduction of gluconeogenesis and glycogenolysis (Shaw *et al*., 2005; Hawley *et al*., 2002). Up-regulation of GLUT 4 in skeletal muscles and adipocytes is also thought to occur in response to metformin administration (Kip and Leiter, 1990).

3 **Thiazolidinediones,** e.g., Rosiglitazone, pioglitazone

This class of drugs mediates their function through binding to the PPARγ receptor, predominantly expressed in adipocytes. Binding to this receptor stimulates interaction with the retinoic X receptor, which hetero-dimerizes and activates genes that play roles in carbohydrate and lipid metabolism (Stafylas *et al*., 2008).

4 **Alpha-glucosidase inhibitors,** e.g., Acarbose

Acarbose inhibits the activity of alpha-glucosidases, a family of membrane-bound enzymes in the intestine which assist in the digestion of carbohydrates, thereby slowing the absorption of ingested carbohydrates and reducing post-prandial hyperglycemia in diabetic patients (Chiasson, 2006).

5 DPP IV- inhibitors e. g. Alogliptin, Linagliptin

Incretin hormones such as Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1) contribute to glucose-dependent insulin secretion, an increase in β cell mass and a decrease in glucagon secretion. These gut hormones are sensitive to degradation by dipeptidyl peptidase IV (DPP-IV), a serine protease which cleaves polypeptides containing proline and alanine residues at the penultimate N-terminal position and thereby decreases the efficacy of the hormones. DPP-IV inhibitors attenuate the degradation of incretin, thereby increasing the half-lives of incretins and enhance the stimulation of pancreatic insulin secretion, β cell growth, and so on (Ahren, 2007).

1.8.2. Limitations of conventional anti-diabetic therapy

A variety of adverse effects are associated with the use of most conventional anti-diabetic drugs that tend to limit their use, or complicate the morbidity status of diabetic patients. In some cases, the adverse effects of anti-diabetic drugs have led to mortality in early or long-term stages of their use.

a. Adverse effects of Insulin therapy

- Hypoglycemia due to prolonged effect, after normal glycemic levels have been achieved

(Bott *et al.*, 1997; Allen *et al.*, 2001).

- Weight gain, especially at the start of insulin treatment. The mechanisms associated with weight gain are complex, but is thought to be related to a reversal of the catabolic state induced by insulin deficiency, and also, in part, due to a decrease in glucosuria (Lebovitz, 2001).

- Lipoatrophy (loss of adipose tissue) or lipohypertrophy (increased accumulation of fat deposits) around the sites of injection resulting from the need to frequently administer insulin in diabetic patients (Bhatia and Aggarwal, 2007).

b. Adverse effects of oral hypoglycemic drugs

- Metformin has been associated with increases in lactate production in the splanchnic bed and portal venous system due to a reduction in the activity of the pyruvate dehydrogenase enzyme, thereby causing lactic acidosis (Bosenberg and van Zyl, 2008). Metformin administration has also been associated with hepatic impairment, renal and/or heart failure. Metformin may also produce abdominal discomfort and diarrhoea.

- Reported side effects of sulfonylureas include hypoglycemia, weight gain, skin reactions, acute porphyria and hyponatraemia (DeFronzo, 2000; Garratt *et al*., 1999).

- Acarbose produces side effects including flatulence and diarrhoea, likely due to excessive bacterial fermentation of undigested carbohydrates in the colon (Sonmez et al., 2005).

- Thiazolidinediones have been associated with the pathophysiology of fluid-retention and weight gain. Fluid retention may be due to mechanisms including increased vascular

permeability, decreased urinary sodium excretion, increased sympathetic tone and altered interstitial ion transport. The overall effects of fluid retention include edema and complications of existing cardiac failure, liver toxicity and anemia, most likely due to hemodilution (Semple and Krishna, 2006).

1.8.3. Metabolism of Anti-diabetic drugs

The Cytochrome P450 superfamily of drug metabolizing enzymes is critical to the bio-transformation of a large number of drugs, including anti-diabetic drugs. The cytochrome P450 (CYP 450) superfamily comprises a group of heme-containing mono-oxygenases that exhibit an absorption maximum at wavelength 450 nm in the presence of carbon monoxide. The members of this superfamily are sub-divided based on amino acid identity, phylogenetic criteria and gene organization. The primary site of bio-transformation and activity of these enzymes is the liver, although CYPs are also significantly expressed in extra-hepatic tissues, including the small intestinal walls, which plays a major role in pre-systemic metabolism and the elimination of many drugs (Fasinu *et al.*, 2014).

A detailed knowledge of drug metabolism (metabolism of anti-diabetic drugs) has become very important in recent years due to the need for combination therapy, rather than monotherapy in the management of diabetes. Combination therapy, however, has the potential for harmful drug-drug interactions, mediated by the effects of these drugs and their metabolites on the CYP enzymes. Drugs that inhibit CYP enzymes can greatly raise the plasma concentrations of certain other drugs metabolized by these enzymes. In the process, they may enhance the latter's pharmacological and/ or toxicological effects. On the other hand, the induction of CYP enzymes can lower the plasma concentration and effects of some substrates of CYP enzymes. For example, macrolide antibiotics form inactive metabolic intermediates with certain CYP enzymes and thereby inhibit their catalytic activities (Lindstrom *et al.*, 1993). Also,

the antibiotic, Rifampicin, induces several CYP enzymes by binding and activating the pregnane X receptor (PXR), leading to increased CYP 3A4 and p-glycoprotein synthesis (Geick *et al.*, 2001).

CYP 3A4 is considered the most important drug-metabolizing CYP isoform in man. It is involved in an estimated 50% of all clinically used drugs (Wrighton and Thummel, 2000; Fasinu *et al.*, 2014). CYP 3A4 is the most prominently expressed (almost 30%) isoform in the liver. This is also the dominant CYP enzyme in the small intestinal mucosa, particularly the proximal small intestine (Zhang *et al.*, 1999).

1.9. Alternative approaches to management of diabetes mellitus

The effective management of diabetes mellitus is currently viewed as necessitating the combination of two or more drugs from the different categories, together with diet modification and physical exercise. However, none of the current paharmacological interventions are free of disadvantages as they are all associated with adverse effects. Although regular exercise has been proven to prevent and/or delay the onset of various metabolic diseases, the sustained benefits are often difficult to achieve as day-to-day living often affects our ability to adhere to an exercise regimen. As a result, dietary approaches (modification and supplementation) remain a crucial tool to achieve the goal of cost-effective management with minimal complications and maximum quality of life (Evert *et al.*, 2013).

In recent years, evidence from epidemiological, pre-clinical and clinical trials have indicated that a lower risk of diabetes with improved glucose and lipid metabolism appear to be positively correlated with a lower consumption of carbohydrates, saturated fat, processed food and a higher consumption of fruits, vegetables, legumes, coffee and tea. It is now well known that plants are rich in compounds collectively referred to as phytochemicals, such as flavonoids,

phenolic acids, lignans and stilbenes, which have been shown to improve glucose homeostasis in several organs including the liver, gastrointestinal tract, adipocytes and skeletal muscles (Vinayagam and Xu, 2015).

1.9.1. Plants with Anti-diabetic activity

Both in vivo and in-vitro techniques have been employed to assess a wide variety of plants for their anti-diabetic activities. Excellent reviews on plants identified with potential anti-diabetic activity from different parts of the world abound in literature (Marles and Farnworth, 1995; Bnouham *et al.,* 2006; Chauhan *et al.,* 2010; Mamun-or-Rashid *et al.,* 2014).

In South Africa, ethno-botanical surveys have revealed many plants traditionally used to treat diabetes mellitus (Erasto *et al.,* 2005; Oyedemi *et al.,* 2009; Deutschlander *et al.,* 2009; Afolayan and Sunmonu, 2010; Semenya *et al.,* 2012). A few South African plants have well-characterized anti-diabetic effects and are available as herbal supplements, possessing the NAPPI code, a unique coding identifier for medicines, surgical products and medical procedures. For example, Probetix (Nappi code: 711050-001) is a herbal supplement developed from the leaf extract of the indigenous shrub *Sutherlandia frutescens* (major active ingredient: pinitol), shown to cause reversal of insulin resistance as well as reducing intestinal glucose uptake (Chadwick *et al.,* 2007). Manna DFM43 (Nappi code705846-001) developed from the pods of *Prosopis glandulosa* (major active ingredient: galactomannan) is known to delay absorption of glucose and also reduces the glycemic index of foods (Huisamen *et al.,* 2013)

On the basis of reports existing in previous ethnobotanical surveys and local reports from traditional healers in the Eastern Cape, we selected the two plants: *Dianthus thunbergii* and *Hypoxis argentea* for the present studies in an attempt to provide scientific justification (if any) for their use as traditional anti-diabetic remedies.

1.10. *Dianthus thunbergii* (Caryphyllaceae)

Dianthus thunbergii S.S. Hooper (Hooper, 1961) (also called 'wild pink', from the color of its flowers) belongs to the genus *Dianthus*, family *Caryophyllaceae*. The Caryophyllaceae is a large family of flowering plants containing about 88 genera with about 2,000 species. The genus *Dianthus* is made up of about 300 species. *Dianthus thunbergii* is included in the group of plants described as Carnations, a family of clove plants. *Dianthus thunbergii* is included in a published list of Xhosa plants from the Eastern Cape, South Africa (Russell et al., 1987; Dold and Cocks, 1999). Its local names in South Africa include Inkomoyentaba, Ungcana, Indlela-zimhlope and unkomentaba (Dold and Cocks, 2002; Oyedemi *et al.*, 2009).

1.10.1. Identification and Distribution

The name 'Dianthus' was believed to be coined by the Greek botanist Theophrastus, and is derived from the Greek words for divine (dios) and flower (anthos). The term 'carnation' was originally applied to one of the most popular species called *Dianthus caryophyllus*. The name comes from coronation or corone (flower garlands), as it was one of the flowers used in Greek ceremonial crowns. Other *Dianthus* species are also now referred to by the name, carnation.

Most *Dianthus* species are perennial herbs, although some are annuals. The leaves are slender, almost always opposed and whole, with or without stipules. The flowers are usually bisexual and have five petals and five sepals, but sometimes, only four petals. The flowers of Caryophyllaceae are usually pink, reddish pink, red or white. The mature *D. thunbergii* is about 30 cm tall. The flowers are pale pink with bracts about 4 cm long with thin, blue grey leaves at the base (Fig. 1.6).

The over 300 *Dianthus* species are distributed throughout Asia, Europe, Africa and North America. Most of the species are based in the temperate regions with the largest number in the

Mediterranean. About 16 species, including *D. thunbergii* are concentrated in South Africa and occur mostly in the Eastern part of the country (Pooley, 1998).

Fig. 1.6: Whole plant and separated roots of *Dianthus thunbergii*

1.10.2. Medicinal and other traditional uses

Plants of the *Dianthus* species are used by African tribes for traditionally magical and medicinal purposes in the form of infusions for the treatment of chest complaints, unclear vision, severe colic and soothing of wound stitches. The root of *D. thunbergii* is the part most commonly used for medicinal purposes. The infusion from the roots of *Dianthus thunbergii* are used by traditionalists as a ritual wash against evil spirits or bad luck and is taken orally to facilitate communication with ancestors through dreams (Dold and Cocks, 2012). *Dianthus* species are popular in Chinese herbal medicine with a wide range of indications including treatment of difficulty in urination, urolithiasis, amenorrhea, swollen eyes, boils and carbuncles, eczema and itching; the recommended dosages usually being 3–10 grams in decoction. Extracts from the fresh crushed roots are reportedly used to treat diabetes and other conditions and is taken as 2 teaspoonfuls orally three times daily after meals (Oyedemi *et al.*, 2009).

1.10.3. Phytochemistry and Bioactivity

As yet, no reference has been made to the phytochemical composition and bioactivity of *D. thunbergii* in scientific literature. Other species in the genus, e.g., *D. caryophyllus* and *D. superbus* (Shin *et al.,* 2012*;* Chandra *et al.,* 2016*),* have been relatively well characterized and they offer some insight into the bioactive components and pharmacological activities of plants from the genus. The compounds Dianthalexin and Dianthramine found in *D. caryophyllus* have been reported to possess antifungal activity (Harbone *et al.*, 1999). Anticancer, antiviral, antibacterial and insecticidal properties have also been reported (Chandra *et al.,* 2016). The fragrance of *D. caryophyllus* is mainly due to the presence of eugenol, β-caryophyllene and benzoic acid derivatives (Zuker *et al*., 2002), while similar compounds are also present in scent of other *Dianthus* spp. flowers (Jurgens *et al*., 2003). Some flavonoid glycosides with antifungal activity have also been isolated from *D. caryophyllus* (Galleoti *et al.*, 2008).

1.11. *Hypoxis argentea* (Hypoxidaceae)

Hypoxis argentea Harv ex Baker (Watt and Breyer-Brandwijk, 1962) is one of several species of the genus *Hypoxis*, the largest genus of the family Hypoxidaceae. The word *Hypoxis* comes from the Greek words ‘hypo’ meaning below and ‘oxy’ which means sharp, referring to the pointed base of the rootstock. In South Africa, the *Hypoxis* species are commonly referred to as African potato or locally as Ilabatheka, Inongwe and inkomfe.

1.11.1. Identification and Distribution

Hypoxis species are recognized by their bright yellow, often star-shaped flowers, the number of flowers varying from 2–12 per inflorescence. The leaves arise directly from the rootstock, sometimes enclosed in a sheath, in what appears to be a false stem. The leaves are usually hairy and this distinguishes the *Hypoxis* from other closely related species. All species of the *Hypoxis* are perennial herbs, possessing an underground adventitious rootstock (corms) of

varying sizes from species to species, which enable them to survive in unfavorable conditions, such as drought (Nair *et al.*, 2006; Singh *et al.*, 2007). The corms are dark brown in color externally and range from white, yellow to yellowish green internally (white for *H. argentea*). When sliced, the corms exude a resinous juice which also varies in color from species to species (greyish in the case of *H. argentea*) (Fig. 1.7).

There are about 90 species of *Hypoxis*, with an almost a world-wide distribution, occurring in the warm temperate and tropical regions of the world. Many of these species are indigenous to South Africa. They are mostly found in grassland regions, preferring sunlight, although a few species are able to tolerate the shaded conditions of forest regions.

Fig. 1.7: Whole plant and corms of *Hypoxis argentea*

1.11.2. Medicinal uses

Traditionally, the corm of the *Hypoxis* species, including *H. argentea*, is the part of the plant that is sought after, as it is believed to contain medicinal compounds. For the treatment of diabetes and other ailments, the corms of *H. argentea* are reportedly stamped, boiled in water and administered orally till the patient is healed. The plant is also used in traditional veterinary practice to treat cracked teats of cows and wounds on horses (Bizimana, 1994).

Since the elucidation of two major compounds from the *Hypoxis* species, namely hypoxoside (a phytosterol diglucoside) and rooperol (a hydrolysed aglycone), the public interest in the use of

this plant species has grown considerably for their possible wide range of medicinal uses in curing various ailments. The South African public has enthused *Hypoxis* preparations as the 'miracle muthi', referring to a herbal remedy capable of curing a number of ailments. *Hypoxis hemerocallidea* is the prototype species of the *Hypoxis*, a medicinal plant that is used to treat almost all kinds of human and veterinary diseases in South Africa (Ojewole, 2006) and one of the most researched medicinal plants in Africa. The wide range of medicinal uses of the *Hypoxis* species include the treatment of intestinal parasites, urinary infections, infertility, vomiting, nausea, cough, palpitations, weakness, impotency, anxiety, insanity, lice, common-cold, wounds, arthritis, cancers, conditions related to HIV/AIDS, hypertension, diabetes, cancer, psoriasis, gastric and duodenal ulcers, tuberculosis, asthma, epilepsy, childhood convulsions, depression, laxative, burns, prostatitis, benign prostatic hyperplasia and prostate adenoma (Ncube *et al.*, 2013).

1.11.3. Phytochemistry and Bioactivity

Hypoxis argentea remains one of the least investigated species among the *Hypoxis*. Literature reveals very little with regards to its phytochemistry and pharmacological activities. Much can be learnt, however, from the properties of other species in the group. Different reports have identified a nor-lignan glycoside called hypoxoside [(E)-1, 5-bis (4′-β-D-glucopyranosyloxy- 3′-hydroxyphenyl) pent-4-en-1-yne as the most important and common phytochemical constituent in the *Hypoxis* species (Albrecht *et al.*, 1995; Nair and Kanfer, 2007; Drewes *et al.*, 2008). Hypoxoside, when hydrolysed by the enzyme β-glucosidase readily converts to its aglycone, rooperol, a biologically active compound that has therapeutic values. Other glycosides discovered in *Hypoxis* species include nyasol, nyaoside, nyaside, mono-nyasines, obtusides and interjectin (Ncube *et al.*, 2013). Several sterols including β-sitosterol,

stigmasterol and their glycosides and stanols such as sitostanol have also been discovered in *Hypoxis* species.

The different compounds isolated from *Hypoxis* species have been reported to have anti-inflammatory activity (Lim *et al.*, 2009), anti-diabetic activity (Ojewole, 2006) antioxidant and anti-atherogenic activities (Song *et al.*, 2007), as well as anticancer activity (Steenkamp and Gouws, 2006).

1.12. Problem statement

Diabetes mellitus continues to constitute a global public health challenge as a significant chronic non-communicable disease. It is especially becoming an increasing health concern in developing countries where the people have adopted more modernized, sedentary lifestyles with diets that are "diabetes-risks". Anti-diabetic therapy with conventional drugs is often not a single- dose program as most drugs require frequent injections, sometimes for the entire life of the diabetic patient. This creates a huge financial burden and a major handicap to proper anti- diabetic care, especially in developing sub-Saharan Africa. In addition, many of these drugs have inadequate efficacy and serious adverse effects. These limitations have largely prompted the exploration of management strategies involving the use of herbs and other traditional remedies.

Available information from ethno-medicinal plant use has shown promise for the development of cheaper, cost-effective anti-diabetic agents with fewer side effects that will prove more affordable for patients. However, despite the great reputation of many plants used in traditional medicine to treat diabetes mellitus, their efficacy and safety remains to be scientifically proven. *Dianthus thunbergii* and *Hypoxis argentea* are among such plants used

traditionally to treat diabetes in the Eastern Cape Province of South Africa. Their ethno-botanical uses against diabetes are yet to be validated in any known study.

1.13 Hypothesis

Null hypothesis: Neither the roots of *Dianthus thunbergii* nor the corms of *Hypoxis argentea* possess anti-diabetic properties.

Alternative hypothesis: The roots of *Dianthus thunbergii* and/or corms of *Hypoxis argentea* used traditionally to manage diabetes mellitus in the Eastern Cape possess anti-diabetic properties.

1.14 Aims and Objectives

Overall aim

To investigate the anti-diabetic and toxicological properties of the root and corm extracts of *Dianthus thunbergii* and *Hypoxis argentea* and determine possible mechanisms of anti-diabetic action.

Specific objectives

- To determine the phytochemical contents and antioxidant activities of the aqueous and ethanol extracts of *D. thunbergii* and *H. argentea.*
- To perform metabolite profiling using Gas chromatography-mass spectrometry (GC-MS) and Liquid chromatography-mass spectrometry (LC-MS) techniques, in order to identify bioactive compounds present in the essential oils and extracts of *D. thunbergii* and *H. argentea.*
- To evaluate the potential of *D. thunbergii* and *H. argentea* extracts for cytotoxicity in different cell lines.

- To evaluate the potential of extracts of *D. thunbergii* and *H. argentea* for herb-drug interactions by the measurement of effects on drug metabolizing enzymes.
- To determine the in vitro anti-diabetic activities and possible mechanism(s) of action of extracts of *D. thunbergii* and *H. argentea,* using enzymatic and cell-based assays.
- To evaluate the potential of extracts of *D. thunbergii* and *H. argentea* for the induction of cellular proliferation and/or apoptosis.

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CHAPTER 2

Plant selection, Extraction, Phytochemical analysis and Antioxidant activities of Dianthus thunbergii and Hypoxis argentea extracts

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Chapter 2

PLANT SELECTION, EXTRACTION, PHYTOCHEMICAL ANALYSIS AND ANTIOXIDANT ACTIVITIES OF *DIANTHUS THUNBERGII* AND *HYPOXIS ARGENTEA* EXTRACTS

2.1. Background

The pathogenesis of most chronic diseases, including diabetes mellitus, usually involves oxidative and inflammatory mechanisms, arising from the production of excessive amounts of reactive oxygen species (ROS) (Sylvie *et al.,* 2014). ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$), as well as non-radicals such as hydrogen peroxide (H_2O_2) and sinlget oxygen ($^1O_2$). These non-radicals have the potential to participate in reactions that eventually give rise to free radicals. Although the human and animal body is equipped with antioxidant defense mechanisms (enzymatic and non-enzymatic) that neutralize the oxidative and tissue-damaging effects of ROS, these defenses are insufficient to counteract the severity of the oxidative challenge that characterize many disease states (Wang *et al*., 2013).

Dietary supplementation with herbal preparations which have anti-oxidative and anti-inflammatory properties is viewed, in recent times, to represent an effective protective strategy to complement the activities of intrinsic antioxidant defense systems (Shadidi and Naczk, 2003). Consumption of fruits and vegetables rich in phenolics, flavonoids, tannins and alkaloids has been linked to a decreased risk of chronic diseases via a reduction of oxidative stress and inhibition of oxidation in macromolecules such as proteins, lipids and DNA (Wang *et al.,* 2013). Plant-derived antioxidants, including flavonoids, act by different mechanisms including direct scavenging of free radicals before they can attack cellular components as well as the chelation of metal ions that are capable of catalyzing free radical reactions and the donation of hydrogen

atoms to radicals, thereby interfering with the oxidation of lipids and other macromolecules. There has been considerable interest in exploring the antioxidant properties of various herbal extracts, as several synthetic antioxidant agents such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) have become associated with toxicity to animals and humans (Bursal and Gulcin, 2011).

To date, no studies have investigated the free radical scavenging or antioxidant activities of *Dianthus thunbergii* and *Hypoxis argentea.* It was therefore necessary to undertake a preliminary screening of extracts of the plants for their phytochemical composition and antioxidant activities in order to understand their potential for therapeutic use over established standard antioxidant compounds.

2.2. Materials and methods

2.2.1 Plant collection, identification and extraction

Whole plant samples of *Dianthus thunbergii* and corms of *Hypoxis argentea* were collected in May, 2015 from Alice, Eastern Cape, South Africa. They were identified and authenticated at the Giffen Herbarium, University of Fort Hare, South Africa, where voucher specimens CRY-2502 for *Dianthus thunbergii* and HYP-1230 for *Hypoxis argentea* were kept.

The roots of *D. thunbergii* and corms of *H. argentea* were separated from the rest of the plant, washed with clean tap water to remove soil residues and were then oven-dried to a constant weight at 30°C. The dried plant materials were milled into fine powder using an electric blender (Commercial Blender type GB27, Hamilton Beach Brands, Inc. China).

This study utilized food-grade solvents (water and ethanol) for extraction because

1. Water is the most commonly used solvent for traditional medicinal use of these plants,

2. Ethanol is very similar to water in terms of polarity, when considering other commonly used solvents.
3. Both solvents are considered safe for human consumption and can be removed completely from the extracts.

200 g of each powdered plant material was extracted separately in distilled water and 99.99% ethanol maintained on an orbital shaker (Labcon laboratory service (Pty), South Africa) for 24 hours. The ethanol extract was thereafter filtered using Whatmann No. 1 filter papers in a Buchner funnel and the filtrate was concentrated using a rotary evaporator (Heidolph Laborata 4000, Heidolph instruments, GmbH & Co, Germany). The aqueous extracts were initially filtered through a thick layer of sterile cotton wool before their re-filtration with filter papers. The filtrate in this case was immediately frozen at -40°C in a chilling machine filled with acetone and then desiccated for about 48 hours using a freeze dryer. The different extracts were reconstituted in their respective solvents to give the required concentrations used for the assays in this study. The yield of the extracts was calculated using the formula:

$$\text{Yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of plant material}} \times 100$$

2.2.2. Phytochemical analyses of extracts

2.2.2.1. Total Phenolic content

The content of phenols in the different extracts was determined spectrophotometrically by the Folin Ciocalteu reagent according to the method of Ozkok *et al.,* (2010). A calibration curve **(see Appendix I)** was prepared with Gallic acid as standard (0.025–0.125 mg/ml in 70% methanol v/v). To 0.5 ml of each of Gallic acid concentrations or extracts (1 mg/ml), 2.5 ml Folin Ciocalteu reagent (previously prepared as 10% v/v dilution in distilled water) was added. Thereafter, 2 ml anhydrous sodium carbonate (7.5%) was added, producing a blue-colored solution. The mixtures were vortexed thoroughly and placed in a water bath for 30 minutes at 45˚C. The absorbance was then read at 765 nm. The equation of the calibration curve obtained ($Y = 14.885x$; $R^2 = 0.9961$) was used to establish the Gallic acid equivalence (mg/ml). The total phenolic content was calculated using the formula: $T = C \times V/m$, where T is the total phenolic content; V is the volume of the extract (ml) used in the assay, C is the Gallic acid equivalent (mg/ml) and m is the weight of the pure plant extract used in the assay. Values were expressed as Gallic acid equivalent per gram of dry plant extract (mg GAE/g). All assays were performed in triplicate.

2.2.2.2. Total flavonoid content

Flavonoid contents in the extracts were determined using the Aluminium chloride method as described by Ozkok *et al.,* (2010). A calibration curve **(see Appendix I)** was prepared with quercetin (0.025-0.125 mg/ml in 80% methanol v/v). Briefly, 0.5 ml of the extract (prepared at a concentration of 1 mg/ml) or the standard at the different concentrations was mixed with 3 ml of 95% ethanol, 0.2 ml aluminium chloride (prepared as a 10% aqueous dilution), 0.2 ml of 1M potassium acetate and the whole mixture was made up to 10 ml with distilled water. The resulting solutions, prepared in triplicate, were yellow in color and were thoroughly vortexed and

allowed to stand for 30 minutes at room temperature, after which the absorbance was read at 420 nm. The equation of the calibration curve obtained (Y = 11.922x; R^2 = 0.9955) was used to establish quercetin equivalence (mg/ml) and the total flavonoid content was calculated using the formula: T = C x V/m, where T is the total flavonoid content; V is the volume of the extract (ml) used in the assay, C is the quercetin equivalent (mg/ml) and m is the weight (g) of the pure plant extract used in the assay. Values were expressed as quercetin equivalent per gram of dry plant extract (mg Qe/g).

2.2.2.3. Total flavonol content

Total flavonols were determined using the method of Wintola and Afolayan (2011) with slight modifications. A calibration curve **(see Appendix I)** was prepared with quercetin (0.025-0.125 mg/ml in 80% methanol v/v). Plant extracts were prepared at a final concentration of 1 mg/ml. 2 ml of extract or standards was mixed with 3 ml of 95% ethanol, 0.2 ml of aluminium chloride (10% w/v) and 0.2 ml of sodium acetate (50 g/liter). The resulting mixture was made up to 10 ml with distilled water and was vortexed thoroughly. All assays were done in triplicate and were allowed to stand for 2.5 h at room temperature, after which absorbance was read at 440nm. The equation of the calibration curve obtained (Y = 13.128; R^2 = 0.9990, where y is the absorbance and x is the concentration) was used to establish quercetin equivalence (mg/ml). The total flavonol content was calculated using the formula: T = C x V/m, where T is the total flavonol content; V is the volume of the extract (ml) used in the assay, C is the quercetin equivalent (mg/ml) and m is the weight (g) of the pure plant extract used in the assay. Values were expressed as quercetin equivalent per gram of dry plant extract (mg Qe/g).

2.2.2.4. Total tannin content

The method described by Wintola and Afolayan (2011) was used to quantify the tannin content in the plant extracts. A standard curve **(Appendix I)** was prepared using tannic acid (0.002–0.010mg/ml in distilled water) as standard. Briefly, 0.2g of each extract in triplicate was dissolved in 20 ml of 50% methanol. This was placed in a water bath at 80˚C for 1 h, after which the mixtures were filtered into 100 ml volumetric flasks. To the standard concentrations of tannic acid as well as the filtrates from the extracts, 20ml distilled water, 2.5 ml of Folin Ciocalteu reagent and 10 ml of 17% sodium carbonate were added in that order. All mixtures in triplicate were then made up to 100 ml with distilled water and were allowed to stand for 20 minutes. A bluish green color was developed at the end of the reaction and the absorbance of the mixtures was read at 760 nm. Tannic acid equivalence (mg/ml) was established from the equation of the standard curve ($Y = 154.45x$; $R^2 = 0.9585$), where y is the absorbance and x is the tannic acid equivalent in mg/ml. Total tannin content (T) in milligram tannic acid equivalent per gram of dry extract was calculated using the formula: $T = C \times V/m$, where V is the volume of the extract (ml) used in the assay, C is the tannic acid equivalent (mg/ml) and m is the weight (g) of the pure plant extract used in the assay.

2.2.2.5. Total proanthocyanidins

Total proanthocyanidins were measured using the method described by Oyedemi *et al.* (2010). The extracts were prepared at a final concentration of 1 mg/ml. To 0.5 ml of the extracts, in triplicate, 3 ml of 4% vanillin-methanol solution and 1.5 ml of hydrochloric acid were added. The mixtures were thoroughly mixed and allowed to stand for 15 minutes at room temperature. The absorbance was read at 500 nm. Total proanthocyanidin content as catechin equivalent was evaluated from the equation $Y = 0.5825x$; $R^2 = 0.9277$, where y is the absorbance and x is the catechin equivalent (mg/ml). The amount of proanthocyanidin in the extracts in mg/g was

calculated with the formula: T = C x V/m, where V is the volume of the extract (ml) used in the assay, C is the catechin equivalent (mg/ml) and m is the weight (g) of the pure plant extract used in the assay.

2.2.2.6. Alkaloids

Alkaloid content was determined using the method of Harbone (2005) with slight modifications. Briefly, 0.5 g of the dried extract was dissolved in 20 ml of 20% acetic acid in ethanol v/v. The mixture was allowed to stand for 4 h, after which it was filtered. The filtrate was then placed in a water bath for about 30 minutes at boiling temperature. Thereafter, concentrated ammonium hydroxide was added drop-wise which produced some effervescence and precipitation. The collected precipitate was washed with dilute ammonium hydroxide and then filtered with already weighed filter papers. The residues left in the filter papers were then dried in an oven and the resulting dried papers with residue were also weighed. The alkaloid content was determined using the formula: Alkaloid (%) = final weight of the residue / initial weight of the extract used x 100. All the assays were done in triplicate.

2.2.3. Assay of antioxidant activities

2.2.3.1. Ferric Reducing Antioxidant Power

The method described by Aiyegoro and Okoh (2010) was used for the determination of the ferric reducing activities of the plant extracts. The assay is based on the reduction of ferric tripyridyl triazine (Fe^{3+} - TPTZ) complex by the action of electron-donating antioxidants at low pH to the ferrous form. Each extract or standard was initially prepared in distilled water in increasing concentrations from 0.025–0.5 mg/ml. 1 ml of each extract or the standard at the different concentrations were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide. The mixture was incubated for 20 minutes at 50˚C. This was followed by the addition of 2.5 ml of TCA (10% w/v) and centrifugation at 3000 rpm for 10 min.

Thereafter 2.5 ml of the supernatant was withdrawn and mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1% w/v). The absorbance was read at 700 nm with distilled water as blank. An increase in absorbance with increasing concentration of extract or standard corresponds to the formation of the bluish-green color of the reduced form of TPTZ. The average absorbance of the reactions performed in triplicate was obtained and was plotted against the different concentrations of each extract and standard.

2.2.3.2. DPPH (1, 1 diphenyl-2- picryl hydrazyl) radical scavenging activity

The method described by Liyana-Pathiana and Shadidi (2005) was used in this assay where 1 ml of the extracts or the standard at different concentrations (0.025–0.50 mg/ml), prepared in triplicate was mixed with 1 ml of DPPH (0.135 mM) prepared in methanol. The mixtures were vortexed thoroughly and left in the dark for 30 minutes at room temperature. The absorbance was then measured spectrophotometrically at 517 nm. The percentage DPPH scavenging activity of the extract or standard was calculated with the formula: % DPPH radical scavenging activity = $[(A_C - A_S)/A_C] \times 100$; where A_C is the absorbance of the control and A_S is the absorbance of the test samples (extract or standard).

2.2.3.3. ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity

This assay was performed according to the method of Adedapo *et al.,* (2008). It is based on the reaction between ABTS and potassium persulfate, to produce the ABTS radical cation ($ABTS^+$), a bluish-green chromogen, which is converted to a colorless solution in the presence of an antioxidant reductant (Sahoo *et al.*, 2013). A working solution was prepared by mixing equal amounts of 7 mM ABTS and 2.4 mM potassium persulphate. These were allowed to react for about 12 h in the dark at room temperature and 1 ml of the resulting solution was mixed with 60 ml methanol and the absorbance was adjusted to 0.706 ± 0.001 units at 734 nm by addition of drops

of the original ABTS/potassium persulphate solution. Thereafter, 1 ml of each extract and the standard prepared at different concentrations (0.025–0.50 mg/ml) in methanol was mixed with 1 ml of the ABTS/methanol solution. The absorbance of the resulting solutions was read at 734 nm after about 7 minutes. ABTS radical scavenging activity was indicated by different degrees of decolorization of the dark-green color of the ABTS solution. All assays were done in triplicate. The percentage of ABTS radical scavenging activity was calculated from the following equation:

% ABTS scavenging activity = $[(A_C - A_S)/A_C] \times 100$; where A_C is the absorbance of the control (ABTS + methanol) and A_S is the absorbance of the test samples (extract or standard).

2.2.3.4. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method described by Wintola and Afolayan (2011). Hydrogen peroxide in the presence of an antioxidant is converted to water and oxygen, decreasing the concentration of hydrogen peroxide as the concentration of antioxidant increases. 1 ml of extract or the standard prepared at different concentrations (0.025–0.5 mg/ml) in distilled water was mixed with 0.6 ml of 40 mM H_2O_2 (prepared in 0.1 mM phosphate buffer; pH 7.4). This was left to react for 10 minutes after which the absorbance was read at 230 nm using a UV–VIS 3000 PC spectrophotometer, against a blank containing phosphate buffer without H_2O_2. The inhibition of hydrogen peroxide was calculated as the percentage of hydrogen peroxide remaining in the solutions (Ruch *et al.*, 1989) using the formula: % hydrogen peroxide remaining = $[(A_C - A_S)/A_C] \times 100$; where A_C is the absorbance of the control (Phosphate buffer + H_2O_2) and A_S is the absorbance of the test samples (extract or standard).

2.2.3.5. Nitric oxide scavenging activity

The method described by Wintola and Afolayan (2011) was used for the assay of the nitric oxide radical scavenging activity of the extracts. The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. The nitrite ions are detected in solution by the Griess reagent which contains sulfanilamide and naphtylethylenediamine dihydrochloride. Compounds that scavenge nitric oxide compete with oxygen, leading to a reduced production of nitrite ions (Ebrahimzadeh *et al.*, 2010). 0.5 ml of the extracts or the standards was mixed with 2 ml of 10 mM sodium nitroprusside (prepared in 0.5 mM phosphate buffered saline; pH 7.4). The mixture was incubated for 2.5 h at 25˚C. 0.5 ml of the mixture was mixed with 0.5 ml of Griess reagent (prepared by mixing 1 ml sulphanilic acid (0.33% in 20% glacial acetic acid) with 1 ml of naphthylenediamine dihydrochloride (0.1% w/v). The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 540 nm. The percentage of nitric oxide scavenging ability of the plant extracts and standard compounds was calculated using the formula: %NO scavenged = $[(A_C - A_S)/A_C] \times 100$; where A_C is the absorbance of the control reaction and A_S is the absorbance of the test samples (extract or standard).

2.2.4. Statistical analysis

All the data obtained were analyzed using Microsoft® Excel Statistical package and were expressed as mean ± standard deviation. IC_{50} values for antioxidant activities were also determined using Microsoft® Excel. Significant differences ($p<0.05$) were determined using Student's t-test to compare the mean values between two sets of data.

2.3. Results and Discussion

2.3.1. Yields of extracts

The yields of the extracts obtained are: *Dianthus thunbergii* aqueous extract (DTA; 16.6%); *Dianthus thunbergii* ethanol extract (DTE; 2.4%); *Hypoxis argentea* aqueous extract (HAA; 3.2%); *Hypoxis argentea* ethanol extract (HAE; 1.8%).

2.3.2. Quantification of Phytochemical constituents

The relative amounts of major phytochemicals in the extracts of *D. thunbergii* and *H. argentea* are presented in Table 2.1. There were significant differences in the yield of the extracts as well as in the content of the different phytochemicals between the two plants and between the extraction solvents used in this study, water and ethanol. It is generally known that the yield of chemical extraction depends on different factors including the type of solvents with regards to varying polarities, extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples (Dai and Mumper, 2010).

Table 2.1: Phytochemical composition of aqueous and ethanol extracts of *Dianthus thunbergii* and *Hypoxis argentea*

Phytochemical constituents	DTA	DTE	HAA	HAE
Total phenols (mg GAE/g)	9.71±0.98^{a,c}	28.01±0.37^{a,d}	18.01±0.44^{b,c}	66.71±2.71^{b,d}
Total flavonoids (mg Qe/g)	16.56±1.31^{a,c}	62.21±1.75^{a,d}	27.70±2.20^{c}	28.39±1.17^{d}
Total flavonols (mg Qe/g)	14.71±1.85^{c}	13.06±0.21^{d}	25.51±1.92^{b,c}	10.09±0.92^{b,d}
Total tannins (mg TAE/g)	0.72±0.04^{a,c}	1.07±0.07^{a}	0.89±0.01^{b,c}	1.18±0.07^{b}
Total proanthocyanidins (mg Ca/g)	14.31±3.57^{a,c}	432.62±2.43^{a,d}	38.63±3.64^{b,c}	311.87±3.57^{b,d}
Alkaloids (%)	14.33±1.74^{a,c}	25.42±2.25^{a}	15.70±4.44^{b,c}	24.15±2.17^{b}

Values are expressed as mean ± standard deviation of three replicates; mg GAE/g = milligram Gallic acid equivalent per gram of extract; mg Qe/g = milligram quercetin equivalent per gram of extract; mg TAE/g = milligram tannic acid equivalent per gram of extract; mg Ca/g = milligram catechin equivalent per gram of extract; DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract. Superscripts represents significant differences between [a] (DTA vs DTE); [b] (HAA vs HAE); [c] (DTA vs HAA) and [d] (DTE vs HAE) at P <0.05.

Generally, traditional herbal extractions from plant parts utilize water as the most common solvent to produce decoctions and infusions. Water extraction usually produces high yields from original plant material and the solvent does not pose any threat of adverse effects for human consumption. However, due to differences in the polarities of polyphenols from polar to non-polar, a wide range of solvents, including ethanol, methanol, acetone, ethyl acetate,

chloroform or their combinations are sometimes also employed for extraction. Ethanol or water-ethanol mixtures are considered to be the most suitable solvent systems for polyphenols due to range of polarities and the acceptability of the two solvents for human consumption (Shi *et al*., 2005; Dent *et al.*, 2013).

For both plants in this study, the ethanol extracts had higher total phenols, flavonoids, tannins, proanthocyanidins and alkaloids, compared to their respective aqueous extracts. Among all the extracts, the ethanol extract of *Dianthus thunbergii* contained the highest amounts of flavonoids (62.21±1.75 mg Qe/g), proanthocyanidins (432.62±2.43 mg Ca/g) and alkaloids (25.42±2.25)%, while the ethanol extract of *Hypoxis argentea* contained the highest amounts of total phenols (66.71±2.71 mg GAE/g) and tannins (1.18±0.07 mg TAE/g). The content of flavonols, on the other hand, was significantly higher ($P < 0.05$) in the aqueous extracts than the ethanol extracts.

Previous studies evaluating the effects of extraction solvents on total phenol and flavonoid contents of plant extracts have also indicated that extraction with 100% ethanol gave highest yields of total phenols and flavonoids, compared to 100% acetone, 100% methanol or water. In fact, it was observed that the total phenol or flavonoid content of extracts decreased with increasing water content in aqueous-acetone, aqueous-methanol and aqueous-ethanol mixtures (Do *et al*., 2014).

2.3.3. Antioxidant activities

2.3.3.1 Ferric reducing antioxidant power of the extracts

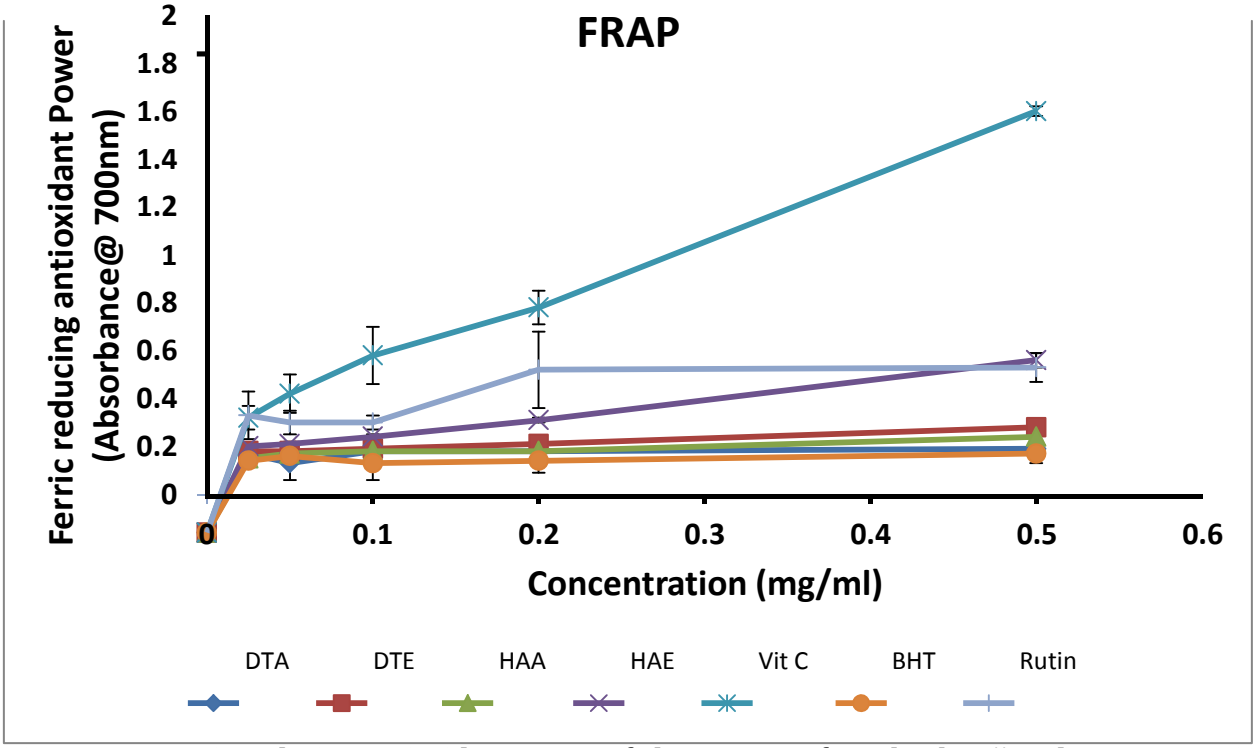

Figure 2.1: Ferric reducing antioxidant power of the extracts of *D. thunbergii* and *H. argentea* in comparison with Vitamin C, BHT and Rutin. Values are presented as mean±standard deviation (n=3). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract

As depicted in Figure 2.1, most of the extracts and standards produced a concentration-dependent response in the ferric reducing abilities. The ferric reducing antioxidant potential of compounds, as measured in this assay, represent the ability of the extracts and/or standards to reduce the ferric tripyridyl triazine (Fe^{3+}-TPTZ) by electron donation, to the ferrous form which has an intense blue color (Wintola and Afolayan, 2011). The ability of compounds to convert the ferric ion to the ferrous form is a good indicator of their antioxidant potential (Rao *et al.*, 2010). In this study, the Vitamin C standard produced a much more significant antioxidant activity

compared to the other compounds. Of those tested, the ethanol extracts of *D. thunbergii* and *H. argentea* possessed better ferric reducing abilities than the respective aqueous extracts. At 0.5 mg/ml, the overall order of decreasing ferric reducing power was Vitamin C > HAE > Rutin > DTE > HAA > DTA > BHT. This trend bears a direct relationship to the content of total phenols and flavonoids in the plant extracts and suggests that these compounds are largely involved in the antioxidant activities promoted by the plant extracts.

2.3.3.2. Scavenging activities of the extracts against ABTS radicals

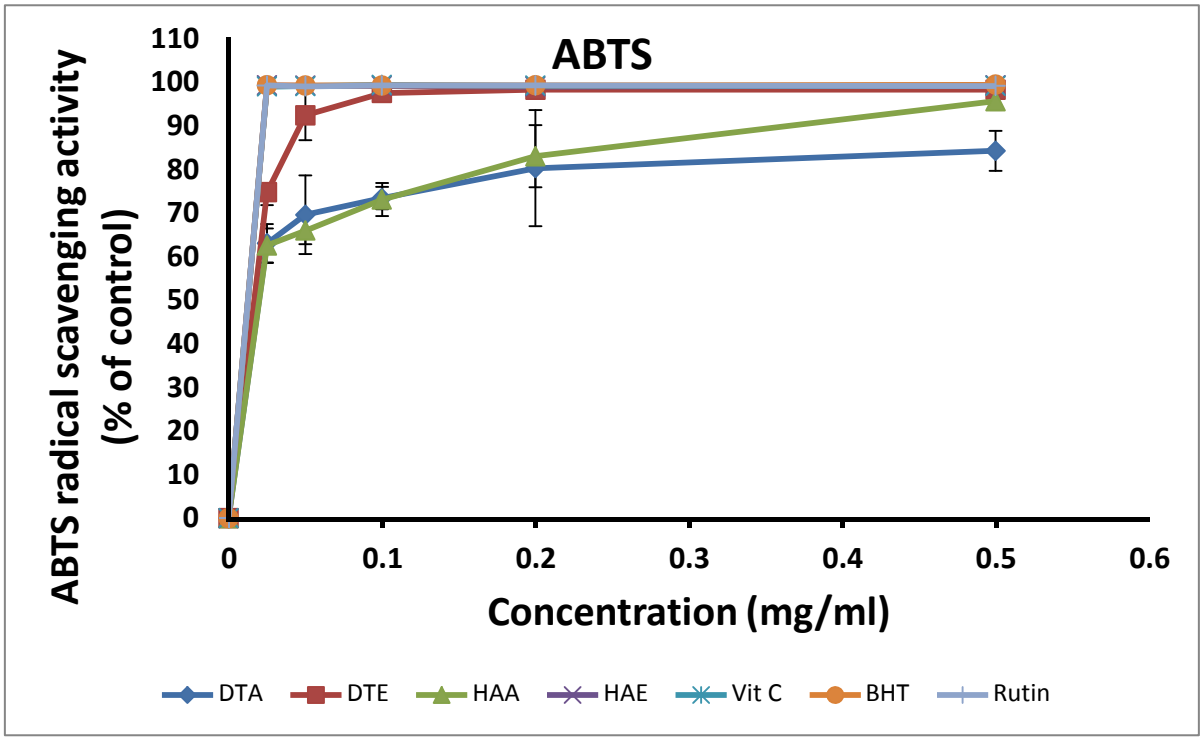

Figure 2.2: Percentage scavenging activities of the extracts of *D. thunbergii* and *H. argentea* against ABTS in comparison with Vitamin C, BHT and Rutin. Values are presented as mean±standard deviation (n=3). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract

The antioxidant activities of the extracts and standards against ABTS radical are shown in Figure 2.2. The data indicate a largely concentration-dependent increase in the antioxidant

activities of all the extracts. The ethanol extracts were more effective as ABTS radical scavengers than the aqueous extracts, having IC_{50} values: 0.0125 and 0.0175 mg/ml for HAE and DTE, respectively. It is noteworthy that both ethanol extracts compared very favorably with the standards at virtually all the concentrations tested. The high percentage of ABTS scavenging activities of the different extracts, even at low concentrations, suggests a high degree of antioxidant potency. The ABTS assay is based on a reaction between ABTS and potassium persulfate to produce the ABTS radical cation ($ABTS^{+}$) which is a bluish-green chromogen. In the presence of an antioxidant, the colored radical is converted back to a colorless ABTS (Sahoo *et al*., 2013). The various degrees of decolorization of the ABTS-potassium persulfate solution directly reflect the antioxidant activity of the test compound.

2.3.3.3. DPPH radical scavenging activities of the extracts

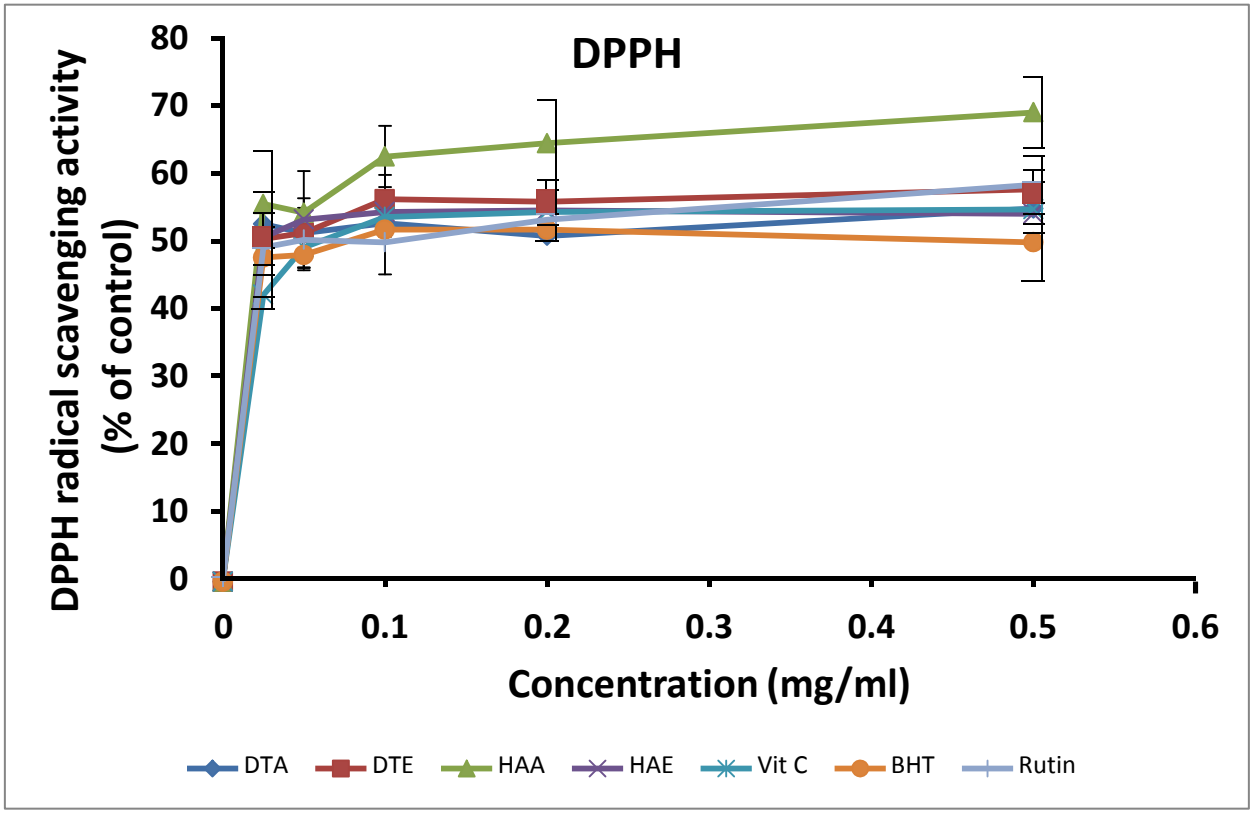

Figure 2.3: Percentage DPPH radical scavenging activities of the extracts of *D. thunbergii* and *H. argentea* in comparison with Vitamin C, BHT and Rutin. Values are presented as mean±standard deviation (n=3). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract

According to Figure 2.3, the DPPH radical scavenging activity of the extracts and standards (at 0.5 mg/ml) was in the order: HAA > Rutin > DTE > DTA > Vitamin C > HAE > BHT. However, DTA and HAE had the most effective antioxidant activity with similar IC_{50} (0.005 mg/ml) (Table 2.2). Hydrogen-donating antioxidants react with the stable free radical 1, 1´ diphenyl-2- picryl hydrazyl radical (deep violet colour), donating a hydrogen atom and converting it to the 1, 1'diphenyl 2-picrylhydrazine (yellowish in colour) (Bhagat *et al.*, 2013) The discolouration indicates the pairing of the lone electron in DPPH and, hence the scavenging

potentials of the sample antioxidants, which occurs to varying degrees depending on their antioxidant capacities. Phenolic compounds, especially flavonoids have the ability to easily donate the hydrogen atoms in their hydroxyl groups (Lugasi *et al.*, 2003) and they might have played a major role in the DPPH radical scavenging activities of the extracts in this study.

2.3.3.4. Hydrogen peroxide scavenging activities of the extracts

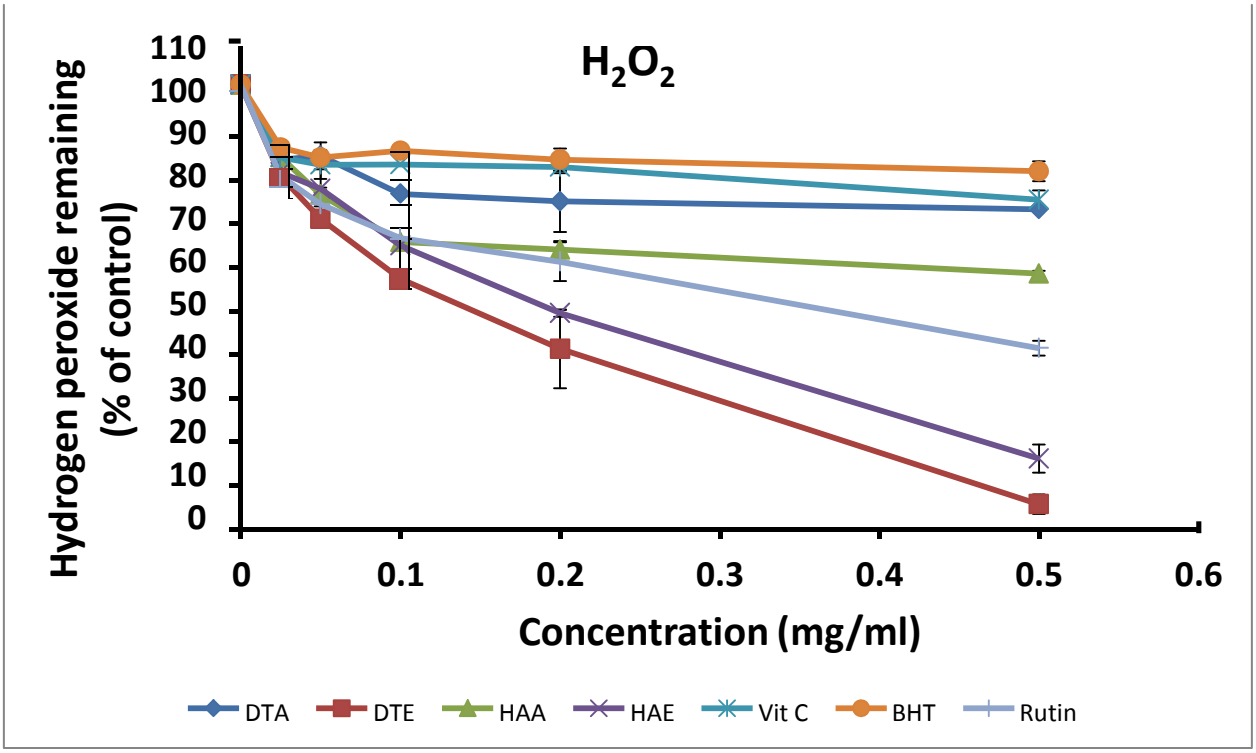

Figure 2.4: Percentage Hydrogen peroxide scavenging activities of the extracts of *D. thunbergii* and *H. argentea* against ABTS in comparison with Vitamin C, BHT and Rutin. Values are presented as mean±standard deviation (n=3). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract.

Figure 2.4 shows the hydrogen peroxide scavenging activities of the extracts in comparison to various standards. The scavenging activities of the different compounds was largely concentration-dependent, with the ethanol extracts producing the most effective antioxidant activity and DTE and HAE having the lowest IC_{50} values of 0.13 and 0.19 mg/ml,

respectively (Table 2.2). Hydrogen peroxide, in the presence of transition metal ions can be converted to the highly reactive hydroxyl radical, which has the ability to cause oxidative degradation of cell membrane lipids to give rise to the occurrence of carcinogenesis, mutagenesis, and cytotoxicity (Cho *et al*., 2011). In the presence of an antioxidant, hydrogen peroxide is converted to water and oxygen (Chang *et al.*, 2002), causing a decrease in hydrogen peroxide concentration in assay mixtures as the concentration of antioxidant increases.

2.3.3.5. Nitric oxide radical scavenging activities of the extracts

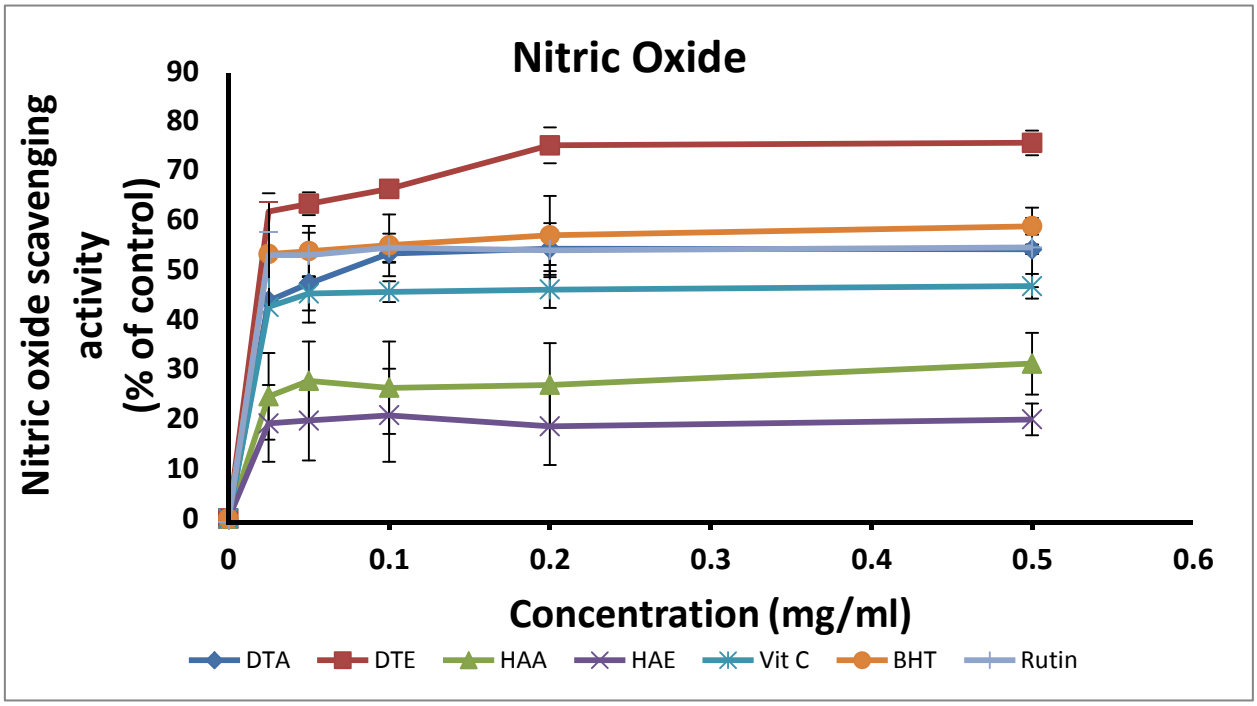

Figure 2.5: Percentage Nitric oxide scavenging activities of the extracts of *D. thunbergii* and *H. argentea* against ABTS in comparison with Vitamin C, BHT and Rutin. Values are presented as mean±standard deviation (n=3). DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract.

In Figure 2.5, the percentage of nitric oxide inhibitory activities of the extracts and standards were in the order: DTE > BHT > DTA > Rutin > Vitamin C > HAA > HAE at the

highest concentration (0.50 mg/ml). The nitric oxide radical is produced in vivo by phagocytes and endothelial cells and is very important in inflammatory processes. However, its overproduction can result in the tissue damage seen in many inflammatory conditions including diabetes and arthritis, etc. (Tayloe *et al.*, 1997). This radical can combine with the superoxide radical to form the highly reactive peroxynitrite anion ($ONOO^-$) (Hiue and Padmaja, 1993). This finding suggests that *Dianthus thunbergii* may be more beneficial than *Hypoxis argentea* in counteracting inflammatory signaling processes involving nitric oxide.

Table 2.2: IC_{50} values for antioxidant activities of the extracts

Extract/standard	**IC_{50} (mg/ml)**			
	DPPH	**H_20_2**	**Nitric oxide**	**ABTS**
DTA	0.0050	0.9400	0.0700	0.0200
DTE	0.0150	0.1300	0.0200	0.0175
HAA	0.0100	0.5500	0.9800	0.0200
HAE	0.0050	0.1900	***	0.0125
Vit. C	0.0600	1.1500	0.4250	0.0125
BHT	0.0750	1.7300	0.0250	0.0125
Rutin	0.0350	0.3400	0.0250	0.0125

IC_{50} = concentration of extract or standard producing 50% antioxidant activity; DTA = *Dianthus thunbergii* aqueous extract; DTE = *Dianthus thunbergii* ethanol extract; HAA = *Hypoxis argentea* aqueous extract; HAE = *Hypoxis argentea* ethanol extract. ***Values too high and not relevant. Note that a low IC_{50} value indicates a high antioxidant activity of a compound.

2.4. Conclusions

Overall, the results of in vitro, non-cellular assays of antioxidant activities in this study indicate that the ethanol extracts of *D. thunbergii* and *H. argentea* demonstrated better antioxidant activities than the aqueous extracts, with respect to ABTS, nitric oxide and H_2O_2 scavenging activities, as well as the ferric reducing power. On the other hand, the aqueous extracts showed better antioxidant activities against DPPH radical. The observed trend of antioxidant activities had a direct correlation to the contents of phytochemicals in the extracts. The higher content of total phenolics, flavonoids, proanthocyanidins, tannins and alkaloids in the ethanol extracts seem to have been responsible for their marked antioxidant activities as assayed through the various in-vitro models. The findings of this study are in accordance with observations from several other studies (Deighton *et al*., 2000; Abdille *et al.*, 2005; Hossain and Shah, 2015).

It is interesting to note that some of the in vitro assays employed (DPPH, nitric oxide and hydrogen peroxide scavenging assays) clearly show that the extracts of either *D. thunbergii* or *H. argentea* demonstrated better antioxidant activities than the established standards included in the assays. This observation identifies the extracts of both plants as promising natural alternatives for the management of conditions involving oxidative or inflammatory mechanisms, while eliminating the adverse effects associated with established synthetic antioxidants. The results of this study represent the first attempt at investigating the phytochemical composition and antioxidant activities of *Dianthus thunbergii* and *Hypoxis argentea.* The identification and/or isolation of particular phenolics in the various extracts will be necessary for further work.

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CHAPTER 3

Evaluation of the potentials of D. thunbergii and H. argentea for cytotoxicity, induction of cell proliferation and herb-drug interactions

Results from this chapter are part of data being submitted for publication in Journal of Ethnopharmacology and Biomed Research International.

Chapter 3

EVALUATION OF THE POTENTIALS OF EXTRACTS OF *D. THUNBERGII* AND *H. ARGENTEA* FOR CYTOTOXICITY, INDUCTION OF CELL PROLIFERATION AND HERB-DRUG INTERACTIONS

3.1 Background

Herbal medicines include both plant-derived materials and preparations intended for therapeutic use against various ailments and those administered with the hope of promoting health in humans (WHO, 2005). Despite the advent of modern medicine, the popularity and use of these herbal products continues to predominate in many populations around the world. In the traditional use, most herbs are ingested raw or as decoctions and infusions (often concentrated preparations), as teas or sometimes as topical applications to the skin in the form of paste or powder. There is often a general assumption that herbal products are non-toxic and safe due to their natural origin and history of long use in traditional medicine (Fennell *et al.*, 2004). However, many studies have revealed that as with orthodox medicines, many medicinal herbs are therapeutic at certain doses and toxic at another.

Despite the considerable growth in research and market demand for herbal medicines, the majority (up to 90%) of herbal products are yet to be truly standardized with respect to their active constituents and very little is known about their toxic constituents (Winston and Maimes, 2007). In many cases, herbal medicines are not subjected to the same regulatory and quality control testing as conventional orthodox drugs in terms of their efficacy and safety. There are, therefore, serious concerns about the safety implications of the use of herbs as medicines in terms of adverse clinical signs observed during short-term applications, and for their potential cytotoxic, genotoxic and carcinogenic effects when used chronically (Ernst, 2004; Rietjens *et al.*, 2005).

The adverse effects of herbal medicines arise due to a variety of factors, including their inherent content of toxic secondary metabolites, contamination of herbs with heavy metals, micro-organisms, pesticides, industrial chemicals or other toxic herbs and surreptitious addition of synthetic drugs and the subsequent toxic effects of potential herb-drug interactions (Kadan *et al.*, 2013). Plants have evolved an array of secondary metabolites described as phytoanticipins and phytoprotectants, which are released in response to specific environmental stimuli such as damage due to herbivores, pathogens or nutrient depletions. Some of these chemicals are harmful for human consumption (Kennedy and Wightman, 2011). A good number of alkaloids produced by plants are known to deter herbivores via agonistic or agonistic activity on neurotransmitter systems (Wink, 2003). Others, such as pyrrolizidine alkaloids, are known to be hepatotoxic. Cardio-active glycosides produced by some plants such as the *Aconita* species are known to produce cardiac effects, while the *Ephedra* species, caffeine, ginseng, and gingko all have stimulant effects. Poisoning with cyanogenic glycosides such as linamarin and loutastralin has also been reported (Ko, 1999; Nelson and Perrone, 2000). A complication of the problem of the inherent toxicity of medicinal herbs arises from the variation in the content of the chemical constituents as a result of differences in the soil type they are grown in, rainfall and sunshine, the season of harvesting, as well as the parts of the plants harvested for use. Large batch-to-batch variations in chemical composition can result in toxicities even in finished products (Ye *et al.*, 2004).

Cytotoxicity evaluation of plant extracts is usually employed as a first phase of anti-diabetic screening to evaluate the suitability of the extracts for *in vitro* cell-based assays. Cytotoxicity assays are also essential in identifying potential artefacts that may compromise the accuracy of true positive results and to prioritize samples for further in vitro testing. The tests for cytotoxicity are usually based on cell density assays that measure the effects of the test samples

on cell proliferation and/or cell death after exposure for specific time periods. In addition, other assays measuring end-points such as cell membrane integrity, oxidative stress, glucose metabolism and cellular morphology are simultaneously included to comprehensively characterize the nature of test plant extracts and increase the capacity of the pre-anti-diabetic cytotoxicity screening.

A major goal of current research in management of both Types 1 and 2 diabetes is the induction of proliferation in human β cells (Berna-Mizrachi *et al*., 2014). Progressive deterioration of β cell function in Type 2 diabetes is usually accompanied by a reduction of β cell mass of up to 60% in some cases (Kjems *et al*., 2001 Butler *et al*., 2003). Restoration or enhancement of β cell function is, therefore potentially, an ideal strategy for regulation of blood glucose concentration in diabetic patients. It is believed that β cells can regenerate through a replication of pre-existing β cells or neogenesis from stem cells inside or outside the islets (Bouwens and Rooman, 2005). Incretin hormones such as GLP-1, apart from producing an enhancement of glucose-dependent insulin secretion from the pancreas, also influence β cell protection by reducing β cell apoptosis and stimulating β cell proliferation and neogenesis. Recently developed anti-diabetic therapies termed GLP-I agonists, for example, lisaglutide, appear to offer promise for durable glycemic control due to their beneficial effects on β cell function (Garber, 2011). GLP-1 agonists can activate cell surface receptors and their cellular signal transduction pathways involved in GLP-1 secretion (Kim and Jang, 2015). Recently, some medicinal plants have also been reported to have the potential to produce regeneration of β cell function via various mechanisms including the direct stimulation of GLP-1-seccreting cells, antioxidant effects and the reduction of β cell-apoptosis (Hosseini *et al*., 2015).

One of the most commonly used techniques for evaluating cell density is the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells (Holst-Hansen and Brünner, 1998). Additional assays include the 2,3bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) assay, lactate dehydrogenase (LDH) leakage assay, crystal violet assay, neutral red and protein (Bradford) assays, many of which are indirect assays of determination of cell viability, although they help to overcome major limitations associated with the MTT assay.

Apart from direct cytotoxicity by plant extracts, the frequent concomitant use of herbal medicines and conventional antidiabetic drugs has been shown to elicit clinical effects. As certain antidiabetic drugs have narrow therapeutic indices, herbal remedies frequently used by antidiabetic patients are of special concern regarding herb–drug interactions (Boullata, 2005, Chavez *et al*., 2006; Izzo and Ernst, 2009). Drug interactions can result in therapeutic failure due to the inhibition or induction of drug metabolism. The most common result of the interactions of herbs with antidiabetic medication is a rise or fall in blood glucose concentrations, thereby disrupting the control of diabetes. The potential for the positive interaction of antidiabetic drugs co-administered with herbs has been reported for CYP 2C9 substrates such as glibenclamide and rosiglitazone, as well as CYP 3A4 substrates such as pioglitazone and repaglanide (Bertram, 2012).

This chapter describes the screening of the extracts of *D. thunbergii* and *H. argentea* for the first time, for their potential to induce cytotoxicity and/or proliferation in the human hepatocellular carcinoma cell line, HepG2 cells and the rat insulinoma cells, INS-1 cells. In addition, the potential effect of the extracts on CYP 3A4 drug metabolism was also evaluated.

3.2. Cytotoxicity of *D. thunbergii* and *H. argentea* extracts in HepG2 cells by MTT assay

3.2.1. Principle

This assay relies on the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by metabolically active cells to insoluble dark blue or purple formazan crystals via the cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. When cells die, they lose the ability to convert MTT to formazan (Riss *et al.*, 2004). Due to its impermeability to cell membranes, the formazan product accumulates in healthy cells and the absorption maximum near 570 nm can then be read spectrophotometrically. The reduction of MTT is believed to be due to its reaction with NADH or similar reducing equivalents (Figure 3.1). As a result, reducing compounds such as reduced glutathione and dithiothreitol can produce a non-enzymatic reduction of MTT, thus interfering with MTT reduction assays by producing a false positive increase in absorbance values.

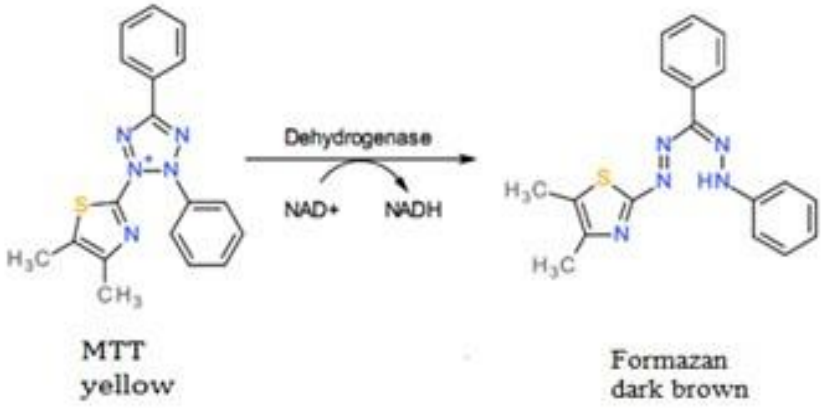

Figure 3.1: Reduction of MTT to formazan by NADH within the mitochondria

3.2.2 Materials and methods

Cell culture conditions: HepG2 cells were maintained routinely in 10 cm culture dishes without antibiotics in a culture medium consisting of RPMI-1640, containing 25 mM HEPES, 2 Mm

glutamine and supplemented with 10% fetal bovine serum. The cells were incubated at 37°C in a humidified 5% CO_2 incubator.

Treatment: HepG2 cells were seeded at a density of 6 000 per well at a volume of 100 µl per well. The cells were treated by adding 100 µl of the aqueous and ethanol extracts (dissolved in distilled water and absolute ethanol, respectively) at double the desired concentrations: 50, 100 and 200 µg/ml for aqueous extracts and 12.5, 50, 100 and 200 µg/ml for ethanol extracts. The initial doubling of the concentrations ensures that the desired concentrations are obtained as the final concentrations in the wells which already contain 100 µl of culture medium. The cells were allowed to attach overnight at 37°C in a humidified 5% CO_2 incubator. On the following day, all spent media were initially removed by aspiration. The medium was replaced with 100 µl EMEM containing 10% fetal calf serum and 0.5 mg/ml MTT (dissolve 25 mg MTT in 50 ml complete culture medium). The plates were gently rocked and then incubated at 37°C for 3 hours. At the end of incubation, the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystals formed in the cells. The absorbance of the wells was read at 540 nm with a multi-plate reader (BioTek® PowerWave XS spectrophotometer). Cytotoxicity of the plant extracts was expressed as percentage of control (medium only), which was taken as zero and IC_{50} values were calculated.

% cell death = 1- (test absorbance/average absorbance of untreated control) X 100

3.2.3. Results and Discussion

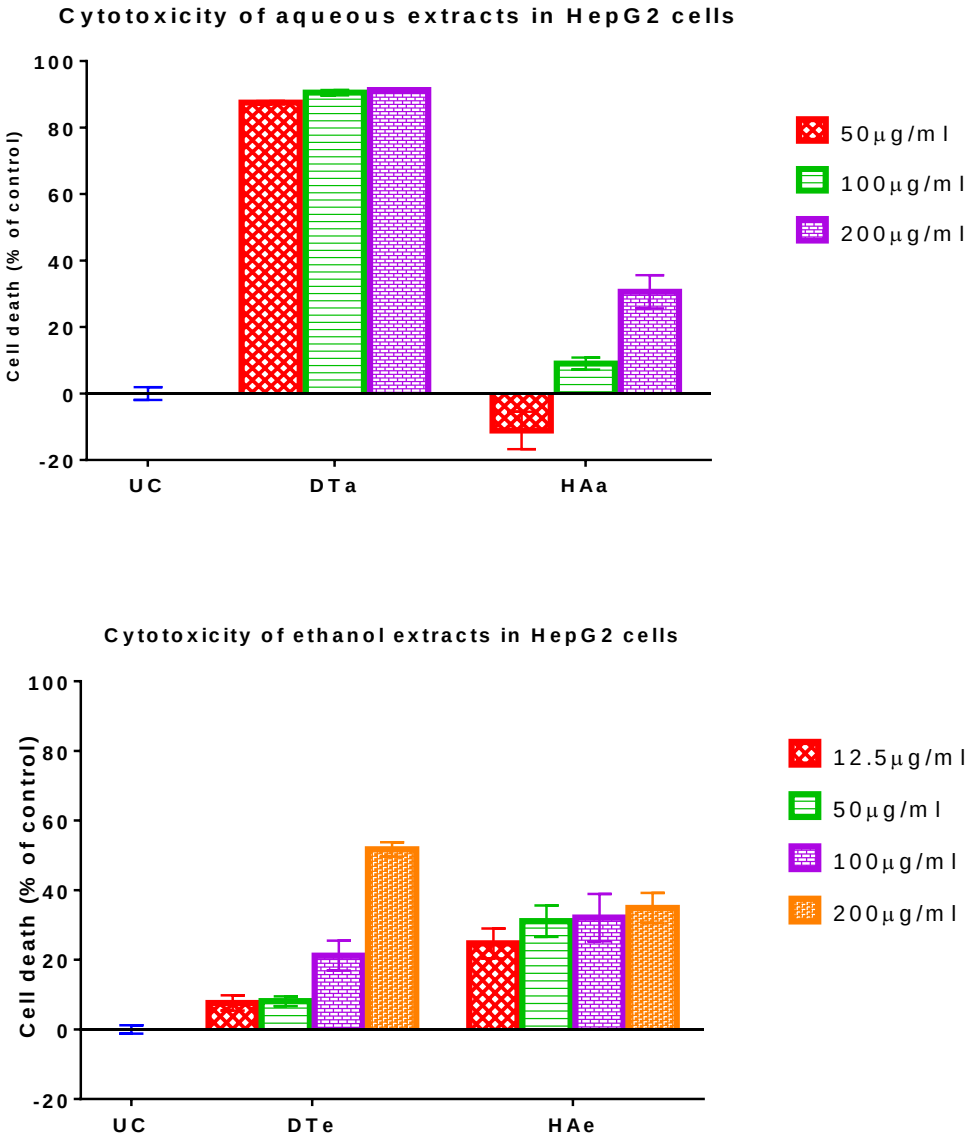

Figure 3.2: MTT cytotoxicity (expressed as % of control ± standard deviation; n = 4) of aqueous and ethanol extracts of *Dianthus thunbergii* roots and *Hypoxis argentea* corms in HepG2 cells. UC = Untreated Control. DTa = *Dianthus thunbergii* aqueous extract; HAa = *Hypoxis argentea* aqueous extract ; DTe = Dianthus thunbergii ethanol extract; HAe = Hypoxis argentea ethanol.

After 24 hours of exposure to the extracts, MTT viability assay in HepG2 cells revealed the pattern of cytotoxicity as shown in Figure 3.2. The IC_{50} values are summarized in Table 3.1. The aqueous extracts of *D. thunbergii* produced considerably high toxicity to the cells at all the

concentrations tested, while the ethanol extract was not significantly toxic. Due to this very high cytotoxic activity against HepG2 cells, IC_{50} could not be determined for the aqueous extract of *D. thunbergii*. According to the American National Cancer Institute, crude plant extracts are considered cytotoxic in an in vitro assay when concentrations 20 µg/ml and below produce 50% inhibition of tumor cells, after an exposure time of 72 hours (Boyd, 1984; Vijayarathna and Sasidharan, 2012). The significant death of cells in the presence of the aqueous extracts of *D. thunbergii* is a concern for its ethno-medicinal use and merits further investigation. Similar to the results obtained in this study, other members of the Caryophyllaceae family have been shown to induce cytotoxicity to different cell lines. The ethanol extracts of *Dianthus chinensis* were found to suppress cell viability and induce apoptosis in HepG2 cells (Nho *et al*., 2012). *Dianthus caryophyllus* contains a flavonol, Kaempferol triglycoside, which has been shown to exhibit inhibitory properties against the human colon cancer cell lines (Martineti *et al.*, 2010).

In contrast, both extracts of *H. argentea* exhibited very low toxicity against HepG2 cells, with the lowest concentration tested (50 µg/ml) stimulating the growth of the cells. Cellular viability and proliferation are important functional characteristics of healthy growing cells, which may be useful in maintaining the integrity of tissues involved in the control of glucose metabolism such as the pancreatic tissue. The relatively low toxicity of the extracts of *H. argentea* suggests that these extracts may pose low risk in their use for anti-diabetic treatment. The results of this study concurs with the work of Verschaeve *et al.* (2013) where a lack of in vitro cytotoxicity or genotoxicity in human hepatoma HepG2 cells was reported for aqueous extracts of four *Hypoxis* species namely *H. acuminata, H. colchicifolia, H. hemerocallidea* and *H. rigidula.* Cytotoxicity screening of aqueous extracts of *H. hemerocallidea* in another study involving prostate carcinoma (DU145) and non-malignant breast (MCF-12A) cell lines showed

stimulation, rather than inhibition of cell growth (Steenkamp and Gouws, 2006). Hypoxoside, a non-toxic metabolite of *Hypoxis sp.* is known to be converted by β-glucosidase to its active metabolite, Rooperol, which possess toxic properties (Albrecht *et al*., 1995; Steenkamp and Gouws, 2006, Boukes and van de Venter, 2011). Therefore, the lack or complete absence of cytotoxicity in extracts of *Hypoxis sp*., as is the case with *H. argentea*, may be due to very low concentrations of hypoxoside.

Table 3.1: IC_{50} values (µg/ml) of plant extract treatment of HepG2 cells after 24 hours

Treatment	***D. thunbergii***	***H. argentea***
Aqueous	< 50	268.00
Ethanolic	204.00	500.00

3.3. Measurement of cell density in INS-1 cells exposed to extracts of *D. thunbergii* and *H. argentea*.

3.3.1 Principle

The Crystal Violet assay is a simple assay that gives quantitative information about the relative density of cells adhering to multi-well culture plates. The cells are quantified after being stained with a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N, N-dimethyl-aniline), which accumulates in the nucleus of viable cells. The amount of dye taken up by the monolayer and intensity of the color produced correlates with the nuclear DNA and is proportional to the adherent cell density (Feoktistova et al., 2016).

3.3.2 Procedure

Cell treatment: INS-1 cells were seeded at a density of 8 000 cell/well in 96-well micro-titer plates with 100 µl of complete medium (RPMI 1640: 10% fetal bovine serum). Aqueous extracts of *D. thunbergii* and *H. argentea* were re-suspended in DMSO (0.25%, v/v) and were added to the wells already containing complete medium to reach concentrations of 12.5, 25 and 50 µg/ml of the extracts. The plates were incubated for about 48 hours at 37ºC in a humidified incubator and 5% CO_2. GABA (100 µM) and Glucose (11.1 mM) were used as positive controls, while DMSO (0.25%, v/v) served as the vehicle (untreated) control. Briefly, for the performance of the crystal violet assay, the treatments together with spent culture medium were removed by aspiration and were replaced with 100 µl of formaldehyde in PBS (10 %, v/v) to fix the cells. After about 1 hour, the fix solution was removed and 100 µl crystal violet solution, 0.1%, w/v (0.1 g of crystal violet dissolved in 100 ml distilled water and filtered through Whatman No 1 filter paper) was added to each well, followed by incubation of the plates at room temperature for about 30 minutes. Excess dye was removed by washing the plates with distilled water and the plates were dried by tapping on a paper towel. The dye taken up by the cells was extracted with 10% acetic acid (100 µl/ well). The absorbance of the wells was read at 595 nm using a BioTek® PowerWave XS spectrophotometer. Cell viability was expressed as a percentage relative to the untreated control, which was taken as 100% using the formula:

% cell viability = 100-% cell death

% cell death = 1- (Test absorbance/average absorbance of untreated control) X 100

3.3.3. Results and discussion

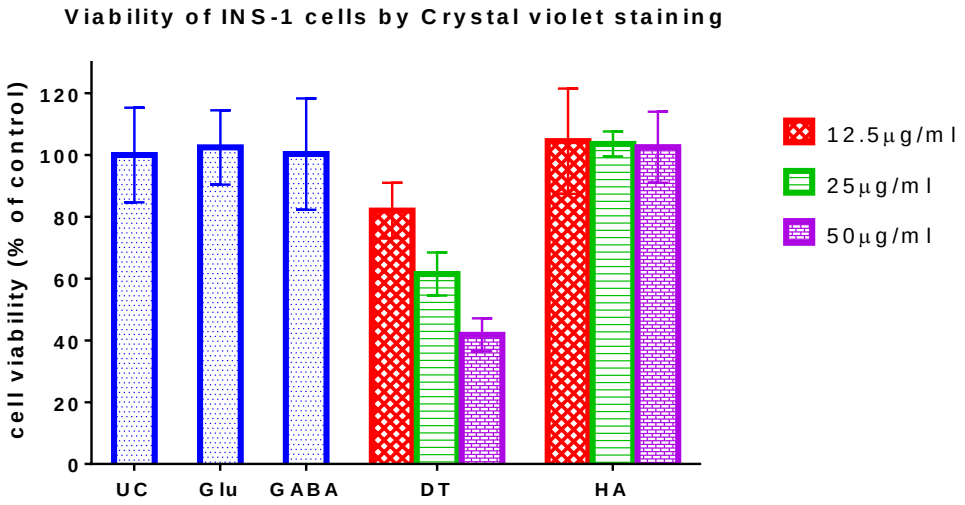

Figure 3.3: Crystal violet assay of induction of cell proliferation/cytotoxicity (expressed as % of control ± standard deviation; n = 8) by aqueous extracts of *Dianthus thunbergii* roots and *Hypoxis argentea* corms in INS-1 cells. UC = Untreated Control; Glu = Glucose; GABA = gamma-amino butyric acid; DT = *Dianthus thunbergii* aqueous extract; HA = *Hypoxis argentea* aqueous extract

The proliferation or otherwise of INS-1 cells after exposure to the extracts of *D. thunbergii* and *H. argentea* is shown in Figure 3.3. Treatment of INS-1 cells with *H. argentea* extract resulted mainly in maintenance of cell viability with a marginal increase in percentage cell viability of 4.47%, 3.57% and 2.52% (above the untreated control) at the respective concentrations of 12.5, 25 and 50 µg/ml. Similarly, the positive controls, GABA and glucose also maintained cell viability at values close to 100%. However, treatment of INS-1 cells with D. thunbergii extract produced a concentration dependent loss of cells with 17.96%, 38.50% and 58.19% of cells killed at increasing extract concentrations of 12.5, 25 and 50 µg/ml, respectively.

The results of this assay lend further credence to those demonstrated by MTT cytotoxicity in HepG2 cells (Section 3.2.3). The maintenance of INS-1 cell viability by the aqueous extract of *H. argentea* further strengthens its low risk status. The apparent tendency of the extract to stimulate cell proliferation requires further investigation. Stimulation of cell

proliferation was also detected when extracts from other *Hypoxis sp*. (*H. stellipilis*) were exposed to HeLa and HT-29 cancer cell lines (Boukes and Van de Venter, 2011). In contrast, cell death induced by the aqueous extracts of *D. thunbergii* further raises concerns about its safety for anti-diabetic use.

3.4 CYP 3A4 enzyme inhibition assay

3.4.1. Principle

The ability of the aqueous extracts of each plant to inhibit the activity of human cytochrome P450 reductase was determined using a Vivid® CYP 3A4 fluorescent screening kit consisting of CYP 3A4 Baculosomes® Plus reagent, reaction buffer, BOMR substrate, Red fluorescent standard, a regeneration system and $NADP^+$. The assay is based on the metabolism (oxidation) of the BOMR substrates by the specific CYP 450 enzyme, in the presence of $NADP^+$, into products that are highly fluorescent in aqueous solution. The assay may be run in a kinetic mode or end-point mode.

3.4.2. Materials and methods

The manufacturer's instructions in the kit were followed in this assay. A Master pre-mix was first prepared by diluting 50 µl of CYP 3A4 Baculosomes® Plus reagent and 100 µl of Vivid® regeneration system (100x) in 4850 µl of 1x Vivid® reaction buffer to give a master pre-mix concentration of 10 nM (or 2x the required screening concentration of 5nM). A substrate and $NADP^+$ mixture was then prepared by mixing 15 µl of reconstituted Vivid® substrate (reconstitution was done by diluting 0.1 mg of the Vivid® substrate in 150 µl of anhydrous acetonitrile) with 100 µl of Vivid® $NADP^+$ (100X), both of which were diluted in 885 µl of 1x Vivid® reaction buffer (this preparation represents 10x the screening concentration). The extract solutions, a solvent control and a positive control (Ketoconazole) were prepared at 2.5 times the test concentration within 1x reaction buffer. The final extract concentration in the wells was 100

µM, while Ketoconazole was prepared to reach final concentrations of 92 µM and 230 µM in wells of the respective columns.

To perform the assay, 40 µl of the 2.5x solutions of extracts, Ketoconazole and solvent control was added to respective wells of a 96-well plate in four replicates. 50 µl of the Master pre-mix was then added to each well, after which the plate was incubated for 10 minutes at room temperature to allow the test compounds and Ketoconazole to interact with the CYP 3A4 enzyme. The reaction was started by the addition of 10 µl of the substrate/$NADP^+$ mixture. In an end-point assay format, the plate was incubated for 30 minutes at room temperature, after which the reaction was stopped by the addition of 50 µl of 0.5 M Tris base. The fluorescence was measured at an emission of 590 nm following excitation at 550 nm using a Biotek® Synergy MX fluorimeter. CYP 3A4 inhibition was calculated as a percentage relative to the solvent control as follows:

% CYP 3A4 inhibition = 1- (test fluorescence/average fluorescence of solvent control) X 100.

3.4.3. Results and discussion

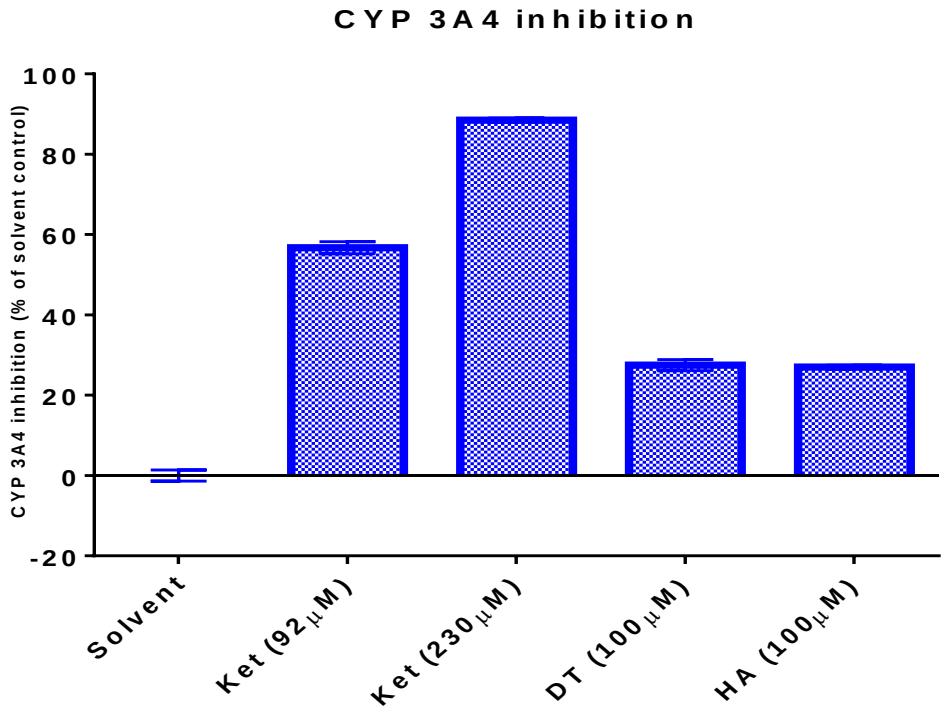

Figure 3.4: CYP 3A4 inhibitory activities of the aqueous extracts *Dianthus thunbergii* roots and *Hypoxis argentea* corms. Ket = Ketoconazole; DT = *Dianthus thunbergii* aqueous extract; HA = *Hypoxis argentea* aqueous extract.

CYP 3A4 is regarded as a promiscuous enzyme, having multiple binding sites in the active site which enables it to metabolize a wide range of structurally diverse compounds (Gwaza *et al.*, 2009). Figure 3.4 shows the single point inhibitory effects of the aqueous extracts of *D. thunbergii* and *H. argentea* on the activity of CYP 3A4. Both extracts produced a similar degree of inhibition at (27.5 ± 1.4) % for DT and 27.0 ± 0.5) % for HA. This level of inhibition was lower than that exhibited by Ketoconazole at both concentrations tested. This result indicates that the extracts of both plants are not likely to produce significant interactions when administered concurrently with drugs metabolized by CYP 3A4. Concentrations up to 1 mg/ml employed in CYP 450 inhibition assays in a previous study involving *Hypoxis sp.* produced strong inhibition of different CYP isoforms, including CYP 3A4, *in vitro* (Gwaza *et al.*, 2009).

implying that higher doses of the extracts used in the present study may result in greater inhibition. Furthermore, Gwaza *et al.* (2009) noted that methanol extracts of the plants tested exhibited greater amount of CYP inhibition than corresponding water extracts, an observation that corroborates the results of the present study.

3.5. Conclusions

Cell viability studies in this chapter have revealed differences in the potentials of the extracts of *Dianthus thunbergii* and *Hypoxis argentea* to induce cytotoxicity or cell proliferation. MTT assay and Crystal Violet indicated significant toxicity of the aqueous extract of *D. thunbergii* to both HepG2 and INS-1 cells, while both extracts of *H. argentea* were either non-toxic or capable of inducing cell proliferation. The observed effects for *D. thunbergii* raises some concern with regards to its use for anti-diabetic therapy, although it should be noted that *in vitro* cellular toxicity does not necessarily equate to whole animal (*in vivo*) toxicity, due to interactions with components of the gut, metabolism and their eventual bioavailability. Further studies are, however, required to evaluate the significance of the toxicity observed in relation to specific targets of anti-diabetic drugs. The results of this study offers information suggesting that both plants may appear not to significantly interfere with CYP 3A4 metabolism, compared to the established inhibitor, Ketoconazole. This observation may exclude the development of severe herb-drug interactions when the plants are used in combination therapy, although the effect of the extracts when sufficient concentrations are achieved *in vivo,* requires investigation. Further, the inhibitory activity of the extracts against other isoforms CYP 450 still needs to be investigated.

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CHAPTER 4

In-vitro anti-diabetic screening and mechanism(s) of action of Dianthus thunbergii and Hypoxis argentea

Data from this chapter are being submitted for publication in the Journal of Ethnopharmacology.

Chapter 4

IN-VITRO ANTI-DIABETIC SCREENING AND MECHANISM(S) OF ACTION OF *DIANTHUS THUNBERGII* AND *HYPOXIS ARGENTEA*

4.1. General background

Conventional antidiabetic agents are categorized according to proven mechanisms by which they lower blood glucose concentrations. The main mechanisms of action include inhibition of glucose digestion and/or absorption from the intestines, increasing insulin secretion from the pancreatic β cells, increasing insulin responsiveness in peripheral tissues, enhancement of glucose uptake by adipose and skeletal muscle tissues and reduction of hepatic glucose production by inhibiting gluconeogenesis (El-Abhar and Schlaalan, 2014). Table 4.1 summarizes the pharmacological relevance of different *in vitro* assays employed in the present studies to evaluate various targets for core antidiabetic activity and the prevention of diabetic complications.

In vitro anti-diabetic assays may be employed for two main purposes. First, they may serve as complementary tools to explore findings obtained from *in vivo* models (Frode and Medeiros, 2008). Second, *in vitro* assays may be used as first phase screening models to identify plant extracts with specific anti-diabetic mechanisms, as well as a basis for prioritizing samples for further studies. The obvious limitation of *in vitro*-screening, however, is that they are designed to target a single cell type, metabolic pathway or enzyme per time, compared to whole body systems in *in vivo* models (van de Venter *et al.*, 2008). Nevertheless, ethical and animal welfare considerations make it difficult to perform screening of large numbers of plants *in vivo.*

The objective of the studies in this chapter was to screen the extracts of *D. thunbergii* and *H. argentea* against various targets involved in antidiabetic therapy, in an attempt to validate

their ethno-medicinal use in the management of diabetes mellitus. These studies have focused on aqueous extracts, as ethno-medicinal information collected from collaborating herbal practitioners in the Eastern Cape indicate that water is most commonly used as a vehicle for the preparation of both plants for antidiabetic remedies. This information is also supported by previous work on other plants (Grierson and Afolayan, 1999; Kelmanson *et al.*, 2000; Inngjerdingen *et al.*, 2004;). The extract concentrations employed in the studies was largely guided by those used in cytotoxicity studies in the previous chapter.

Table 4.1: Overview of in-vitro anti-diabetic assays used in this study and their pharmacological relevance

S/N	In-vitro assays	Pharmacological relevance
1	**α-amylase and α-glucosidase inhibition**	Inhibition of carbohydrate digesting enzymes decreases the post-prandial increase in blood glucose levels.
2	**Lipase inhibition**	Inhibition of fat digestion by pancreatic lipase slows the production of free fatty acids, which are known to contribute to insulin resistance in Type-2 diabetes.
3	**DPP-IV inhibition**	DPP IV degrades incretin hormones such as GLP-1, which are known to enhance β-cell function. Inhibition of DPP IV leads to increased half-life and circulating levels of the incretins.
4	**Protein Glycation inhibition**	Advanced glycation end-products (AGEs) formed during protein glycation are involved in the development of several diabetic complications. Inhibition of protein glycation may help to reduce the development of these complications.
5	**Collagenase inhibition**	Collagenases are metalloproteinases capable of cleaving collagen and other connective tissues involved in wound healing. Inhibition of collagenase activity represents a useful mechanism to promote healing of wounds that develop as a complication of diabetes.
6	**Hepatic and Skeletal**	Increased utilization of glucose by tissues may attenuate fasting

	muscle glucose utilization	and post-prandial hyperglycemia.
7	**Nitric oxide inhibition**	Inhibition of nitric oxide production by macrophages may be useful in reducing adipose tissue inflammation that gives rise to insulin resistance.
8	**β cell glucose metabolism**	Mitochondrial ATP accounts for the majority (> 90%) of glucose metabolism in β cells and is the main effector for insulin secretion. Stimulation of β cell glucose metabolism may, therefore, enhance insulin secretion.
9	**Adipocyte differentiation**	Stimulation of adipocyte differentiation results in the formation of increased numbers of smaller metabolically-active adipocytes with increased triglyceride accumulation and restoration of dyslipidemia and insulin sensitivity.

4.2 Inhibition of carbohydrate digesting enzymes (Alpha amylase and alpha glucosidase)

4.2.1. Alpha amylase inhibition assay

4.2.1.1 Assay Principle:

Alpha amylase activity was measured by the amount of starch hydrolyzed into monosaccharides in the presence of the enzyme. The reaction incorporates an iodine reagent which gives a blue color in the presence of starch. In the presence of an enzyme inhibitor, the intensity of the color, measured spectrophotometrically, indicates the amount of starch remaining in the reaction mixture, and hence, the extent of alpha amylase inhibition.

4.2.1.2 Reagents

i. *Starch solution (2 mg/ml):* 0.2 g of starch in 100 ml distilled water. Solubilization was facilitated by heating the starch solution until boiling in a glass beaker directly on a stirring plate and maintained at this temperature for 15 minutes. The starch was allowed to cool to room temperature and the volume made up to 100 ml with distilled water.

ii. *Phosphate buffer:* 1.2 g NaH_2PO_4 and 0.2 g NaCl were dissolved in 450 ml distilled water. pH was adjusted to 6.0 using 1M NaOH, and volume was made up to 500 ml.

iii. *1M Hydrochloric acid:* 10 ml HCl (10N) was added to 80 ml distilled water, made up to 100 ml with same.

iv. *Iodine reagent:* 0.127 g I_2 and 0.083 KI were mixed together in 100 ml of distilled water.

v. *Alpha amylase enzyme:* 10 mg porcine pancreatic amylase was solubilized in 100 ml phosphate buffer. The preparation was made just before use and kept on ice.

vi. *Acarbose stock solution (positive control):* A 500 µM solution was initially prepared by mixing 3.2 mg acarbose dissolved in 10 ml phosphate buffer and aliquots of 1 ml were made into eppendorf tubes. This was stored at -20ºC until use. The aliquots were diluted five-fold to give a working concentration of 100 µM.

4.2.1.3 Procedure:

Plant extracts were prepared at the concentrations: 50, 100 and 200 µg/ml in a phosphate buffer. 5µl of enzyme solution was pipetted into appropriate wells of a 96-well plate. Thereafter, 15 µl of test samples, phosphate buffer (the blank, enzyme without inhibitor) or positive control (acarbose, 100 µM) were added to the enzyme in respective wells. The plate was pre-incubated for 10 minutes at 37ºC to allow interaction of the enzyme with the different compounds. The reaction was started by the addition of 20 µl starch solution to the wells, and the plate was again incubated for 30 minutes at 37ºC. The reaction was terminated by adding 10 µl of 1M HCl to each well, followed by 75 µl iodine reagent. Absorbance was measured at 580 nm using a BioTek® PowerWave XS spectrophotometer. Alpha amylase inhibition was measured as a percentage of the enzyme control using the formula:

% α-amylase inhibition = 1- (B/A) X 100.

Where A = *absorbance of test well (plant extract or acarbose)*

B = *Average absorbance of enzyme control*

Controls without enzyme (no-enzyme) and without starch (no-starch) were also included in the assay to be certain that no reaction occurred when one of either the enzyme or substrate was absent. This was done to exclude false positive results, as some plants extracts have been reported to contain traces of α-amylase or starch.

4.2.2 Alpha glucosidase inhibition assay

4.2.2.1 Assay Principle

The assay is based on the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (PNP-GLUC) specifically by α-glucosidase into a yellow colored product, p-nitrophenol (PNP) and D-glucose, with absorbance maximum at 405 nm. Inhibition of α-glucosidase results in reduced formation of PNP.

4.2.2.2 Reagents:

i. Potassium phosphate buffer (67 mM; pH 6.8)

ii. p-Nitrophenyl-α-D-glucopyranoside (PNP-GLUC) solution (10 mM)

iii. Sodium carbonate solution (100 mM)

iv. α-glucosidase solution

v. Epigallocatechin gallate; EGCG, 10 µg/ml (positive control)

4.2.2.3 Procedure

Within wells of a 96-well micro-tire plate, the reaction mixture consisted of 5 µl of plant extract (prepared at concentrations of 50, 100 and 200 µg/ml) or the positive control (EGCG) and 20 µl of α-glucosidase solution. This was pre-incubated at 37ºC for 5 minutes and initial background absorbance was read at 405 nm. 10 µl of PNP-GLUC was then added and the reaction mixture was incubated again for 20 minutes at 37ºC. The reaction was terminated by the

addition of 25 µl of sodium carbonate solution. The absorbance was measured again at 405 nm using a multiplate reader (BioTek® PowerWave XS spectrophotometer). Controls without enzyme and without the substrate (PNP-GLUC) were also included in the assay.

The percentage inhibition of α-glucosidase was calculated as follows:

% α-glucosidase inhibition = 1- (B/A) X 100.

Where A = *absorbance of test well (plant extract or positive control)*

B = *Average absorbance of enzyme control*

4.2.3 Results and discussion

α-amylase and α-glucosidase are key small intestinal enzymes that mediate carbohydrate digestion in humans and have both been recognized as therapeutic targets for modulating post-prandial hyperglycemia (Shobana *et al.*, 2009; Manaharan *et al.*, 2012). Figure 4.1 summarizes the results of the α-amylase and α-glucosidase inhibitory activities of the aqueous extracts of *D. thunbergii* and *H. argentea*, with Acarbose and Epigallocatechin gallate (EGCG) as respective positive controls. Acarbose is used in the clinical management of early diabetes (Heo *et al.*, 2009), while catechins (including EGCG) are well reported to exhibit strong α-glucosidase inhibitory activities (Zhu *et al.*, 2010; Xu *et al.*, 2013).

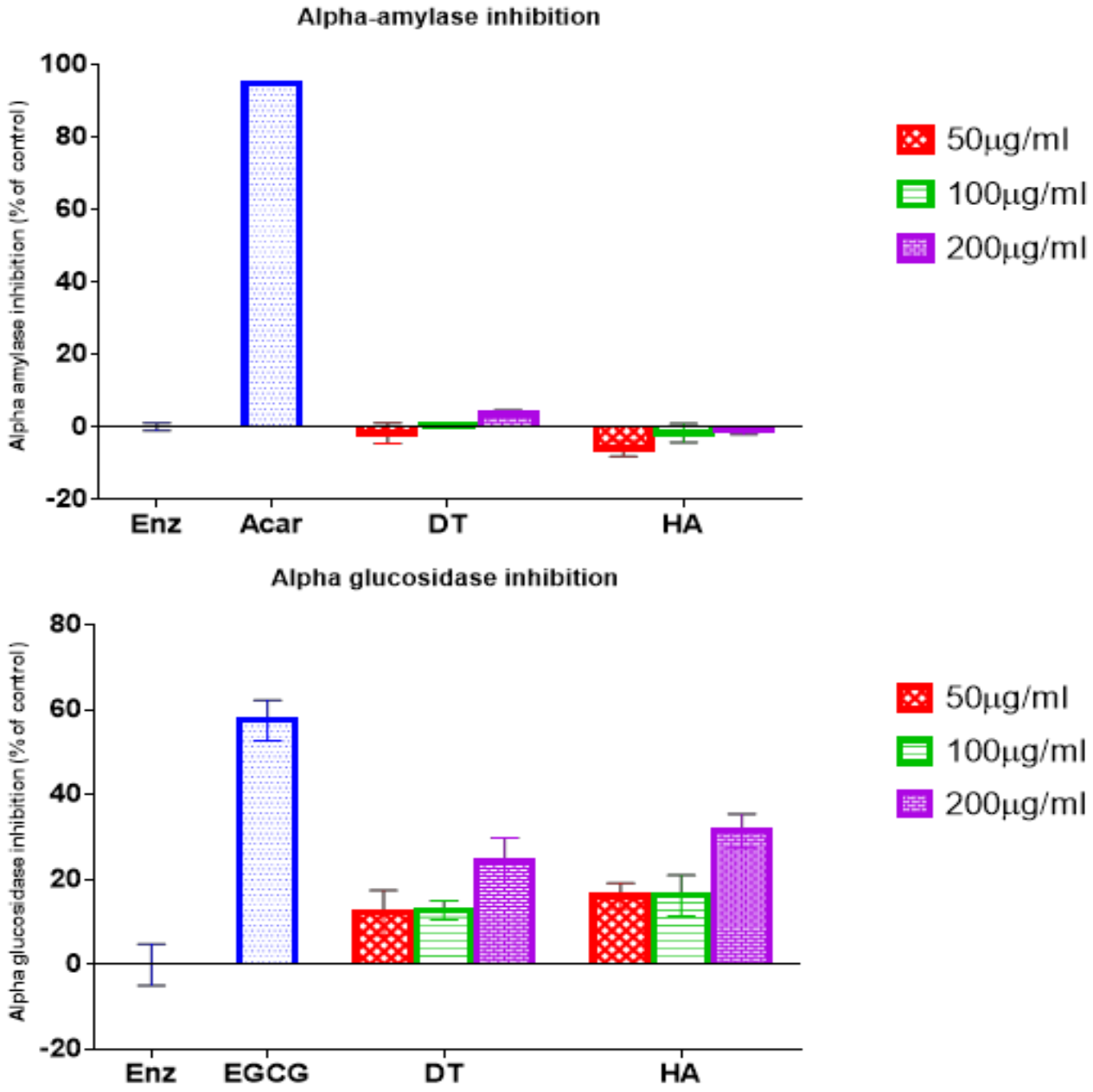

Figure 4.1: α-amylase and α-glucosidase inhibitory activities of the aqueous extracts Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. Acar = Acarbose; EGCG = Epigallocatechin gallate. Values are presented as mean±standard deviation (n = 4).

The results indicate that both extracts exhibited some appreciable degree of inhibitory activities against α-glucosidase, but very weak inhibitory effects on α-amylase. At concentrations

of 50, 100 and 200 µg/ml, the % α-glucosidase inhibition produced by *D. thunbergii* was 12.2%, 12.8% and 24.5%, respectively, while the values for *H. argentea* were 16.1%, 16.3% and 31.5%, respectively. Acarbose and EGCG, as positive controls, were far more effective in the respective assays than the extracts from both plants, exhibiting percentage inhibitory activities of 94.7 % and 57.5% against α-amylase and α-glucosidase, respectively.

The observed results indicate that inhibition of α-amylase may not represent a potential mechanism by which these plants will exert any anti-diabetic action. However, considering that these are crude extracts from the plants, the results here provide evidence that more effective α-glucosidase activities may be obtained with purification of specific active fractions from the crude extracts. This investigation is recommended for the extract of *H. argentea*, as previous cytotoxicity evaluation in the previous section, using the same concentrations, did not show significant toxicity to cell lines.

Previous studies have indicated that plant phytochemicals are stronger inhibitors of α-glucosidase than α-amylase (Kwon et al., 2007). The findings in this study are similar to those of a recent study by Kazeem and Ashafa (2015), using similar concentrations who also observed stronger alpha-glucosidase inhibitory activity than alpha-amylase inhibition by the aqueous whole plant extract of *Dianthus basuticus*, another member of the Caryophyllaceae family. Previous reports have also indicated the potential of the aqueous extracts of other *Hypoxis* species, e.g., *Hypoxis hemerocallidea*, in lowering blood glucose levels *in vivo* (Ojewole, 2006; Oguntibeju *et al.*, 2016). However, no studies were found in literature regarding possible *in vitro* inhibition of these carbohydrate-hydrolyzing enzymes by extracts of the *Hypoxis sp.*

4.3. Inhibition of Lipase activity

4.3.1 Principle

The enzyme lipase plays a key role in fat digestion by hydrolyzing dietary triglycerides to monoglycerides and free fatty acids. Free fatty acids are known to contribute to the development of insulin resistance in Type 2 diabetes (Abel *et al.*, 2001). Inhibition of lipase activity has been explored as a possible mechanism for reducing total caloric intake and therefore, a possible means of interfering with the development of obesity-related Type-2 diabetes (Gondoin *et al.*, 2010; Lewis and Liu, 2012). In this assay, the conversion of the substrate, para-Nitrophenol palmitate (pNPP) is used to study lipase inhibition in a single phase system.

4.3.2 Reagents:

i. *Solution A (pNPP):* 2 mg pNPP was dissolved in 2 ml isopropanol of 10% isopropanol.

ii. *Solution B:* consisted of 20 mg gum arabic, 40 mg sodium deoxycholate and 100 µl of Triton X-100 all dissolved in 18 ml of Tris-HCl buffer (pH 8.0).

iii. *Substrate solution:* Solution A was added to solution B and stirred until all was dissolved.

iv. *Lipase solution (10 mg/ml):* 50 mg of porcine pancreatic lipase added to 5 ml of 50 mM Tris-HCl buffer.

4.3.3. Procedure:

10 µl of extracts (prepared at concentrations of 50, 100 and 200µg/ml), positive control (Orlistat, 100 µM) and DMSO (negative control vehicle used to dissolve the extracts) were pipetted into respective wells of a 96-well plate. Freshly prepared porcine pancreatic lipase was added at four times the volume of the test samples, positive and negative controls (40 µl). The plates were initially incubated at 37ºC for 15 minutes. Thereafter, 170 µl of the substrate solution

was added to the wells. The plate was then incubated at 37°C for 25 minutes and the absorbance was read at 405 nm using a Biotek® Power Wave XS spectrophotometer.

Percentage lipase inhibition was calculated as:

% Lipase inhibition = 1- (A/B) X 100.

Where A = *absorbance of test well (plant extract or orlistat)*

B = *Average absorbance of enzyme control*

4.3.4 Results and Discussion

Recent efforts to prevent the chronic positive energy balance that occurs in obesity and obesity-related diabetes have included the exploration of natural products for their inhibitory activities against pancreatic lipase, a key enzyme for the digestion of dietary triglycerides. Plant polyphenols have been shown to exhibit lipase inhibitory activities (Birari and Bhutani, 2007; Slanc *et al.*, 2009). However, most reports of large scale screening of plant extracts have yielded very few plants with very potent lipase activities (Seyedan *et al.*, 2015) when compared with established inhibitors such as Orlistat. Orlistat is an irreversible inhibitor that binds covalently to the putative active site serine residue of pancreatic lipase (Sternby *et al.*, 2002).

In the present study, both extracts of *D. thunbergii* and *H. argentea* showed weak inhibitory activities against porcine pancreatic lipase as shown in Fig. 4.2. The highest inhibition obtained with *D. thunbergii* was 17.5% which was much lower compared to 58.8% obtained with Orlistat used as a positive control. This suggests that the aqueous extracts of the two plants investigated in this study may not be considered to offer anti-diabetic activities via mechanisms related to inhibition of lipase activity. To the best of our knowledge, no previous studies have investigated the lipase inhibiting activities of any type of extracts from these plants.

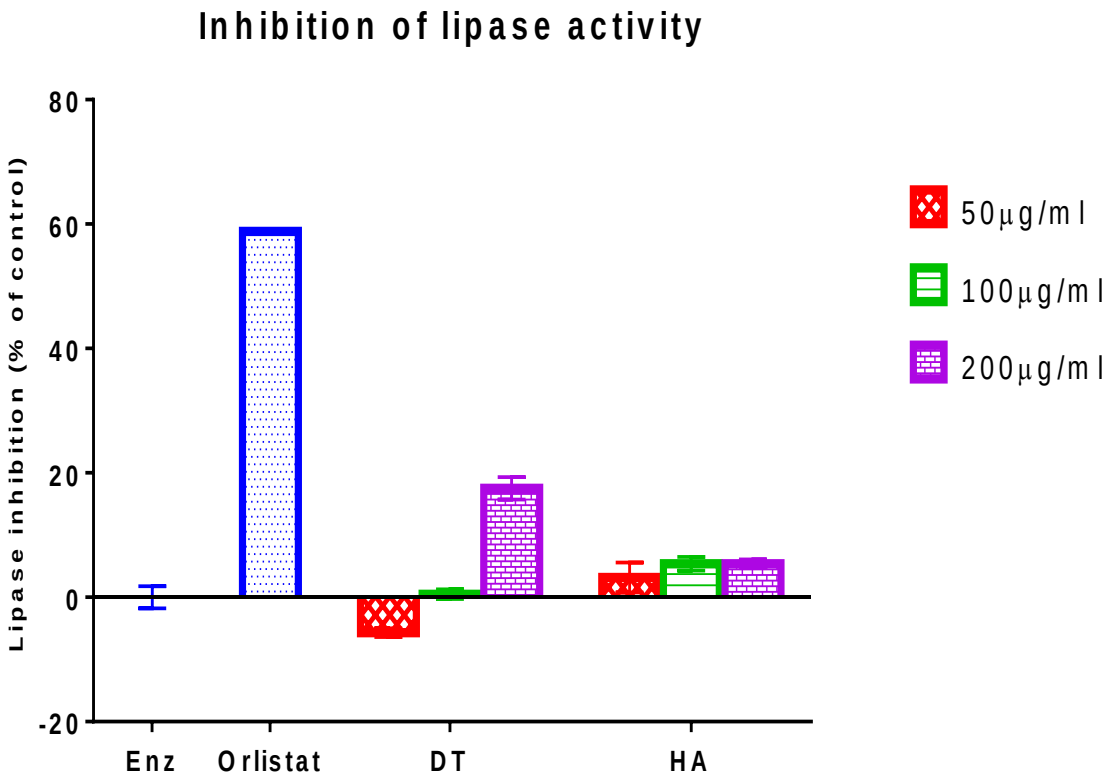

Figure 4.2: Lipase inhibitory activities of the aqueous extracts Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. Values are presented as mean±standard deviation (n = 4).

4.4 Dipeptidyl peptidase (DPP-IV) inhibition

4.4.1 Assay Principle:

The method described by Al-Masri *et al*. (2009), with slight modifications was used in determining DPP-IV inhibition, with slight modifications. The chromogenic substrate Gly-Pro-*para*-nitroanilide (GPPN) is cleaved by DPP IV to release *para*-nitroanilide (pNA), a yellow colored product which can be measured colorimetrically. Acetic acid is added to the reaction mixture to terminate the reaction.

4.4.2 Reagents

i. Tris-HCl buffer (50 mM; pH 8.0): 3.027 g Tris was dissolved in 400 ml distilled water. The pH was adjusted to 8.0 with HCl and the volume was made up to 500 ml with same.

ii. Substrate stock solution (20 mM Gly-Pro-p-nitroanilide): 4.6 mg Gly-Pro-pNA was dissolved in 500 µl DMSO and stored at -80ºC. To prepare a working solution, the DMSO stock was thawed in a 37ºC water bath and diluted in Tris-HCl buffer to a concentration of 0.2 mM.

iii. Diprotin A (Positive control): A stock solution of 5 mg/ml was prepared and stored at -80ºC as 50 µl aliquots. The working solution was prepared by diluting the stock to 50 µg/ml (i.e.100µl into 1000 µl).

iv. DPP IV enzyme solution: Human recombinant DPP IV enzyme was diluted to a final concentration of 50 µU/µl in Tris buffer (approximately 1.5 µl into 1.5 ml Tris buffer, sufficient for one 96-well plate.

v. 25% acetic acid: Concentrated acetic acid was diluted 1:4 with distilled water.

4.4.3 Procedure

35 µl of extracts (prepared at 50 and 100 µg/ml) and the positive control (Diprotin A, 50µg/ml) were initially mixed with 15 µl of the DPP IV enzyme solution in respective wells of a 96-well plate. The assay included a no-sample blank in which the samples were replaced with buffer and a no-enzyme blank in which the enzyme solution was also replaced by buffer. The plate was pre-incubated at 37ºC for 5 minutes to allow interactions of extracts and the enzyme, after which a background absorbance was measured. Thereafter, 50 µl of the substrate solution (Gly-Pro-*p*Na) was added to all the wells and the plate was incubated at 37ºC for 30 minutes. The reaction was terminated by the addition of 25 µl of 25% acetic acid. Absorbance was measured at 410 nm using a Biotek® Power Wave XS spectrophotometer. The background

absorbance was initially subtracted from the final absorbance, after which the percentage inhibitory activity was calculated according to the following formula:

% DPP IV inhibition = 1- (A/B) X 100.

Where A = *absorbance of test well (plant extract or Diprotin A)*

B = *Average absorbance of untreated control*

The assay was conducted in quadruplicates.

4.4.4 Results and discussion

As shown in Fig. 4.3, DPP IV inhibition was virtually non-existent for the plant extracts at the concentrations tested, compared to the positive control which exhibited 75.8% inhibition of DPP IV activity. Some previous studies by other workers on other plants using a similar methodology and range of concentrations as used in this study have reported appreciable inhibition of DPP IV, although it appears that the use of other solvents such as methanol appeared to give better results than aqueous extracts (Calero *et al.*, 2014; Unnikrishnan *et al.*, 2014; Saidu *et al.*, 2016). It is possible, therefore, that the chemical composition of different plant extracts, as well as the type of solvents used for extraction may play a major role in determining potential for DPP IV inhibition. No previous studies were found in literature detailing the DPP IV inhibitory activities of either *Dianthus sp*. or *Hypoxis sp*.

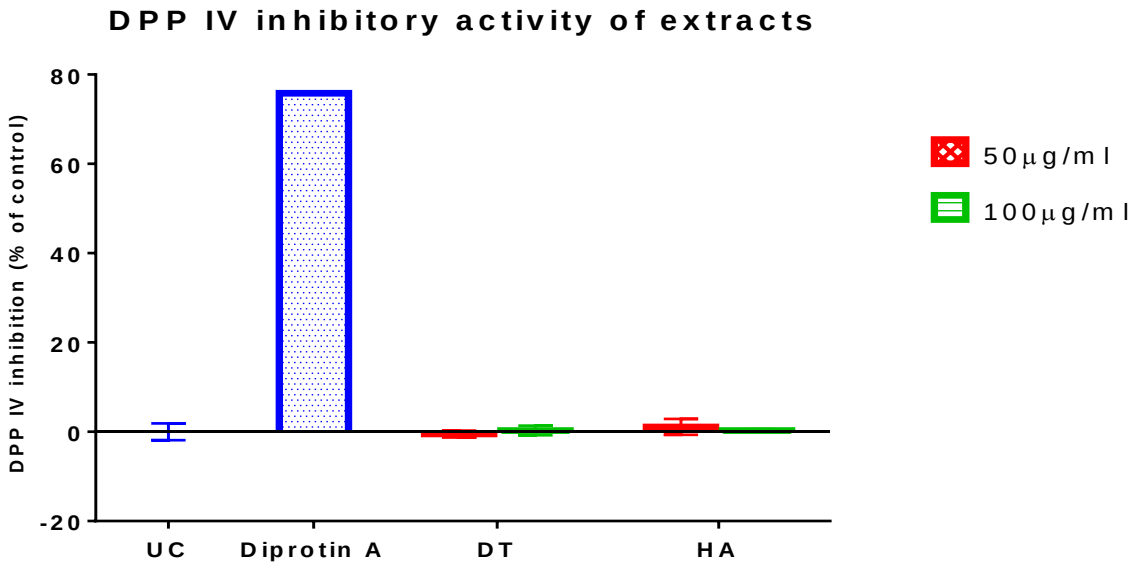

Figure 4.3: Dipeptidyl peptidase (DPP) IV inhibitory activities of the aqueous extracts Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. Values are presented as mean±standard deviation (n = 4).

4.5 Protein glycation inhibition

4.5.1 Assay Principle

This assay is designed to measure fluorescent advanced glycation end-products (AGEs) formed when the protein gelatin is glycated with glyceraldehyde. The fluorescent AGEs can be detected with a fluorescence microplate reader at an excitation wavelength of 370 nm and emission wavelength of 440 nm.

4.5.2 Reagents

i. Glyceraldehyde solution (500 mM): 225 mg of glyceraldehyde was dissolved in 5 ml of distilled water.

ii. Protein (Gelatin) solution; 20 mg/ml: 100 mg gelatin was mixed with 5 ml distilled water. This was heated to facilitate dissolution.

iii. Positive control (Aminoguanidine; 20 mM)

4.5.3 Procedure

Gelatin solution (50 µl) was first pipetted into the wells of a 96- well plate. Then, 40 µl of Aminoguanidine (20 mM) or extracts (at 50 and 100 µg/ml) were added to the respective wells. Thereafter, 10 µl of 500 mM glyceraldehyde solution was added to each well. The plates were sealed and incubated at 37ºC for 24 hours. The fluorescent intensity was measureds at an excitation wavelength of 370 nm and emission wavelength of 440 nm using a Biotek® Synergy MX fluorimeter. The experiment was conducted as quadruplicates and fluorescence quench controls were also included for each sample. Inhibition of protein glycation was calculated as a percentage of untreated control (water) using the formula:

% protein glycation inhibition = 1- (A/B) X 100.

Where A = *absorbance of test well (plant extract or Aminoguanidine)*

B = *Average absorbance of untreated control*

4.5.4 Results and discussion

Protein glycation and the subsequent formation of AGEs in long-standing diabetes cause damage to various organs, resulting in complications such as neuropathy, nephropathy, retinopathy, and cardiomyopathy. AGEs are known to mediate these deleterious effects by direct interference with normal protein conformation and function or by interacting with certain plamsa membrane localized receptors (RAGEs) with stimulation of intracellular signaling, gene expression, release of pro-inflammatory mediators and free radicals. Thus natural products with potent antioxidant activities have been found to possess considerable inhibitory activities against protein glycation (Sadowska-Bartosz and Bartosz, 2015).

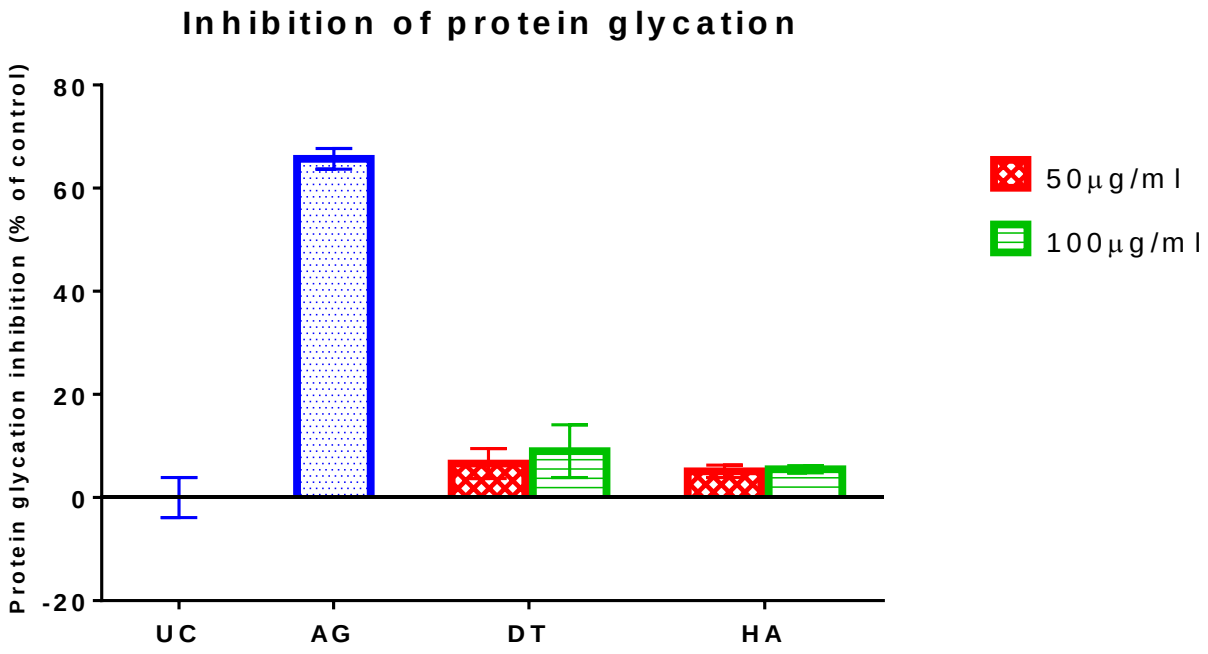

Figure 4.4: Inhibition of protein glycation by the aqueous extracts Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. AG = Aminoguanidine. Values are presented as mean±standard deviation (n = 4).

In the present study, the aqueous extracts of *D. thunbergii* and *H. argentea* produced weak protein glycation inhibition, with the 100 µg/ml concentration producing only 9.0% and 5.5% inhibition respectively (Fig. 4.4). This was in contrast to aminoguanidine which produced 65.7% inhibition. Aminoguanidine was the first AGE-inhibitor discovered in 1986 and its mechanism of action involves the trapping of reactive intermediates such as methylglyoxal, glyoxal, and 3-deoxyglucosone formed during the Maillard reaction, thereby preventing the formation of AGEs (Thornalley, 2003). The results indicate that the extracts of the two plants in the present study probably possess very low potential for prevention of diabetic complications involving glycation of proteins. However, this study represents the first attempt to investigate the anti-glycation properties of the extracts of *D. thunbergii* and *H. argentea.*

4.6 Collagenase inhibition assay

4.6.1 Assay Principle

Gelatin was used in this assay as a substrate for collagenase. Reaction between the enzyme and substrate causes a breakdown of gelatin. In the presence of a collagenase inhibitor, gelatin breakdown occurs to a limited extent, such that the portion broken down solubilizes and remains in supernatant, while intact proteins remain as pellets after centrifugation of the reaction mixture. Coomasie Brilliant Blue (CBB), a protein dye, is used to stain intact proteins, as well as aid their precipitation. With inhibition of collagenase activity, therefore, more gelatin remains as pellets and are stained by CBB.

4.6.2 Reagents

i. Assay buffer consisting of Tris-Hcl (50 mM); $CaCl_2$ (10 mM) and NaCl (0.15 M); pH 7.4.

ii. Gelatin (2 mg/ml) prepared in distilled water: This preparation was initially autoclaved in order to solubilize gelatin.

iii. EDTA (positive control): A stock of 20 mM was prepared and was diluted to 10 mM for use in the assay.

iv. Catechin (positive control): A stock of 1 mM was prepared and diluted to 0.5 mM for the assay.

v. Collagenase enzyme: 40 µg/ml prepared in 1X buffer. Final working concentration was 400 ng / well.

vi. Coomasie Brilliant Blue: 0.25% CBB R-250 prepared in a 40% methanol/10% acetic acid solution. This was filtered in Whatmann No 1 filter paper and heated three times for 3 minutes in a microwave.

4.6.3 Procedure

The reagents (enzyme, sample, gelatin and assay buffer) were initially prepared 4 times the final concentration. Extracts were prepared at final concentrations of 12.5, 25 and 50 µg/ml. To respective wells of a 96-well plate, the reaction mixture contained 10 µl of each of the enzyme, samples or positive controls, gelatin and the assay buffer. The plate was then incubated at 37ºC for 1h. Thereafter, 20 µl of CBB was added and the plate was then shaken by gentle tapping for about 5 minutes. The plate was centrifuged at 500 rcf for 5 minutes. The supernatant was removed by aspiration with a micropipette. The wells were then washed with 50 µl of the washing solution (40% methanol/10% acetic acid) to remove excess CBB. The pellets were then dissolved using 50 µl of DMSO. The absorbance of the wells was measured at 600 nm using a Biotek® Power Wave XS spectrophotometer. The assay was conducted as quadruplicates and the percentage collagenase inhibition was calculated relative to the untreated control as follows:

% collagenase inhibition = 1- (B/A) X 100.

Where A = *absorbance of test well (plant extract or EDTA or catechin)*

B = *Average absorbance of untreated control*

4.6.4 Results and discussion

An increased incidence of wound complications and delayed wound healing has been reported in patients with diabetes mellitus. Hyperglycemia caused by decreased insulin availability can also affect cellular response to tissue injury. Collagenases are extracellular matrix metalloproteinases capable of cleaving collagen, as well as other connective tissue molecules such as elastin, fibronectin and gelatin (Pilcher *et al.*, 1999). These enzymes are involved in the complex response to tissue injury that is controlled by local and migrating cells. The role of collagenases in wound healing is two-fold. On the one hand, collagenase activity is

desirable at a very early stage of wound healing as certain degradation products of collagen, (e.g., collagen-derived peptides), can promote macrophage chemotaxis to wound sites (Agren *et al.*, 1993). In other words, collagenase-induced restructuring of collagen is critical for cell movement and wound repair, particularly as abnormal deposition of collagen due to altered collagen lysis results in formation of excessive scar tissue or keloids.

On the other hand, excessive collagenase activity, especially in chronic wounds, may result in decreased tensile strength of wound tissue, with repeated breakdown of wound connective tissue (Black *et al.*, 1989; Hatz *et al.*, 1995). For this reason, a variety of compounds are being investigated for their anti-collagenase activities. Therefore, finding a balance by selectively enhancing or blocking collagenase activity is critical for the management of wound complications in diabetes mellitus.

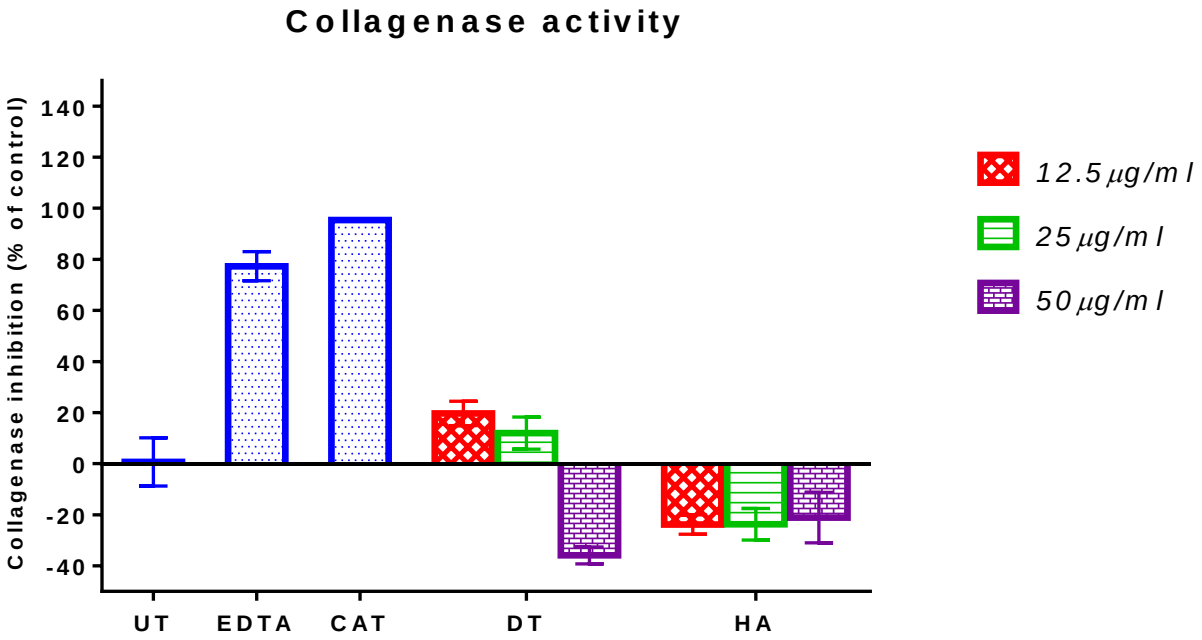

Figure 4.5: Collagenase inhibitory activities of the aqueous extracts Dianthus thunbergii roots and Hypoxis argentea corms. EDTA = Ethylene diamine tetra acetic acid; CAT = Catechin. Values are presented as mean±standard deviation (n = 4).

In this study, *D. thunbergii* produced a concentration-dependent inhibition of collagenase activity with percentage inhibitions of 19.7% and 12.0% obtained at the low concentrations of 12.5 and 25 µg/ml, respectively, while a stimulation of enzyme activity up to -35.9% was obtained with the highest concentration tested (50 µg/ml). However, all the concentration of *H. argentea* showed stimulatory activities of the collagenase enzyme, rather than inhibition (Fig. 4.5). The activities of the extracts were in contrast to those of the standard collagenase inhibitors, EDTA and catechin, which produced 77.3% and 95.4% inhibition of collagenase activity, respectively. As noted earlier, effective wound healing through the early to late stages requires the selective modulation of collagenase activity. It appears from these results that the extracts of both *D. thunbergii* and *H. argentea* would probably offer more benefit in the very early stages of wound healing when higher collagenase activity is often desirable. To have a better view of the effects of the *Dianthus* and *Hypoxis* extracts on collagenase activity, it may be necessary to employ cell lines such as human skin fibrobalsts expressing collagenase.

4.7. Glucose Utilization assays

4.7.1 Background

Glucose is utilized as the major fuel by most cells. Its uptake into cells is tightly regulated and is often the rate-limiting step for glucose utilization (Klip *et al.*, 1994). The disposal of post-prandial glucose is the combination of liver (splanchnic) and peripheral tissue (skeletal muscle, adipose tissue, brain) glucose uptake. Liver glucose uptake includes first-pass extraction via the hepatic portal vein, and uptake from the blood flowing to the liver through the hepatic artery (Woerle *et al.*, 2003). The liver consequently accounts for the clearance of up to one-third of the blood glucose concentrations occurring post-prandially. Glucose uptake in the liver is mediated by the high capacity, low affinity GLUT 2 transporter and does not require insulin to facilitate glucose uptake. However, insulin concentrations are required to regulate glucose output by the

liver via suppression of gluconeogenesis and facilitating glucose storage by glycogenesis. Compounds that stimulate hepatic glucose utilization may thus have potential anti-diabetic applications.

Skeletal muscle and adipose tissues are the primary peripheral tissues that respond to insulin stimulation of glucose uptake. Insulin mediates the uptake of glucose by these tissues via translocation of functional GLUT 4 transporters from the endoplasmic reticulum to the plasma membrane (Klip and Ishiki, 2005). GLUT 4 is one of a family of 14 facilitative transmembrane glucose transporters. It is expressed predominantly in muscle cells and adipocytes and it is a high affinity glucose transporter (Leto and Saltiel, 2012). Studies demonstrating insulin stimulation of GLUT-4 transporters have revealed that in the basal state (without insulin), intracellular microsomes contained virtually all glucose transporters, while plasma membrane fractions did not contain glucose transporters. On the other hand, during insulin stimulation, there is redistribution, such that there is an increase in glucose transporters in the plasma membrane and a decrease in the intracellular microsomal fractions (Holman and Cushman, 1994; Wilson and Cushman, 1994; Dawson *et al.*, 2001). Insulin stimulation of glucose transport has been observed to be markedly impaired in diabetic animals, an effect produced by a combination of marked reduction in GLUT 4 numbers, as well as decreased translocation to the plasma membrane (Karnielli *et al.*, 1981).

Hepatic (human hepatocellular carcinoma cells, HepG2) and muscle cell line (L6 myotubes) were chosen in this study to evaluate the effects of the aqueous extracts of *D. thunbergii* roots and *H. argentea* corms on glucose uptake because they have different glucose transporters and react differently to insulin stimulation. HepG2 cells are widely used as a cell culture model of human hepatocytes as they have the essential morphology and function for the

study of hepatic glucose uptake and glucose production as well as the modulation of the insulin signaling pathway in vitro (Huang *et al.,* 2015). Rat L6 and C2C12 myotubes are the most established *in-vitro* model cell line systems for in-depth studies on skeletal muscle glucose uptake because they have an "intact insulin signaling pathway and express the insulin-sensitive GLUT 4" (Nedachi and Kanzaki, 2006; Gupta *et al.*, 2009). L6 myoblasts usually require prior differentiation into L6 myotubes, which actually appear to exhibit glucose uptake to a higher extent than C2C12 cells (Sarabia *et al.*, 1990).

4.7.2 Glucose uptake in HepG2 cells

4.7.2.1 Cell culture conditions and treatment

HepG2 cells were routinely maintained by incubation at 37ºC in a humidified incubator with 5% CO_2 and were fed fresh growth medium, RPMI 1640 (Highveld Biological, South Africa), supplemented with 10% fetal bovine serum every 2–3 days. For the glucose uptake assay, the HepG2 cells were dislodged by brief exposure to 0.25% Trypsin in phosphate-buffered saline, counted, suspended in new growth medium and then seeded into 96-well culture plates at a density of 6, 000 cells/well (or 30, 000 cells/ml) in a volume of 200 µl per well of growth medium. The cells were allowed to adhere and grow in a humidified incubator (5% CO2 at 37ºC) for three days. Two cell-free rows were included to serve as blanks for the glucose utilization assay. On day 3, without changing the medium, 10 µl aliquots of the plant extracts (prepared at final concentrations of 25 and 100) and the positive controls, Metformin (1 µM) and Berberine (50 µM) were added to each well (six replicates for respective wells). The cells were incubated for a further 48 hours. At the end of incubation, all spent medium was removed and then replaced with 25 µl incubation buffer containing 8 mM glucose (RPMI 1640 diluted with PBS containing 0.1% BSA). The plates were then incubated for 3 hours at 37ºC. 10 µl of the incubation medium from each well were transferred to a new 96-well plate, and the glucose

concentration in the medium was determined by adding 200 µl of glucose oxidase reagent (SERA-PAK Plus, Bayer) and developed for 15 minutes at 37ºC. The absorbance was measured at 510 nm using a Biotek® Power Wave XS spectrophotometer. Thereafter, glucose utilization was calculated as the difference between the cell-free and cell-containing wells. Glucose utilization was expressed as a percentage of the untreated controls according to the formula:

% Glucose uptake = (A-B/C) X 100,

Where A = average absorbance of cell-free blanks

B = absorbance of cell-containing test wells

C = average absorbance of untreated controls

Toxicity assays to determine the cell density of each well in the original culture plate was done by adding MTT (0.5 mg/ml in RPMI 1640 and 10% fetal bovine serum) to the wells. The plate was incubated at 37°C for 3 hours, the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystals formed in the cells. The absorbance of the wells was read at 540 nm. The toxicity results were compared with untreated controls using Student's t-test and differences less than 0.05 were considered significant.

4.7.2.2 Results and discussion

The results obtained for the *in vitro* assay of glucose uptake in HepG2 cells in the presence of the plant extracts at 25 and 100 µg/ml are presented in Fig. 4.6. Metformin and Berberine were chosen as positive controls based on reported anti-diabetic activities (Wiernsperger and Bailey, 1999; Cheng *et al.*, 2006). Among different mechanisms proposed for the anti-diabetic actions of metformin, it is believed to enhance the insulin-stimulated increase in glucose uptake into skeletal muscle by increasing the functional properties of insulin-sensitive

glucose transporters, as well as their movement to the plasma membrane. The precise mechanism of stimulation of glucose uptake by berberine remains unknown, but is thought to be independent of insulin (Zhou *et al.*, 2007).

The results from this study indicate that the corms of *H. argentea* showed some potential in lowering blood glucose levels, producing an enhancement of glucose uptake by 3.85% and 19.58% over the untreated control, (taken as 100% glucose uptake), at 25 and 100µg/ml, respectively. On the other hand, glucose uptake in cells treated with *D. thunbergii* extract was significantly reduced ($p<0.05$) by 35.34% at 25 µg/ml, while no amount of glucose uptake was noticed at the 100 µg/ml concentration of this extract. Metformin (1 µM) and Berberine (50 µM), however, enhanced glucose uptake by 69.71% and 15.11%, respectively, over the untreated control (which was taken as 100%)

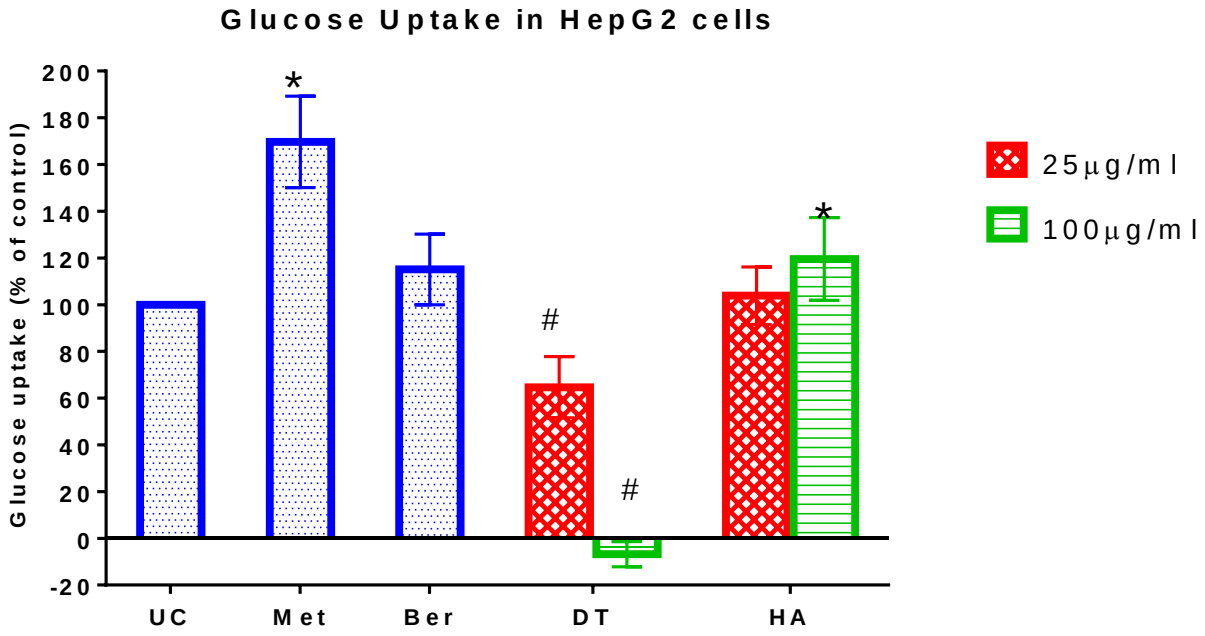

Figure 4.6: Glucose uptake in HepG2 cells (expressed as % of control ± standard deviation; n=6) of the aqueous extracts of Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. UC = untreated control; Met = Metformin; Ber = Berberine. * Significant increase compared to untreated control; # Significant decrease compared to untreated control.

The toxicity assay performed on cells carrying out glucose uptake revealed that the *D. thunbergii* extract was toxic to HepG2 cells, producing 8.35% and 90.53% cell death at the 25 and 100 µg/ml concentrations, respectively (Fig. 4.7). Thus, glucose uptake was lower at both concentrations of *D. thunbergii*, compared to the untreated control. The toxicity of these extracts to the cells most likely explains the significant reduction in glucose uptake in the cells exposed to this extract. The long exposure time of 48 hours could have allowed sufficient interaction of the cells with toxic compounds in the extract. Previous cytotoxicity assays involving *D. thunbergii* in this study (Chapter 3) have also indicated the toxicity of this extract to HepG2 and INS-1 cells. Treatment of HepG2 cells with the *H. argentea* extract as well as Metformin and Berberine did not produce any indication of toxicity in the cells. A concentration-dependent increase in the enhancement of glucose uptake obtained with *H. argentea*, with no toxicity, suggests that it may potentially produce anti-hyperglycemic actions by this mechanism, and therefore, offers promise

for further investigation.

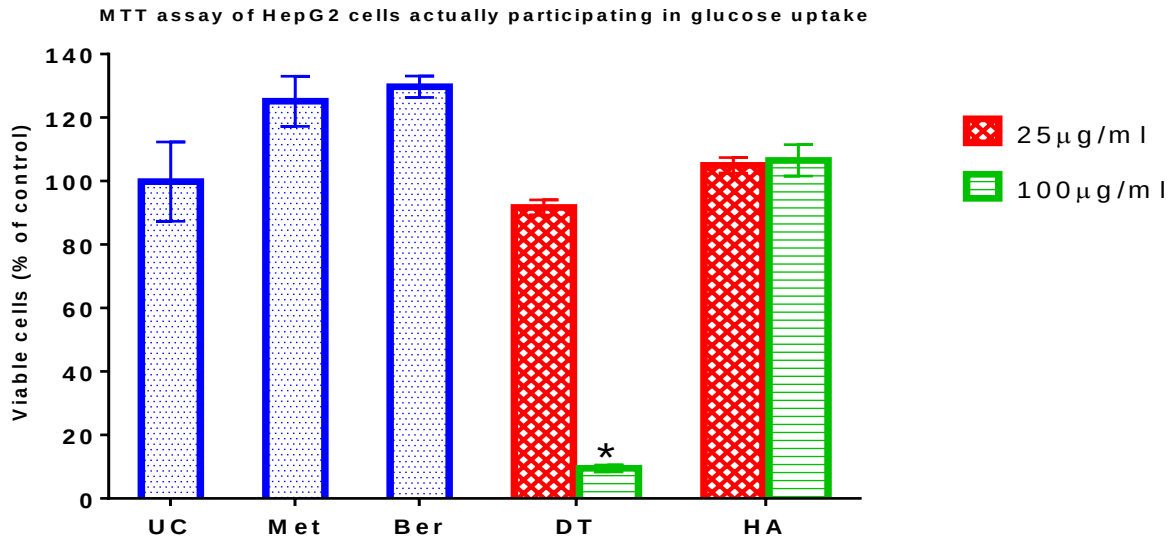

Figure 4.7: Toxicity (expressed as % of control ± standard deviation; n = 6) of the plant extracts to HepG2 cells used for glucose uptake assay. * Significant compared to untreated control (UC); DT = Dianthus thunbergii; HA = Hypoxis argentea; Met = Metformin; Ber = Berberine.

4.7.3 Glucose uptake in L6 myotubes

4.7.3.1 Cell culture conditions and treatment

L6 myoblasts were seeded into flat-bottom 96-well plates at a density of 3, 000 cells per well, maintained in Dulbecco's modified Eagle's Medium (DMEM) (Highveld Biological, South Africa) supplemented with 10% fetal bovine serum and incubated in a 5% CO_2 humidified atmosphere at 37ºC. They were allowed to adhere and grow to about 90% confluence, after which the culture medium was replaced with DMEM containing 2% fetal bovine serum. The culture was then left for an additional five days to allow differentiation into myotubes. Two cell-free rows were included to serve as blanks for the glucose utilization assay. Forty-eight hours prior to the glucose utilization assay, the culture medium was replaced and an aliquot (10 µl) of each plant extract (prepared at final concentrations of 12.5, 25 and 50 µg/ml) and the positive control, insulin (1 µM) were added to the respective wells (5 replicates of each treatment). The cells were incubated in the presence of the extracts or insulin for a further 48 hours. The spent medium was removed and replaced with 25 µl incubation buffer containing 8 mM glucose (RPMI 1640 medium diluted with PBS containing 0.1% BSA) and this was incubated for 3 hours at 37ºC. 10 µl of the medium from each well was transferred into a new 96-well plate and the glucose concentration was determined using a glucose oxidase assay as described earlier. Absorbance was measured at 510 nm using a Biotek® Power Wave XS spectrophotometer and glucose utilization was calculated as the difference between the cell-free and cell-containing wells. The percentage of glucose uptake was calculated in relation to the untreated controls, and MTT assay was performed on the cells in the original plate as previously described.

4.7.3.2 Results and discussion

The results obtained in the in vitro assay of glucose uptake in L6 myotubes for the plant extracts at 12.5, 25 and 50 µg/ml indicated that both plants showed some potential in lowering blood glucose levels. At the concentrations tested, *D. thunbergii* produced 112.01%, 114.31% and 118.39%, while *H. argentea* showed 111.31%, 116.20% and 116.96% with increasing concentrations of the plant extracts (Fig. 4.8). When compared to the untreated control (taken as 100% glucose uptake), these extracts have shown potential to stimulate glucose uptake in these cells. Insulin (1 µM) used as a positive control, produced better stimulation with 133.32% glucose uptake. This study has not evaluated the potential for synergism of the plant extracts in combination with insulin in increasing glucose utilization in muscle cells. However, it is reasonable to suggest that the extracts could exert their hypoglycemic activities by mechanisms that mimic insulin-mediated signaling, possibly via activation of the PI3K, PKB/Akt activity pathway, leading to increased translocation of GLUT 4 molecules to the plasma membrane (Van Dam *et al.*, 2005).

Interestingly, none of the plant extracts exhibited any potential for toxicity to L6 myotubes at the concentrations tested (Fig. 4.9), suggesting that the degree of glucose uptake exhibited by these plants might actually be a true reflection of their hypoglycemic potential. This lack of toxicity to L6 myotubes, coupled with their potential for stimulation of glucose uptake, may offer a basis for further investigation of these plants in relation to their stimulation of glucose utilization, especially in insulin-responsive tissues.

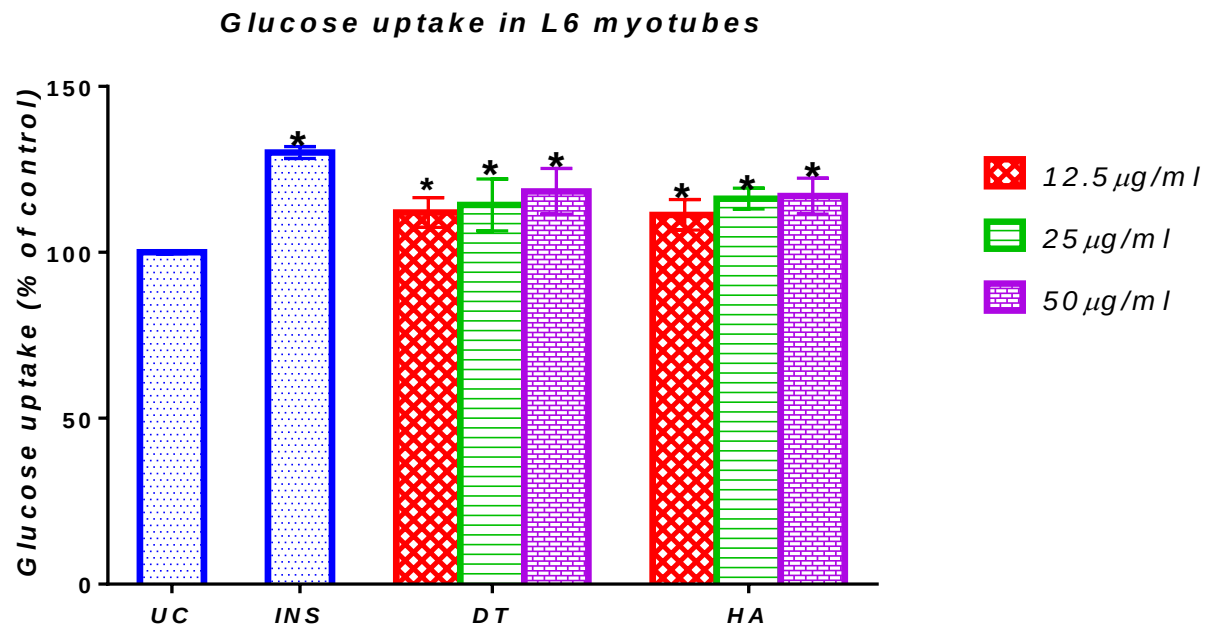

*Figure 4.8: Glucose uptake in L6 myotubes (expressed as % of control ± standard deviation; n=6) of the aqueous extracts of Dianthus thunbergii (DT) roots and Hypoxis argentea (HA) corms. INS = insulin. * Significant compared to untreated control (UC).*

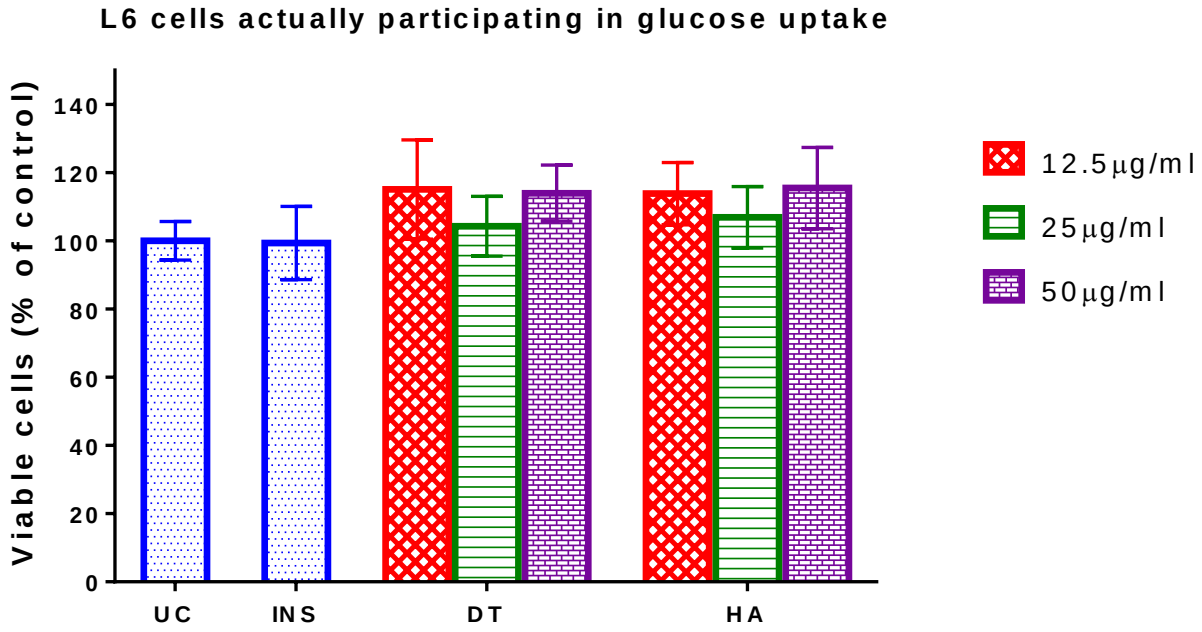

Figure 4.9: Toxicity (expressed as % of control ± standard deviation; n = 6) of the plant extracts to L6 myotubes used for glucose uptake assay. UC = untreated control; INS = insulin; DT = Dianthus thunbergii; HA = Hypoxis argentea

4.8 Inhibition of Nitric oxide production in RAW 264.7 macrophages

4.8.1 Principle

The ability of the extracts to inhibit the production of nitric oxide in a cellular medium (murine macrophage RAW 264.7 cell line) was employed to give an indication of their antioxidant and/or anti-inflammatory activities. Chronic diabetes is usually associated with adipose tissue inflammation, characterized by increased proliferation and recruitment of macrophages to the inflammatory site. Nitric oxide overproduction from activated macrophages may cause injury to tissues, including pancreatic islet cells via inflammatory mechanisms (Miller and Grisham, 1995). This assay employs the stimulation of RAW macrophages by lipopolysaccharides (LPSs) which are components of the cell walls of gram-negative bacteria. LPSs stimulation of NO production occurs via by an induction of inducible nitric oxide synthase (iNOS) transcription in activated cells (Guzik *et al.*, 2003). Plant extracts that inhibit iNOS and NO bioavailability are currently considered to be promising in the treatment of inflammatory diseases.

4.8.2 Cell culture conditions and treatment

Raw 264.7 macrophages were seeded in 96-well plates at a density of 50,000 cells per well in complete medium (RPMI 1640 containing 10% fetal bovine serum) and were allowed to attach overnight. The plates were incubated at 37ºC in a humidified incubator and 5% CO_2. On the following day, all spent culture medium was removed by aspiration. Twice the desired concentrations of the aqueous extracts (12.5, 25, 50 and 100 µg/ml of *D. thunbergii* and *H. argentea* were prepared in fresh medium. 50 µl of these concentrations were added to respective wells in the micro-titer plates. Aminoguanidine was used as a positive control. Thereafter, 50 µl of LPS containing medium (100 ng/ml) was added to all the wells except those of the blank, thus bringing the concentrations of the extracts to the desired concentrations of 12.5, 25, 50 and 100

µg/ml. Simultaneously, another set of cells was treated with the extracts and aminoguanidine, but without LPS. The plates were initially incubated for about 20 hours, after which 50 µl of medium was removed from each well and transferred to a new microplate. This was followed by the addition of 50 µl of Griess reagent, (1:1 mixture, v/v of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid), to the new microplates. The new plates were incubated at room temperature for 15 minutes and the absorbance was read at 510 nm). MTT assay was performed on the remaining cells to normalize cell density, whereby 100 µl of MTT solution was added to obtain a final concentration of 0.5 mg/ml in each well of the original plates. The cells were then returned to the incubator for about 20 minutes. Spent culture medium was removed and the formazan crystals were solubilized using 100 µl DMSO. The absorbance was read at 540 nm.

Nitric oxide production was measured in terms of nitrate production using a sodium nitrate standard curve. The slope of the curve was used to generate nitrate content according to the formula:

$$\textit{Nitrate content} = \frac{\textit{Absorbance of sample/standard} - \textit{Absorbance of blank}}{\textit{Slope of standard curve}}$$

4.8.3 Results and discussion

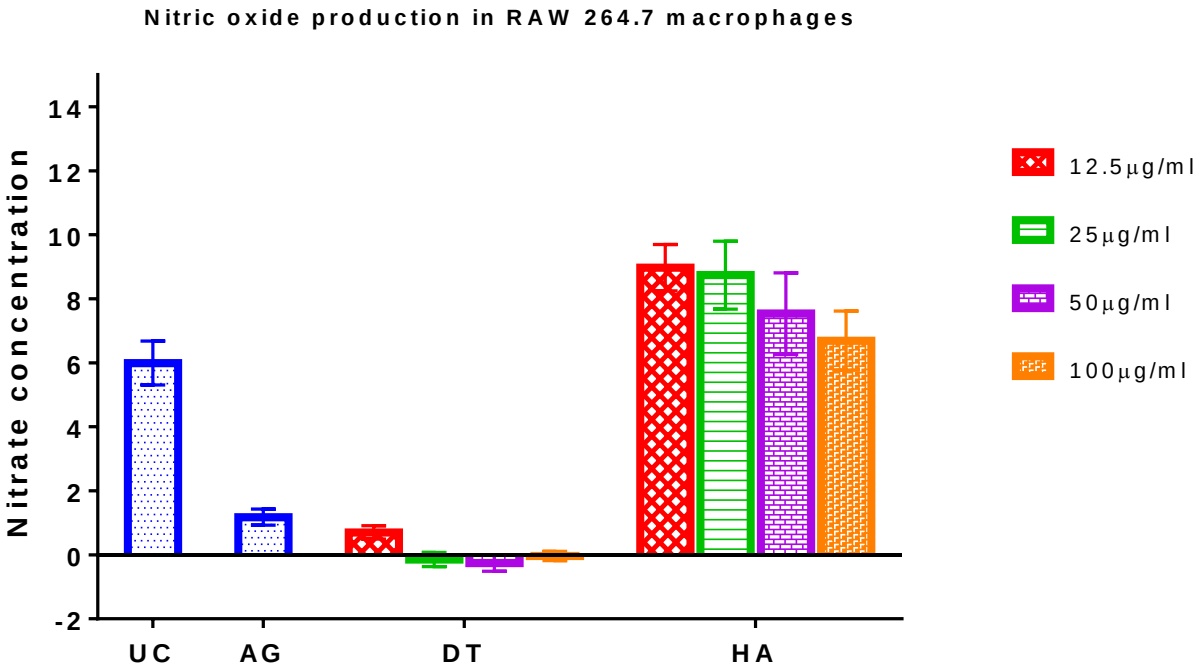

Figure 4.10: Effects of aqueous extracts of D. thunbergii and H. argentea on nitric oxide production in RAW macrophages. UC = Untreated Control (Blank); AG = Aminoguanidine; DT = Dianthus thunbergii aqueous extract; HA = Hypoxis argentea aqueous extract.

The effects of the extracts of *D. thunbergii* and *H. argentea* on nitric oxide production in LPS-stimulated macrophages are presented in Figure 4.10. Aminoguanidine used as a positive control in the assay produced significant reduction ($p < 0.05$) in nitrate content compared to the untreated blanks. Significant loss of cells in the wells treated with *D. thunbergii* extract (indicated by MTT cell viability assay) resulted in a complete lack of nitrate production with increasing concentrations of the extract. On the other hand, cells treated with *H. argentea* exhibited concentration-dependent reduction in nitric oxide production, although the values at the highest concentration tested were not significantly different from that of the blanks. The *Hypoxis* extract may therefore be able to produce significant inhibition of NO production at higher concentrations, as no significant toxicity was observed in the cells.

4.9 INS-1 Glucose metabolism as target for improved insulin secretion

4.9.1 Background

Pancreatic β cells do not express any appreciable levels of lactate dehydrogenase, ensuring that pyruvate is prevented from shunting away from the mitochondrial metabolic pathways. Therefore over 90% of glucose reaching the β cell is channeled into mitochondrial metabolism, which produces the majority of cellular reducing equivalents NADH and $FADH_{2,}$ as well as up to 98% of β-cell ATP. β cell ATP is the main effector for insulin secretion. It acts by displacing bound ADP on K^+ channels, leading to their closure, membrane depolarization with subsequent opening of Ca^{2+} channels, influx of Ca^{2+} into the cytosol and insulin exocytosis (Fu *et al.,* 2013). MTT is reduced to a purple precipitate in the presence of reducing equivalents and can thus be used as a rapid test to measure the effect of plant extracts on β cell glucose metabolism (Janjic and Wollheim, 1992).

4.9.2 Cell culture

INS-1 cells were seeded at a density of 30,000 cells per well in 96-well plates for 3 days. The media was then removed and the plates were pre-incubated with PBS and incubated at 37ºC for about 4 hours to allow the cells to use up the glucose already present in them. Plant extracts prepared at a concentration of 100 µg / ml in growth medium, with and without glucose, were then added to the respective wells. MTT (0.5 mg/ml) was added to the wells and the plates were incubated for 2 hours at 37ºC. At the end of incubation, the medium was aspirated and 200 µl of DMSO was added to solubilize the formazan crystals formed. The absorbance of the wells was read at 540 nm with a multi-plate reader (BioTek® PowerWave XS spectrophotometer).

4.9.3 Results and Discussion

The assay presented in this section was performed as an indirect measurement of the ability to modify β cell function (insulin secretion) by modulating its glucose consumption when INS-cells are exposed to extracellular glucose in the presence of the plant extracts. The results presented in Fig. 4.11 indicate that the presence of extracellular glucose in cultures (irrespective of the presence of plant extracts) ordinarily promoted glucose utilization and, thereby, an increase in cell viability, indicated by increased formazan production, compared to cells without extracellular glucose (Fig. 4.11).

However, the change in absorbance when INS-1 cells were incubated with the plant extracts in the presence of glucose did not show any significant increase compared to the control. This suggests that the plant extracts did not provide any stimulation of glucose metabolism above the level observed in control cultures incubated with glucose. As noted earlier, stimulation of improved glucose metabolism leading to enhanced ATP production in pancreatic β cells could lead to increased secretion of insulin (Fu *et al.*, 2013). On the evidence provided by the present results, it is unlikely that the plant extracts exert any stimulatory effect on the activity of enzymes involved in glucose metabolism.

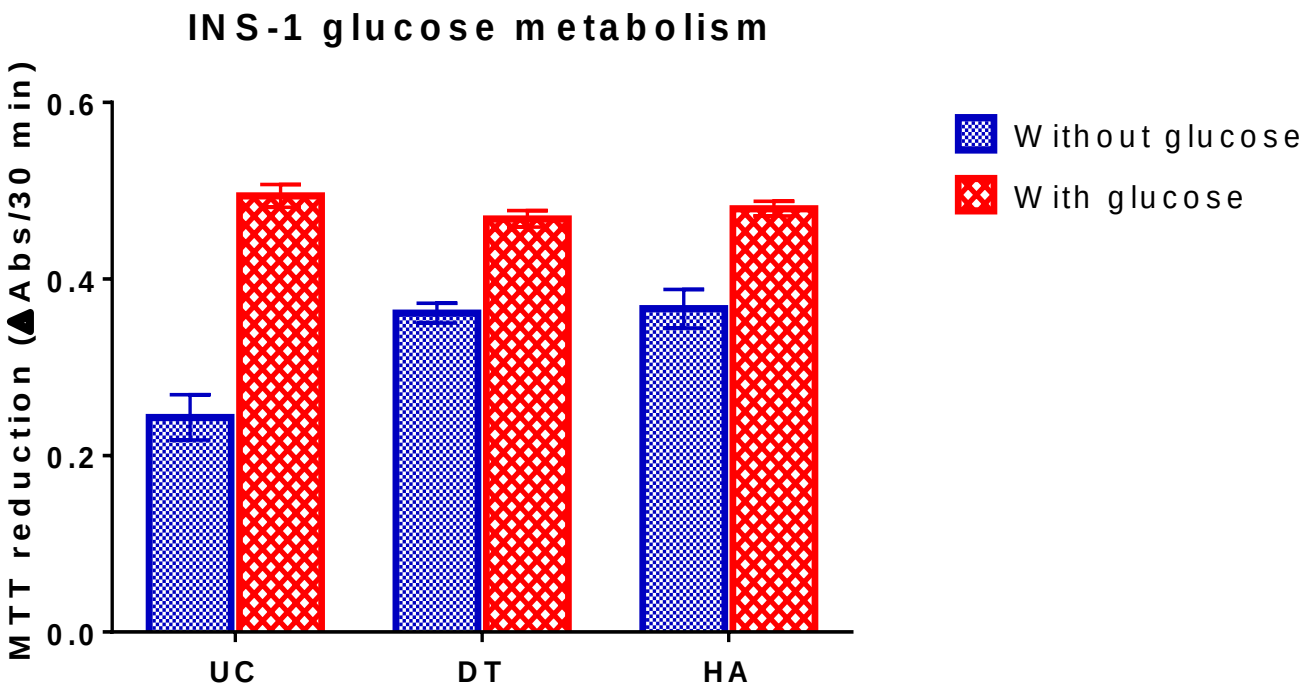

Figure 4.11: Effect of D. thunbergii (DT) and H. argentea (HA) on glucose metabolism indicated by MTT reduction in INS-1 cells. UC = Untreated control.

4.10 3T3-L1 pre-adipocyte triglyceride accumulation

4.10.1 Background

The adipose tissue plays two crucial roles in contributing to normal insulin sensitivity and glucose homeostasis:

i. As an endocrine organ, the adipose tissue secretes adipokines, e.g., adiponectin, leptin, resistin, which affects whole body metabolism and controls feeding behaviors (Ahima *et al.*, 2000; Berg *et al.*, 2002)

ii. As a storage organ, the adipose tissue possesses a unique capacity to sequester large quantities of lipids as triglycerides in adipocytes (Schaffer, 2002).

The latter function ensures that non-adipose tissues are protected from the ectopic deleterious effects of circulating free fatty acids (FFAs). Elevated levels of circulating FFAs and deposition of fatty acids into non-adipose fat stores, (e.g., muscle and liver), have been strongly associated with insulin resistance in peripheral tissues in humans and animals (Unger 1995; 2002). Indeed, elevated levels of triglycerides in peripheral tissues produce lipotoxicity and contribute to the development of obesity-induced Type 2 diabetes mellitus.

Major approaches to preventing lipotoxicity and insulin resistance in peripheral tissues involve the reduction of serum FFA levels and diversion of excess lipid away from these non-adipose tissues, by increasing fatty acid uptake and triglyceride accumulation in the adipose tissue. Drugs such as Rosiglitazone and Troglitazone prevent diabetes by decreasing the ectopic deposition of lipids while increasing adipose tissue triglyceride accumulation (Higa *et al.*, 1999; Zhou *et al.*, 2000). Promotion of triglyceride accumulation in adipocytes usually requires differentiation of pre-adipocytes into insulin-sensitive small adipocytes.

Adipocyte differentiation has been extensively studied *in vitro* using pre-adipocyte cell lines including 3T3-L1 and 3T3-F442A cells (Rosen and Speigelman, 2000). The process involves increments in adipocyte number and size from undifferentiated fibroblast-like pre-adipocytes and is highly regulated by a cascade of gene expression involving two key families of transcription factors: the CCAAT/enhancer-binding proteins (C/EBPα, β and δ) and the peroxisome proliferator-activated receptor γ (PPARγ) (Kim and Choung, 2016). Differentiation of pre-adipocytes in *in vitro* cultures can be induced after growth arrest has been initiated in proliferating pre-adipocytes. On induction of differentiation, the levels of C/EBPβ rapidly increase, causing growth-arrested pre-adipocytes to synchronously re-enter the cell cycle and undergo approximately two rounds of cell division (mitotic clonal expansion). C/EBPβ then regulates the expression of C/EBPα and PPARγ, which together co-ordinates the dephosphorylation of the retinoblastoma protein with subsequent growth arrest and the cessation of mitotic clonal expansion. In addition, these transcription factors also promote the sustained expression of adipocyte-specific genes required for the formation of triglyceride droplets, the main phenotypic feature of mature adipocytes (Rizzati *et al.*, 2013).

Triglyceride accumulation in differentiated adipocytes is directly proportional to the extent of differentiation. This principle is utilized in assays that make use of lipid soluble dyes such as Oil red O stains, which is used to quantify triglycerides. The optical density increases linearly with triglyceride content in 3T3-adipocytes (Ramirez-Zacarias *et al.*, 1997).

4.10.2 Cell culture and treatments

Triglyceride accumulation in 3T3-L1 pre-adipocytes was studied according to the method of Ramirez-Zacarias *et al* (1997), with slight modifications. 3T3-L1 pre-adipocytes (Highveld Biological, South Africa) were seeded into 48-well culture plates at a density of 6, 000 cells and

allowed to grow to 100% confluence. Differentiation was induced in two-day post-confluent cells by treatment with plant extracts (prepared at 12.5, 25 and 50 µg/ml) or the positive control (Rosiglitazone; 1 µM). The cells were then cultured for an additional 10 days in normal culture medium (DMEM supplemented with 10% FBS) and the medium was replaced every 2–3 days.

On the tenth day, the spent culture medium was removed and the cells were washed gently with PBS. The cells were fixed by adding 500 µl per well of 10% formaldehyde in PBS. Fixing was allowed for 1 hour at room temperature. The fix solution was then aspirated and the cells were stained by adding 200 µl of pre-warmed Oil Red O (prepared initially as a stock solution of 0.5% w/v in isopropanol. A working solution was prepared by adding 4 ml of distilled water to 6 ml of the stock solution. Note that the Oil Red working solution is prepared fresh as it is not stable). Staining was allowed for 15 minutes at 37ºC. The plates were then washed extensively with water to remove excess dye. The plates were then dried in an oven at 37ºC. Triglyceride accumulation was quantified by extracting Oil red O stain with isopropanol (250 µl per well and 200 µl was transferred to a 96-well plate) and the absorbance was measured at 520 nm. Excess isopropanol was removed, the plates were washed with water and dried at 37ºC and were then stained with Crystal Violet as previously described to normalize cell density.

4.10.3 Results and discussion

As shown in Fig. 4.12, the positive control, Rosiglitazone, showed the most intense reddish staining, indicating cytoplasmic lipid droplets in the adipocytes. Staining patterns in cultures treated with either *D. thunbergii* or *H. argentea* were much fainter compared to those treated with Rosiglitazone, although the staining intensity obtained with the plant extracts was higher than in the control wells.

The quantification of triglyceride accumulation is presented in Fig. 4.13. *D. thunbergii* increased lipid accumulation in a concentration-dependent manner in 3T3-L1 cells by 19.03%, 20.71% and 23.32%, respectively at 12.5, 25 and 50 µg/ml, above control values (taken as 100%). *H. argentea* on the other hand, exerted non-appreciable increases, with values at 100.10%, 100.17% and 100.44% at 12.5, 25 and 50 µg/ml, respectively. Thus, *H. argentea*, in this study, possessed neither pro-adipogenic nor anti-adipogenic effects, as its addition to 3T3-L1 culture did not alter triglyceride accumulation at any of the concentrations tested. Rosiglitazone produced a 44.63% increase in triglyceride accumulation above the control.

The increase in triglyceride accumulation obtained with *D. thunbergii* extract suggests the presence of an insulin-like property in the extract. Similar effects have been demonstrated with other plants (Alonso-Castro and Salazar-Olizo, 2008). Insulin promotes triglyceride accumulation via a variety of mechanisms including stimulation of differentiation of pre-adipocytes to adipocytes, stimulation of glucose transport and triglyceride synthesis (via upregulation of lipogenic enzymes such as acetyl Co A carboxylase and fatty acid synthase) and inhibition of lipolysis in mature adipocytes, as well as stimulating the activity of lipoprotein lipase in adipose tissue (Kersten, 2001).

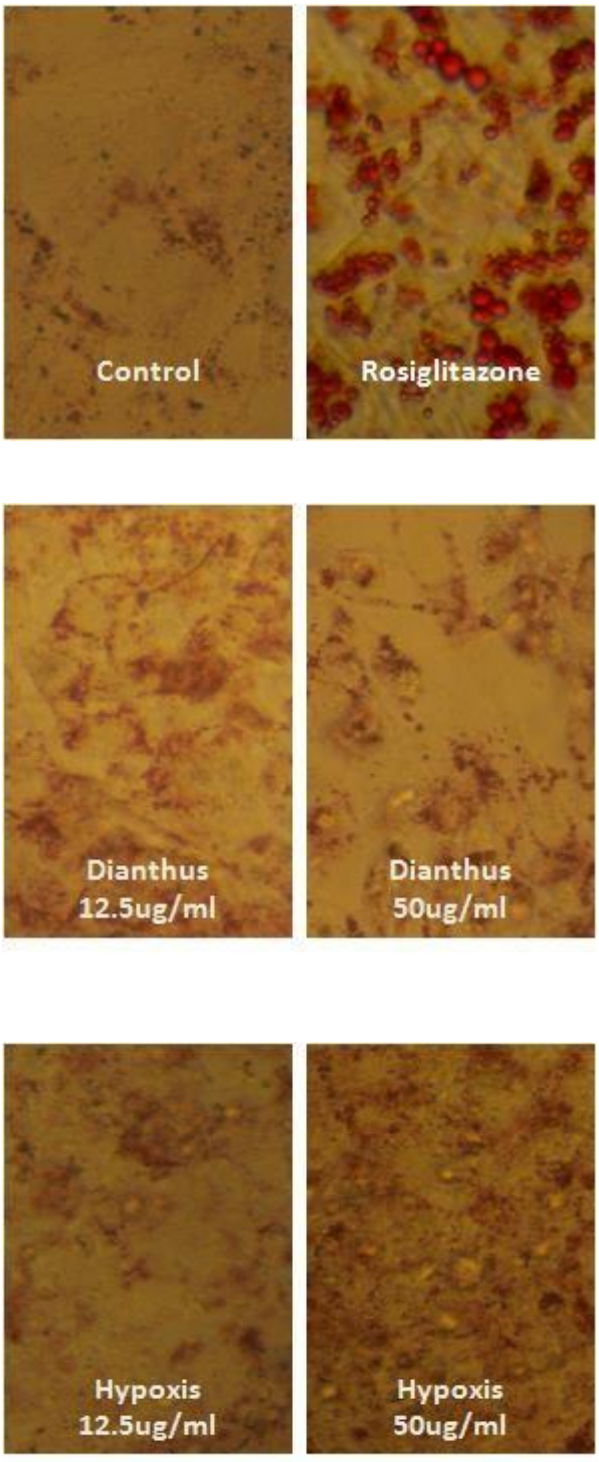

Figure 4.12: Effect of the aqueous extracts of D. thunbergii and H. argentea on 3T3-L1 pre-adipocyte differentiation. Plates show the appearance of Oil Red O stained cells after 10 days of treatment with the plant extracts (12.5 and 50 µg/ml) or Rosiglitazone (1 µM).

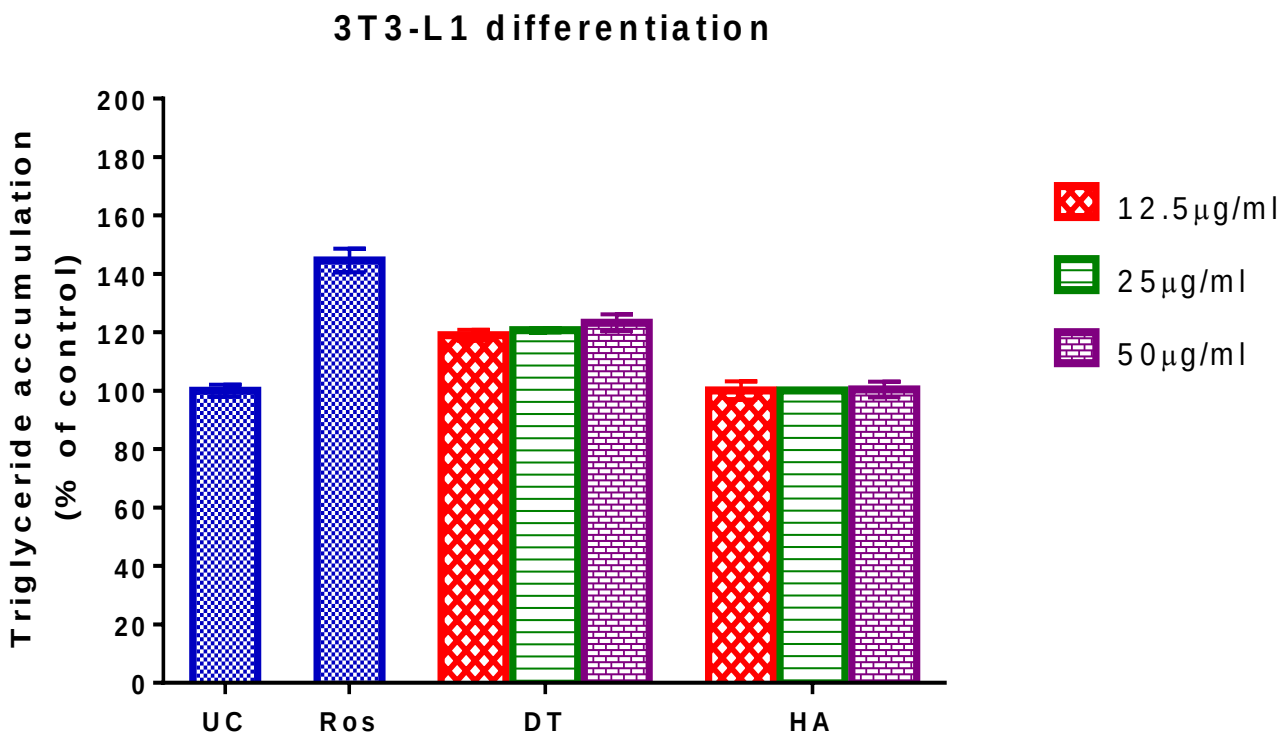

Figure 4.13: Quantitation of the effect of D. thunbergii (DT) and H. argentea (HA) on 3T3-L1 pre-adipocyte triglyceride accumulation. Values are expressed as mean ±standard deviation of four replicates corrected to cell density. UC = Untreated control; Ros = Rosiglitazone.

Increased adipocyte differentiation may be due to mechanisms involving an upregulation of adipogenic transcription factors, including PPARγ and C/EBP proteins, and vice versa (Yang *et al*., 2012; Kim *et al*., 2015). Thiazolidinediones such as Rosiglitazone can bind to PPARγ to mediate the differentiation of pre-adipocytes to adipocytes, thereby increasing the number of small adipocytes, which are known to possess higher numbers of glucose transporters, insulin receptors and a higher capacity to take up fatty acids to facilitate storage of lipids (de Souza *et al*., 2001; Boden *et al*., 2003). Although the diversion of triglycerides from ectopic tissues into the adipose tissue is desirable to prevent insulin resistance, excessive triglyceride accumulation in adipose tissue may lead to adipose hypertrophy, which might also present as a risk factor for

the development of Type 2 diabetes (Weyer *et al.*, 2000; Lonn *et al*., 2010). As a result, recent conclusions by some studies suggest that the ideal antidiabetic agent is one that has neither pro-adipogenic, nor anti-adipogenic effects (Alonso-Castro and Salazar-Olivo, 2008).

4.11 Concluding remarks

The results of the studies presented in this chapter have provided very useful insights into the anti-diabetic potentials of *Dianthus thunbergii* and *Hypoxis argentea,* as well as possible mechanisms of their actions. The aqueous extract of *Hypoxis argentea* demonstrated promising concentration-dependent hypoglycemic potential via stimulation of glucose uptake in the liver carcinoma cell line (HepG2) and skeletal muscle (L6 myotubes), combined with moderate alpha-glucosidase inhibition, as well as a tendency to reduce nitric oxide production in RAW macrophages. Its lack of production of any significant alterations in lipid accumulation in 3T3-L1 pre-adipocytes is also a positive consideration for its antidiabetic potential. In addition, the extract was non-toxic to all the cell lines it was exposed to, suggesting the possibility for better efficacy at higher doses. *Dianthus thunbergii* exhibited some degree of stimulation of glucose uptake in L6 myotubes alone, although this effect was overshadowed by its potential for toxicity in other cell lines employed in these studies. Its tendency to promote triglyceride accumulation may also be undesirable in ectopic tissues such as liver and muscle.

Generally, the inhibition of enzymes involved in glucose homeostasis and diabetic complications does not appear to be a useful mechanism of action with these plants, as they both exhibited very weak inhibitory activities against most of the enzymes investigated in this study. Plants contain many compounds that may be potentially hypoglycemic or cytotoxic, depending on their composition and abundance in different extracts. An investigation of the chemical

composition of the aqueous extracts of *D. thunbergii* and *H. argentea* may help to identify compounds that were responsible for the predominant effects observed with each plant.

Although some studies have reported *in-vivo* anti-diabetic actions for some members of the *Dianthus sp.* and *Hypoxis sp.* (Mohammed and Ojewole, 2003, Ojewole, 2006; Oguntibeju *et al*., 2016*),* very little information was found in literature, which demonstrated the *in-vitro* anti-hyperglycemic effects of plant species from these two genera. The studies conducted here represent the first comprehensive attempts, by *in-vitro* techniques, at investigating the potential anti-diabetic mechanism(s) of actions of *Dianthus thunbergii* and *Hypoxis argentea*, towards validation of their acclaimed traditional use for the management of diabetes mellitus. The aqueous extracts of these plants have been chosen as focus for these studies as water is reportedly the most common solvent used traditionally for their preparations for medicinal use. It is hoped that future investigations will include the use of other solvents in further exploring the anti-diabetic potentials of these plants.

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CHAPTER 5

High-content analysis for induction of cellular proliferation in rat INS-1insulinoma cells by Dianthus thunbergii and Hypoxis argentea extracts

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Results from this chapter are part of data being being submitted for publication in the Journal of Ethnopharmacology

Chapter 5

HIGH-CONTENT ANALYSIS FOR INDUCTION OF CELLULAR PROLIFERATION IN RAT INS-1INSULINOMA CELLS BY *DIANTHUS THUNBERGII* AND *HYPOXIS ARGENTEA* EXTRACTS

5.1 Background

Pancreatic β cell mass undergoes constant renewal achieved by a balance between mechanisms that promote expansion (neogenesis and cell replication) and those causing its involution (apoptosis and/or necrosis) (Figure 5.1). Studies have shown that some degree of increase in β cell mass occurs throughout life (Bonner-Weir, 2000, 2001), although the turnover of β cells declines rapidly following the first few years after birth and, in adults, is usually very slow (Meier *et al*., 2008).

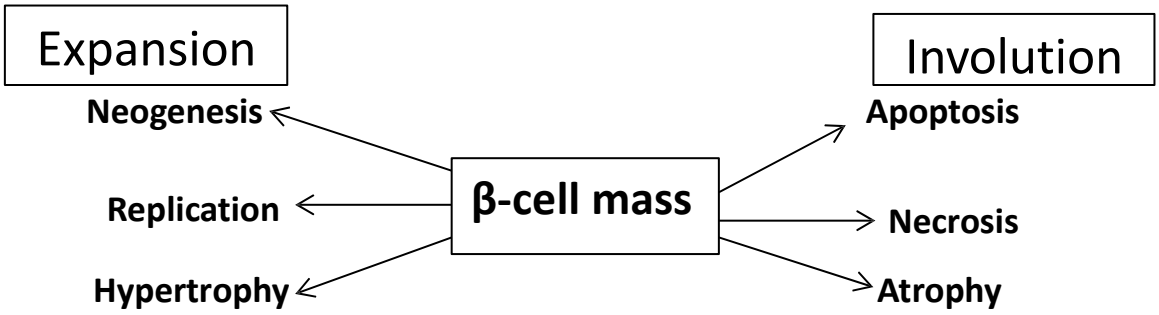

Figure 5.1: Processes controlling the expansion or involution of β cell mass.

Expansion of β cell mass during embryogenesis occurs by means of a population of progenitor cells, which are believed to arise from pancreas-derived stem cells, bone marrow and trans-differentiation of pancreatic precursors (Bonner-Weir and Sharma, 2002). β cell numbers at birth are determined by the self-renewing capacity of these progenitor cells, as well as β-cell differentiation. The regulation of β-cell growth and numbers after birth is then determined by the proliferation of existing β cells (Kassem *et al.*, 2000). The replication of existing β cells as with

other somatic cells, is controlled by a network of finely-tuned cell cycle regulators (cyclins, cyclin-dependent kinases, CDKs, and CDK inhibitors) and is organized into a series of events including growth, DNA synthesis and mitosis phases. β cell replication is, however, reflective of the stage of life of the individual (Heit et al., 2006; Lee and Nielsen, 2009). In adults, there is an increased expression of cell-cycle inhibitors, such as p16, which correlates with the reduction in β-cell replication observed as an individual ages (Kohler *et al.*, 2011). The exit of cells from the cell cycle may occur due to growth arrest. Mitotic stimulation can induce re-entry into the cell cycle in response to metabolic demands, such as during insulin resistance of pregnancy, diabetes or obesity (Georgia and Bhushan, 2004).

Apoptosis refers to programmed cell death that normally serves as a mechanism for tissue remodeling, cell replacement and the removal of infected, damaged or mutated cells from tissues, which regulates cell mass (Lupi and Del Prato, 2008). Physiologically, apoptosis provides a complementary, but opposite role to mitosis and cell proliferation in the regulation of cell population. Pathological forms of apoptosis are caused by abnormalities in cell cycle regulation that either result in suppression of apoptosis (giving rise to over-proliferation of cells such as in cancer), or excessive apoptosis as observed in conditions such as auto-immune diseases and neurodegenerative diseases. The development of diabetes has been linked to excessive apoptosis in pancreatic β cells (Chandra *et al.*, 2001; Butler *et al.*, 2003).

Currently, strategies towards the development of antidiabetic therapy are leaning toward preserving and/or expanding β cell mass either by stimulating increased cell proliferation or decreasing apoptosis in β cells (Vetere *et al.*, 2014). These efforts are based on the understanding that β cell mass declines during the development of diabetes. Decreasing β cell apoptosis will result in the enhancement of their viability and increase in insulin production. Stimuli that

increase β cell apoptosis in Type 2 diabetes include inflammatory cytokines, (e.g., TNF-α, INF-γ and Interleukin-1β), high levels of glucose (glucotoxicity) and free fatty acids (lipotoxicity). These factors induce ER stress, reactive oxygen species production and subsequent oxidative stress (Eizirik *et al.*, 2008; Wang *et al.*, 2005). B cells are particularly vulnerable to oxidative stress, due to their poor intracellular antioxidant capacity (Sharma *et al.*, 2009). Hence, drugs and phytochemicals that ameliorate oxidative stress and enhance tissue antioxidant capacity are believed to prevent lesions to the pancreatic islet cells via their antioxidant effects (Hosseini *et al.*, 2015).

Although the expansion of β-cell mass in adults is slow, strategies that can produce β-cell regeneration (recovery of lost or damaged cells by increasing proliferation of insulin-producing cells) after loss due to Type 1 or Type 2 diabetes, are believed to be effective treatment approaches (Tourell *et al*., 2002). Several flavonoids, (e.g., quercetin, catechin, caffeic acid) are believed to modulate β cell proliferation via their effects in diminishing oxidative stress in β cells (Lapidot *et al.*, 2002; Pinent *et al*., 2008). Other natural products such as genistein have been shown to induce β-cell proliferation and insulin secretion by activation of protein kinase A and extracellular signal regulated kinase (Ohno et al., 1993; Liu *et al.*, 2006).

The studies in this chapter were focused on live cell imaging techniques to evaluate the effects of the extracts of *Dianthus thunbergii* and *Hypoxis argentea* on cell proliferation in INS-1 cells.

5.2 Materials and methods

In this study, the percentages of live and dead cells were analyzed using DNA-fluorescent dyes including Hoechst 33342 for total cell counts and Propidium iodide for dead cell counts. Cell imaging studies were performed using ImageXpress® Micro XLS widefield high-content

analysis system (Fig. 5.2). This is a widefield automated microscope capable of fluorescent, transmitted light and phase-contrast imaging and high-content analysis of fixed- or live-cell assays, tissues and small organisms. The features of the system are presented in Table 5.1.

Fig. 5.2: ImageXpress® Micro XLS system with Transmitted Light option installed

Table 5.1: Features and configurations of ImageXpress® Micro XLS system

Features	Configurations
Camera	4.66 megapixel scientific sCMOS camera with large field-of-view.
Objectives	Nikon 10x Plan Fluor, 10x Plan Fluor DL (phase contrast), 20x Super Plan Fluor ELWD ADM cc 0-2 mm) phase contrast, 40x Super Plan Fluor ELWD ADM cc 0-2mm (phase contrast)
Light Source	Solid state light source, 380 nm (DAPI) – 680 nm (cy5) 3-log dynamic range
Filter cubes	DAPI, GFP, FITC, YFP, TRITC, Cy3, Texas Red, Cy5 etc.

	Colourimetric: Red, Green Blue
Sample compatibility	Slides, multi-well plates, glass or plastic
Analysis software	MetaXpress software
Analysis modules	Cell cycle, Multi-wavelength cell scoring, Translocation, neurite outgrowth, Micronuclei, etc.
Environment control	Temperature (ambient to 40ºC), CO2 (5-10%), humidity
Phase contrast transmitted light	Nikon 100W Pillar Diascopic Illuminator with TE-C ELWD condenser; PhL, Ph1 and Ph2 selectable rings

5.2.1 Cell culture

Mouse pancreatic insulinomas (INS-1) cells were obtained from the laboratory of the Medicinal Plant and Natural Product Research Group, Nelson Mandela Metropolitan University, South Africa). They were routinely maintained in RPMI1640 medium supplemented with 10% fetal bovine serum in the presence of penicillin-streptomycin. They were seeded at a density of 8 000 cell/well in 96-well micro-titer plates with 100 µl of the medium. Aqueous extracts of *D. thunbergii* and *H. argentea* prepared in DMSO (0.25%, v/v) were added to the wells already containing complete medium to reach concentrations of 12.5, 25 and 50 µg/ml of the extracts. The plates were incubated for about 48 hours at 37ºC in a humidified incubator and 5% CO_2. GABA (100 µM) was used as positive control, while DMSO (0.25%, v/v) served as the vehicle (untreated) control.

5.2.2 Staining protocol: Hoechst 33342 and Propidium iodide staining

Hoechst stock solution was prepared as a 10 mg/ml solution in distilled water. The stock solution was further diluted 1:2 000 in PBS. All medium was removed from the cultured cells and replaced with 100 µl of the Hoechst solution. The plates were incubated for about 20 minutes at room temperature away from light using an aluminum foil paper. Propidium iodide (PI) (50 µg/ml) was then added shortly before acquiring the images. Note that PI should be added very close to acquiring images as prolonged contact of the dye with the cells may eventually cause its entry into live cells.

5.2.3 Image acquisition and data analysis

Images were taken with a Molecular Devices ImageXpress Micro XLS microscope using the blue and red filters, as well as phase contrast with 40X objective. Nine fields were acquired per well and cells were scored by defined dimensions, analyzed by the MetaXpress software. Statistical analysis was carried out using Graph Pad prism Version 5.01 and the test of significance was done using Student's t-test (two-tailed). Replicate values for each treatment was compared with replicate values of the control wells. Level of significance ranged from $p<0.001$ to $p<0.05$.

5.3 Results and Discussion

Stimulation of cell proliferation is desirable in conditions that require tissue regeneration, repair, as well as in aging. Herbs that can promote cellular proliferation to reverse tissue damage, rather than the abnormal cell proliferation occurring during cancer progression, are currently considered beneficial in preserving pancreatic β-cell mass. The present study assessed the possibility of proliferation in INS-1 cells following exposure to extracts of *D. thunbergii* and *H. argentea*. Images acquired after Hoecsht 33342 and propidium iodide staining are presented in Figure 5.3.

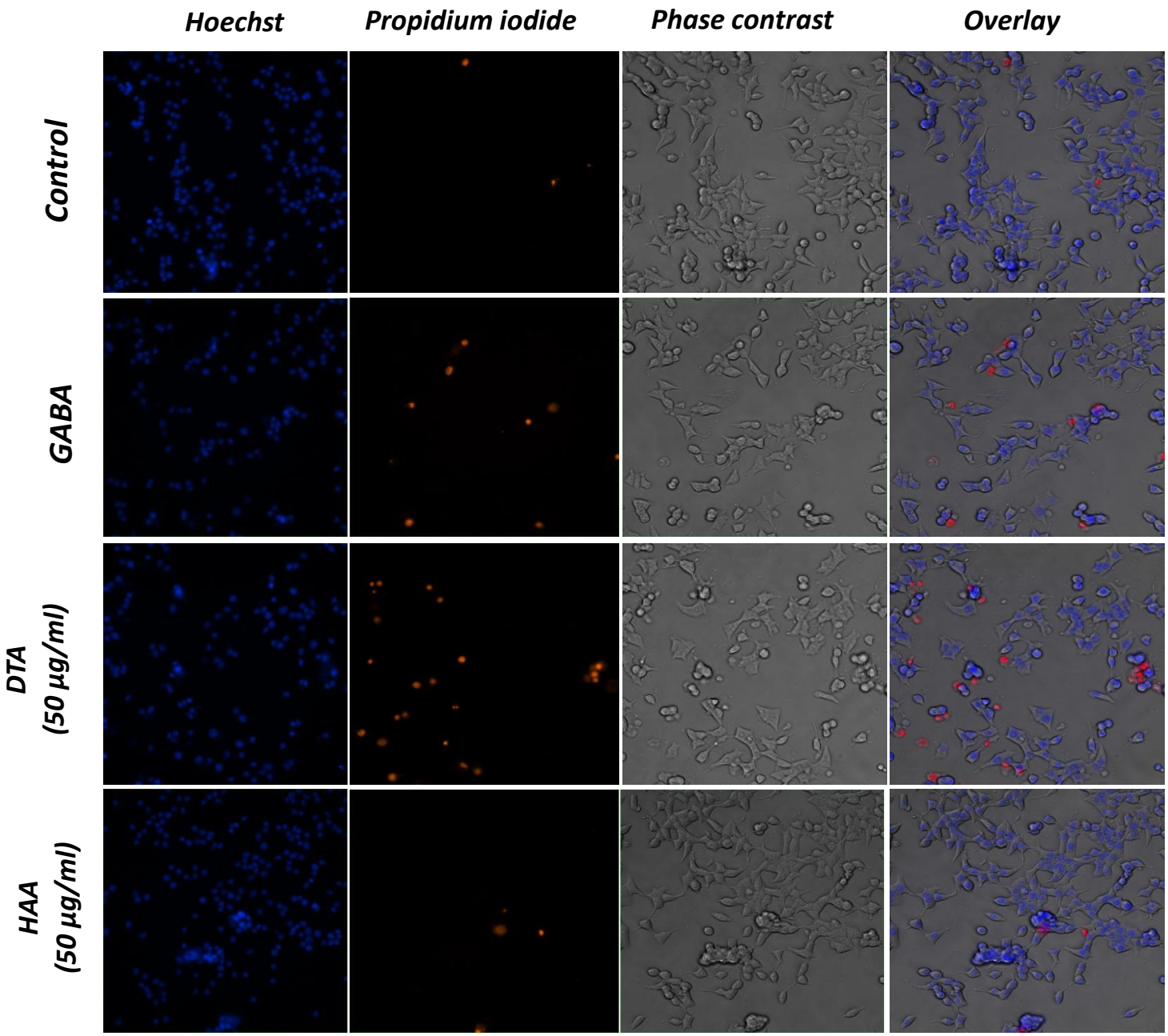

Figure5.3: Hoechst (blue) and Propidium iodide (red) staining of INS-1cells following 48 hours exposure to aqueous extracts of Dianthus thunbergii (50 µg/ml) and Hypoxis argentea (50 µg/ml) and the positive control (GABA; 100 µM). Blue staining indicates live cells; red staining indicates dead cells.

Hoechst® 33342 nucleic acid stain is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA, while Propidium iodide (PI) is a popular red-fluorescent nuclear and chromosome counterstain. As propidium iodide is not permeant to live

cells, it is also commonly used to detect dead cells in a population. PI binds to DNA by intercalating between the bases with little or no sequence preference.

H. argentea produced stimulation of INS-1 cell proliferation as shown in Figure 5.4. After 48 hours, all the concentrations of *H. argentea* tested (12.5, 25 and 50 µg/ml) caused significant increases ($p<0.01$) in total cell numbers to 112.6 ± 2.3%, 115.5 ± 4.3% and 113.1 ± 2.5%, respectively, relative to the untreated control (100%). These results were similar to those of the positive control, GABA, which showed increased proliferation to 116.2 ± 6.2%, relative to the control. Stimulation of β-cell proliferation may be a result of the direct effects of the constituents of the extract on the cells (Schmidt *et al.*, 2013). Several molecular pathways underlying such proliferative action of herbal extracts as observed in this study are thought to eventually lead to the promotion of cell cycle progression via increased expression of cyclins and CDK4 proteins, which play important roles in the regulation of cell cycle progression (Jin *et al.*, 2003).

In contrast to the effect obtained with *H. argentea, D. thunbergii* produced significant growth inhibition ($p<0.01$) with concentration-dependent reduction in total INS-1 cell numbers compared to the control, producing 65.5 ± 2.1%, 51.7 ± 4.2 and 35.3 ± 2.9% at 12.5, 25 and 50 µg/ml, respectively. These results further affirm the observations made in previous chapters with MTT cytotoxicity in HepG2 cells and Crystal Violet assay in INS-1 cells, and suggest that *D. thunbergii*, aside from any potential for anti-diabetic activities, also contains compounds with significant anti-proliferative properties.

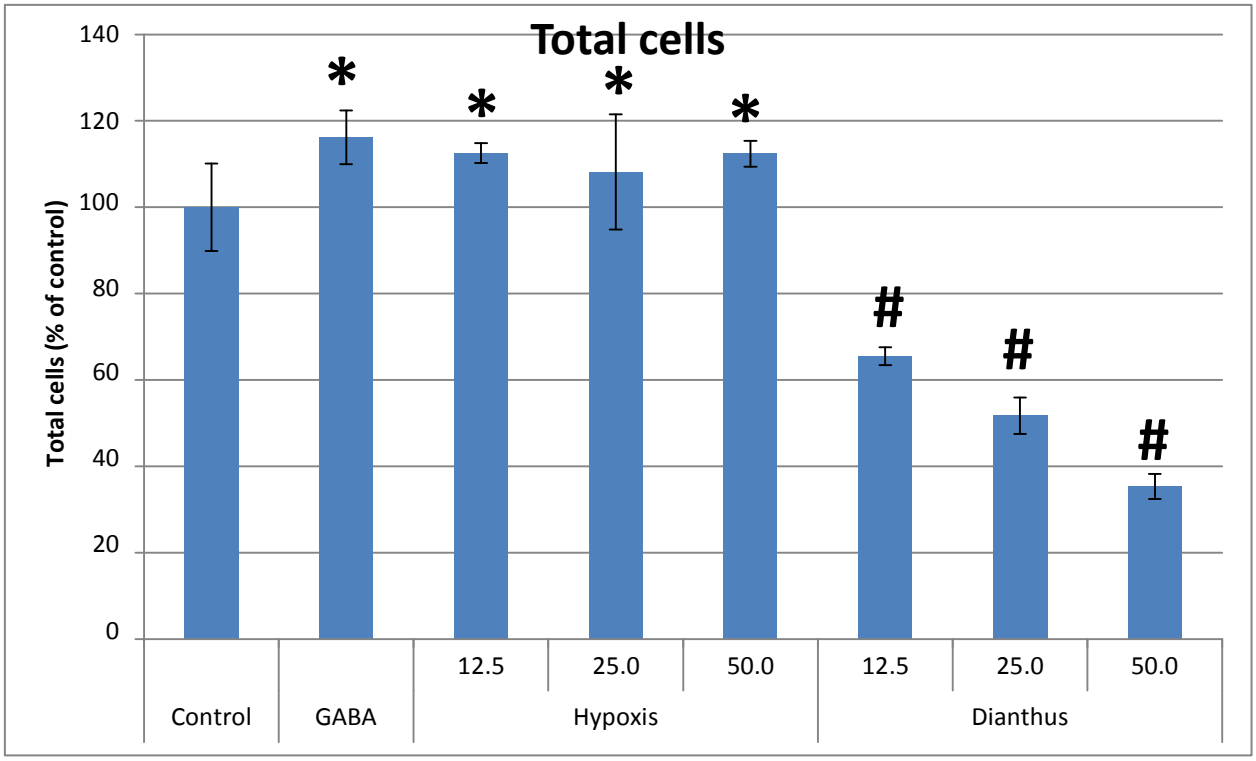

Fig. 5.4: Effects of Dianthus thunbergii and Hypoxis argentea on total INS-1 cell numbers after 48 hours exposure. Values are expressed as percentage of control (mean ± standard deviation; n =8). * Significant increase (p<0.01) compared with control; #Significant decrease (p<0.001) compared with control.

The observed effects of the plant extracts on total cell numbers were also corroborated by the percentage of dead cells stained with propidium iodide (Fig. 5.3). The percentage of dead cells obtained with exposure to *H. argentea* were 0.47, 0.41 and 0.36 at 12.5, 25 and 50 µg/ml, respectively, which were significantly lower ($p<0.05$) than that of control (0.70). On the other hand, *D. thunbergii* produced a higher number of dead cells than the control, which was significant ($p<0.05$) at 50 µg/ml (fig 5.5). The results indicate that the active constituents in *D. thunbergii* which cause cytotoxicity are soluble in water.

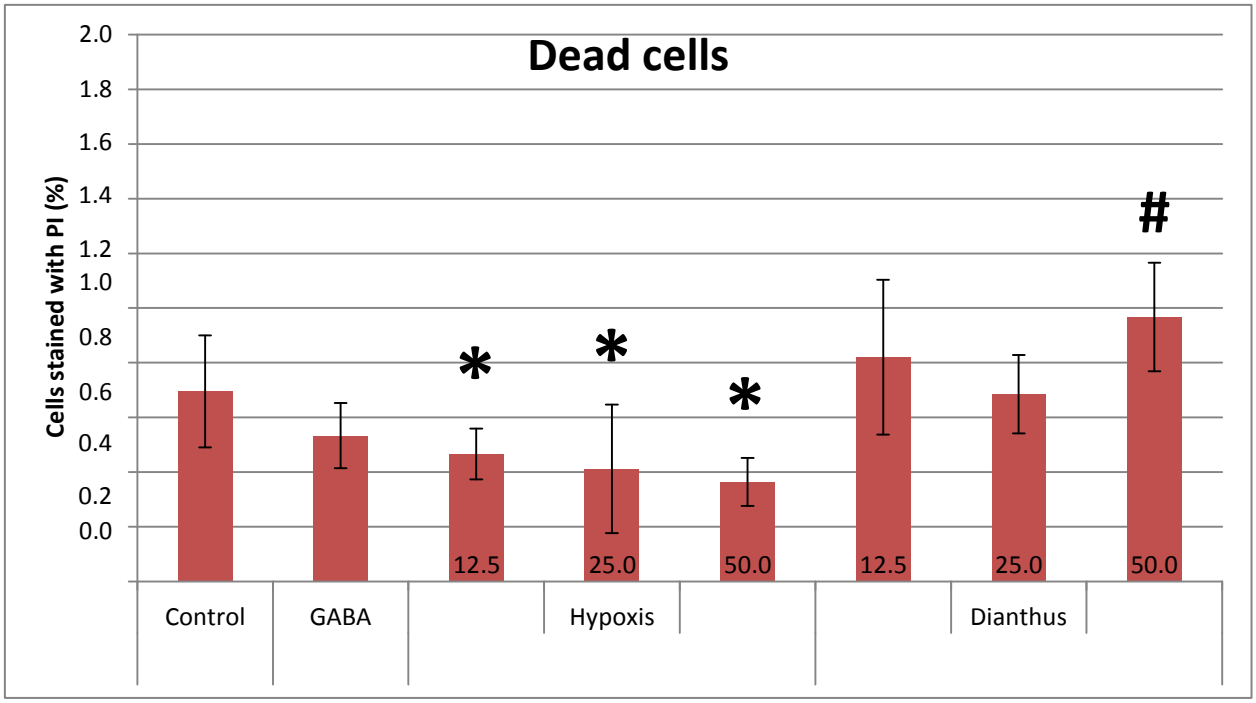

Figure 5.5: Percentage of dead INS-1cells (cells stained with Propidium iodide) after 48 hours exposure to extracts of Dianthus thunbergii and Hypoxis argentea. Values are expressed as percentage of control (mean ± standard deviation; n =8). * Significant decrease (p<0.05) compared with control; #Significant increase (p<0.001) compared with control.

5.4 Conclusions

The results presented in this chapter further corroborate the previous observations of cytotoxicity obtained with aqueous extracts of *D. thunbergii*, highlighting the possibility of risks associated with its use in anti-diabetic therapy. The consistent growth-inhibitory properties of this extract may, however, find application for controlling cancer progression, via reduction in cell proliferation. *H. argentea* has further shown potential to not only be useful in preserving cell populations, but also displays an ability to stimulate β-cell proliferation, a property that could contribute greatly to its acclaimed anti-diabetic use. Future *in vivo* studies are necessary to further evaluate the observed in vitro effects in this study. Metabolomic analysis of the extracts

of these two plants is necessary to identify the bioactive compounds responsible for the observed effects.

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CHAPTER 6

Chemical Profiling of Compounds in the Essential Oils of Dianthus thunbergii and Hypoxis argentea by Gas Chromatography-Mass Spectrometry (GC-MS)

This chapter will be submitted for publication in the Journal of Essential Oil Research

CHEMICAL PROFILING OF COMPOUNDS IN THE ESSENTIAL OILS OF DIANTHUS THUNBERGII AND HYPOXIS ARGENTEA BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

6.1. Background

Essential oils (also called volatile oils because of their tendency to evaporate when exposed to air) are natural complex mixtures of lipophilic compounds, characterized by strong characteristic odours and/or flavors. They are usually stored in different plant parts (roots, stem, leaves, flowers and fruits) in special brittle secretory structures, such as glands, secretory hairs, secretory ducts, secretory cavities or resin ducts (Ahmadi *et al.*, 2002; Bezić *et al.*, 2009). The chemical composition of essential oils (quantitative and qualitative) depends on various factors including geo-climatic location and growing conditions (soil type, climate, altitude and amount of water available), genetic composition, season (for example before or after flowering), time of day when harvesting, condition of plant materials and extraction technique.

Several methods used to extract essential oils from plant parts include hydro-distillation, steam and steam/water distillation, solvent extraction, aqueous infusion, cold or hot pressing, effleurage, supercritical fluid extraction and the phytonic process (Sangwan *et al.*, 2001; Andrade *et al.*, 2011). The majority of compounds in essential oils include hydrocarbons described as ‘terpenes’ and their derivatives called ‘terpenoids’. Both terminologies are now often used interchangeably. Other categories of compounds include fatty acid esters, alcohols, aldehydes and ketones. Terpenoids refer to a group of volatile compounds constructed from two or more isoprenoid units (5-carbon building blocks) and include hydrocarbons (terpenes), with the general formula $(C_5H_8)_n$ and their oxygenated, hydrogenated and dehydrogenated derivatives (Yadav *et al.*, 2014). Based on the value of n, terpenoids can be classified into different groups

as shown in Table 6.1. Many terpenoids have similar molecular formulas, while some show stereoisomerism, and this sometimes make their chemical characterization difficult.

Table 6.1: Classes of Terpenoids

Class of terpenoid	Value of n	Chemical formula
Hemiterpenoids	1	C_5H_8
Monoterpenoids	2	$C_{10}H_{16}$
Sesquiterpenoids	3	$C_{15}H_{24}$
Diterpenoids	4	$C_{20}H_{32}$
Sesterpenoids	5	$C_{25}H_{40}$
Triterpenoids	6	$C_{30}H_{48}$
Tetraterpenoids	8	$C_{40}H_{64}$
Polyterpenoids	>8	$(C_5H_8)_n$

Gas chromatography-mass spectrometry (GC-MS) has developed as the most suitable technique to investigate the components of the complex mixture of volatile compounds in essential oils, as well as their abundance in the oil. GC-MS allows for accurate identification and characterization of volatile compounds even with close structural similarities. Pre-isolation analysis (metabolite profiling) of essential oils is usually carried out by GC-MS, which combines chromatographic and spectral methods (Xue *et al.,* 2015). The chromatography component produces pure or nearly pure fractions of chemical components in a mixture. Spectroscopy produces selective information for identification using standards or library spectra for both quantitative and qualitative analysis of unknown compounds in complex natural product extracts or fractions. Profiling of secondary metabolites in volatile oils by GC-MS provides information on the diversity of chemical constituents and detection of chemically diverse bioactive molecules

and unknown compounds, as well as assessing the possibility of isolation of the major compounds detected (Fiehn *et al.*, 2000; Kopka *et al.*, 2004; Schielmann *et al.*, 2008).

Identification of compounds is achieved by comparing retention indices and mass spectra data with those of certified standard substances in reference libraries or databases. This mode of identification is regarded as tentative, rather than confirmatory, unless the chromatographic and spectroscopic parameters coincide unequivocally with those of a certified standard substance (Kopka et al., 2004). It must be stated, however, that complete separation of compounds with GC-MS is as yet unattainable, as different compounds may have similar retention indices, leading to chromatographic co-elution. The coupling of GC with other techniques such as Fourier transform infra-red (FTIR) and nuclear magnetic resonance (NMR) spectroscopy have been adopted more recently to maximize compound separation (Gomes Da Silva, *et al.*, 2008).

6.1.1 Principle and instrumentation of GC-MS

Gas chromatography is primarily designed for the separation of thermally-stable volatile components of a sample. The separation technique involves the partitioning of the components of the mixture between two phases: a stationary phase and a mobile gas phase. The sample is converted into vapor by heating and is then carried by the mobile phase gas through a column in which the components are separated according to differences in their relative vapour pressure and their relative affinities for the stationary phase (Strehmel *et al.*, 2008). Non-volatile substances to be analyzed by GC-MS normally undergo initial processing by means of chemical reactions, (e.g., Silylation, acylation, esterification or alkylation), generally described as derivatization reactions. These procedures serve to increase the volatility of non-volatile samples, reduce thermal degradation and incorporate functional groups such as hydroxyl, sulfhydryl and carboxyl groups, which lead to higher detection signals (Wang *et al.*, 2001).

The basic instrumentation of a GC-MS system depicted in Figure 6.1 is made up of the following essential components:

1. ***Sample injection system:*** The sample may be injected manually by means of a calibrated micro-syringe or with the aid of a mechanical device mounted on top of the gas chromatograph. Samples are injected as split or split-less injections in the range of a few microliters into a vapourization chamber, which is heated to vaporize the sample and then mixed with a carrier gas that serves as the mobile phase for the chromatographic separation. The carrier gas must be dry and chemically inert (Xue *et al.*, 2015). Helium is the most commonly used carrier gas as it is considered safe and compatible with a wide range of detectors.

2. Chromatographic column: Columns used in gas chromatography are either packed or open-tubular (capillary) columns, although most applications tend to employ capillary columns. In GC, columns as the stationary phase generally determine the separation selectivity as the mobile phase is normally chemically inert and does not react with the sample. Therefore, the most effective way to alter the sensitivity of analysis is to alter the column properties. For instance, the length of the column determines its overall efficiency, as longer columns lead to increased peak efficiency, although longer columns will also increase the analysis time.

GC columns function within thermostated oven environments, providing the opportunity to control the temperature of the column during the separation process. The operations can be performed either in isothermal or temperature-varying programming modes. In the former, the temperature is held constant throughout the entire separation process, while the latter involves increasing the temperature of the column continuously or in steps from a low temperature as the separation progresses. Isothermal programming is ideal for samples with

narrow boiling point range, while variation of temperature is adopted for separating mixtures with a broad boiling point range (Xue *et al.*, 2015).

3. Detection systems: Detection devices are located at the end of the column, which sense the physico-chemical property of the analytes and provide quantitative measurement of the components of the mixture being analyzed, as they elute in combination with the carrier gas in the form of an electronic signal to produce a chromatogram. The mass spectrometer has become the standard, most powerful detector system for gas chromatographic separations, allowing for lower detection limits. The sample components are passed through the inlet of the mass spectrometer where they are ionized and fragmented, typically by electron bombardment from an ion source. The impact of electrons on the molecules eluted causes them to lose electrons through electrostatic repulsion. The ions are then passed into a mass analyzer where they are sorted according to their mass-to-charge (m/z) ratio. The mass spectrometer scans the masses of eluted compounds continuously throughout the separation and the eluted compounds are recorded in order of their retention times. The retention time of a compound refers to the time that elapses between injection of the sample into the chromatographic system to the recording of the peak corresponding to the particular compound in the chromatogram (Sneddon *et al.*, 2007).

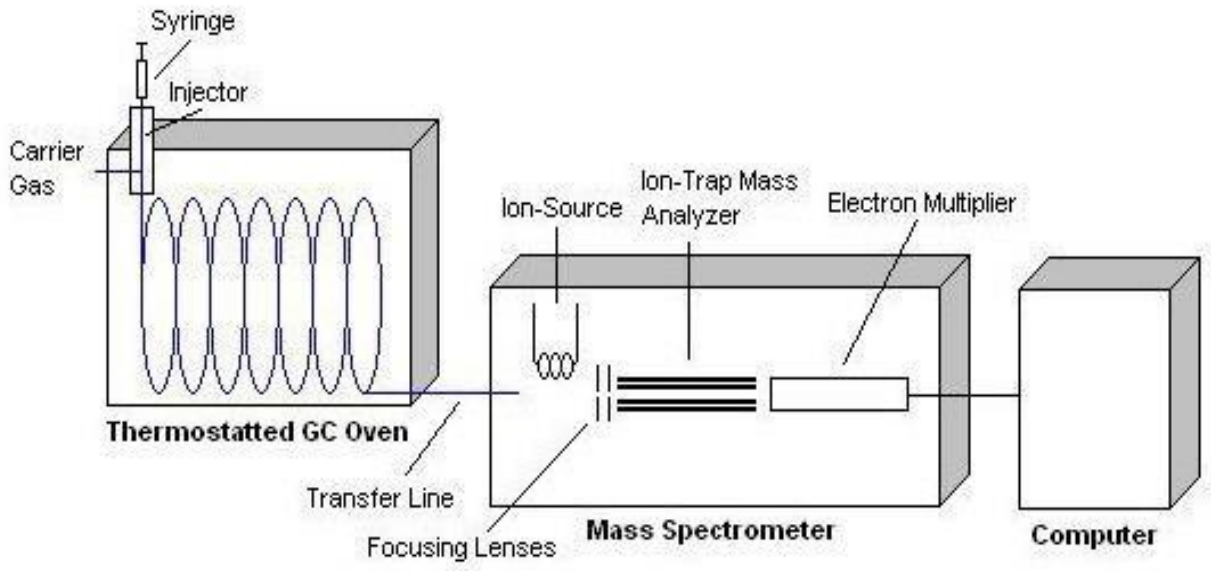


Figure 6.1: Schematic representation of GC-MS instrumentation (adapted from: http://hem.libretexts.org)

6.2 Materials and Methods

6.2.1 Extraction of volatile oils

In this study, a non-targeted analysis of the essential oils of *Dianthus thunbergii* roots and *Hypoxis argentea* corms was performed using GC-MS. The oils were extracted from the fresh and dried roots of *Dianthus thunbergii*, and the fresh and dry corms of *Hypoxis argentea*, respectively. The roots of *D. thunbergii* and corms of *H. argentea* were separated from the rest of the harvested whole plant and then washed with clean tap water to remove soil residues. Fresh plant parts were immediately separated for hydro-distillation, while the remaining portions were dried in an oven to constant weight at 40°C for 24 hours prior to distillation.

About 100 g each of the plant materials were separately subjected to hydro-distillation using a Clevenger-type apparatus fitted with a condenser and connected to a heat-resistant 5-L round bottom flask. The plant materials were heated in boiling water in the flask for about 3 hours, to produce a mixture of gases (oil vapor) which were conveyed with steam into the

condenser, where they were cooled to below 30°C. This produced two non-mixing liquid phases: a lower hydrosol portion and the upper layer consisting of the essential oil. The condensed liquids were gravity fed into a separation funnel, where they were separated. About 3 ml of the oils were obtained and were collected into small glass vials which were completely sealed before their analysis.

6.2.2 Gas chromatography-Mass spectrometry (GC-MS) analysis of volatile oils

The volatile oils were subjected to GC-MS analysis using an Agilent 7890 GC complex equipped with a Agilent 5977A Mass selective detector system at the Department of Botany, University of Fort Hare and a Zebron-5MS (cross-linked 5%- phenyl methyl polysiloxane) column (ZB-5MS 30 m X 0.25 mm X 0.25 µm). GC-grade helium was used as a carrier gas at a flow rate of 2 mL/min; splitless 1 µL injections were used. A needle with the samples was inserted directly into the inlet of the gas chromatograph. Injector temperature and ion source temperature were maintained at 280°C, while the initial oven temperature was 70°C. This was ramped at 15°C/min to 120°C and then ramped at 10°C/min to 180°C and finally ramped at 20°C/min to 270°C and held at this temperature for 3 min. The data obtained were gathered with ChemStation. Identification of the components of essential oils was accomplished by comparison of their retention times and mass spectra with those stored in the NIST11.L library. The chemical classes of the identified compounds were confirmed using the UC SanDiego Metabolomics Workbench database.

6.3 Results and discussion

In this study, attempted was made to compare the composition of volatile oils obtained from the fresh and dried roots of *D. thunbergii*, and the fresh and dried corms of *Hypoxis argentea*, using GC-MS. Our aim was to determine the effects of removal of moisture on the overall composition of the volatile oils from these plants.

For preservation purposes, the moisture levels of fresh herbs are usually lowered by various methods (Diaz-Maroto *et al.*, 2003). Drying is the most commonly applied method of preservation as it inhibits microbial growth and limits certain biochemical changes within the plant tissue (Hossain et al., 2010; Kubra and Rao, 2012). However, drying at very high temperatures can result in breakdown of bioactive compounds (Yi and Wetszein, 2011). Several studies have reported different drying methods (shade-drying, sun-drying and oven-drying), with different exposure temperatures which had significant effects on the yield and chemical composition of oils extracted from dried plant material (Raghavan *et al.*, 1997; Omidbaigi *et al.*, 2004; Sefidkon *et al.*, 2006).

The compounds identified in the volatile oils extracted from the fresh and dried roots of *D. thunbergii* are listed in Tables 6.2 and 6.3, respectively, while Figures 6.2 and 6.3 present a comparison of typical chromatograms of the volatile oils obtained from the same source. A total of 16 (99.96%) and 25 (99.60%) compounds were identified from the volatile oils of the fresh and dried roots of *D. thunbergii*, respectively. The bulk of the oil from the fresh roots was made up of terpenes (77.17% of total oil composition); these were mainly α-pinene (38.22%) and β-selinene (23.95%), while other terpenoids (including β-pinene, Pinocarveol, Verbenone, 3-keto-isosteviol, γ-Muurolene, Spathulenol and cis-2-Thujen-4-ol) made up another 15% of the total oil composition. Total terpenes and terpenoid content in the oil from the dried roots was reduced

considerably to 47.58%. As with the fresh roots, the most abundant terpenes were β-selinene (19.87%), α-pinene (12.82%) and Verbenone (9.85%). The following compounds were common to the volatile oils from both dried and fresh roots: α-pinene, cis-2-Thujen-4-ol, Verbenone, β-selinene. The fragrance of the flowers of *Dianthus sp.* has been associated previously with compounds such as eugenol, β-caryophyllene and benzoic acid derivatives (Zucker *et al.*, 2002). The present study, however, did not show the presence of these compounds in the essential oils from the roots of *Dianthus thunbergii.* This could indicate that these set of compounds may be restricted to the floral parts of the plant. The results presented here represent the first attempt to study the chemical composition of the oils from the roots of *D. thunbergii*, which is the part mostly utilized for medicinal purposes.

Other categories of compounds identified in the oils of *Dianthus thunbergii* roots included alkanes, aldehydes and fatty acid esters. The percentage of alkanes in the oils from the fresh roots decreased from 20.89% to 7.55%, following a similar trend as observed for terpenoids. Octadecane was identified as the alkane common to both oils. While aldehydes were barely detected in the oils from the fresh roots, an abundance of 8.91% was observed in the dried roots. Similarly, fatty acid esters identified in the oils from the fresh roots was 1.90% of the total oil composition, with the abundance increasing to14.78% in the dried roots.

Table 6.2: Chemical composition of the essential oils of the fresh roots of *Dianthus thunbergii*

Sr. No.	Compound name	Chemical formula	Rt(min)	Area (%)	Class of compound
1	1R-α-pinene	$C_{10}H_{16}$	4.007	38.22	Monoterpenoid
2	cis-2-Thujen-4-ol	$C_{10}H_{16}O$	4.177	2.14	Monoterpenoid
3	(-)-β-pinene	$C_{10}H_{16}$	4.372	1.99	Monoterpenoid
4	L-Pinocarveol	$C_{10}H_{16}O$	5.701	2.10	Monoterpenoid
5	Verbenone	$C_{10}H_{14}O$	6.240	3.41	Monoterpenoid
6	β-selinene	$C_{15}H_{24}$	8.723	23.95	Sesquiterpenoid
7	(-)-Spathulenol	$C_{15}H_{24}O$	8.783	1.87	Sesquiterpenoid
8	3-Keto-isosteviol	$C_{20}H_{28}O_4$	8.839	1.28	Diterpenoid
9	Gamma-Muurolene	$C_{15}H_{24}$	9.128	2.21	Sesquiterpenoid
10	1-isopropenyl-1-(tetrahydrofuran-2,5-dion-3-yl)-cyclopropane	N/A	10.315	0.98	Alkane
11	1-nonadecane	$C_{19}H_{40}$	11.650	3.62	Alkane
12	Octadecane	$C_{18}H_{38}$	11.982	3.98	Alkane
13	2,7-dimethyl oct-5-yn-7-en-4-yl ester, 2-ethyl butyric acid	N/A	12.089	1.90	Fatty acid ester
14	1-iodo-octadecane	$C_{18}H_{37}I$	12.367	1.79	Alkane
15	Eicosane	$C_{20}H_{42}$	12.501	8.61	Alkane
16	17-hexadecyl tetratriacontane	$C_{50}H_{102}$	13.399	1.91	Alkane
Total				**99.96%**	

Compounds are listed in order of retention time (Rt); N/A, not available in database.

Table 6.3: Chemical composition of the essential oils of the dried roots of *Dianthus thunbergii*

Sr. No.	Compound	Chemical formula	Rt (min)	Area (%)	Class of compound
1	(1R)-α-pinene	$C_{10}H_{16}$	4.007	12.82	Monoterpenoid
2	cis-2-Thujen-4-ol	$C_{10}H_{16}O$	4.180	2.02	Monoterpenoid
3	2-pentyl Furan	$C_9H_{14}O$	4.404	4.65	Cyclic aldehyde
4	(E)-2-octenal	$C_8H_{14}O$	4.943	1.57	Aldehyde
5	Resacetophenone	$C_8H_8O_3$	5.242	1.29	Phenolic ketone
6	N-methyl-1-octadecanamine	$C_{19}H_{41}N$	5.303	2.02	Amine
7	Cis-Verbenol	$C_{10}H_{16}O$	5.707	3.02	Monoterpenoid
8	3,6-Dimethoxy-2,5-dinitrobenzonitrile	$C_9H_7N_3O_6$	5.873	1.57	Benzenoid
9	5-Benzylidene-3-(4-methoxybenzoyl) rhodanine	$C_{18}H_{13}NO_3S_2$	5.995	1.40	Thiazolidines
10	Verbenone	$C_{10}H_{14}O$	6.240	9.85	Monoterpenoid
11	3-(4-methoxy phenylimino)-1-phenyl-propen-1-ol	$C_{16}H_{15}NO_2$	6.876	2.91	N/A
12	(E,E)-2,4-Decadienal	$C_{10}H_{16}O$	6.924	2.69	Aldehyde
13	β-selinene	$C_{15}H_{24}$	8.217	19.87	Sesquiterpenoid
14	4-Hydroxyphenylacetic acid, ethyl ester	$C_{10}H_{12}O_3$	8.783	1.74	Fatty acid ester
15	N-(3-acetylphenyl)benzene ethanamid	N/A	8.839	4.53	N/A
16	7α-diacetoxy-2-methylene-4b-methyl perhydro-1-phenanthrenepropanenitrile	N/A	9.227	2.13	N/A
17	DL-Cystine	$C_6H_{12}N_2O_4S_2$	10.082	1.23	Amino acid
18	7-methylene-bicyclo[3.3.1] nonan-3-one oxime		10.316	1.74	Imine
19	3-Methoxy-2,4,5-trifluorobenzoic acid, nonadecyl ester	$C_{27}H_{43}F_3O_3$	10.498	0.90	Fatty acid ester
20	1-Bromoeicosane	$C_{20}H_{41}Br$	11.309	1.62	Alkane
21	6,10-dimethyl-2-undecanone	$C_{13}H_{26}O$	11.355	1.96	Ketone
22	Isobutyric acid, tridecyl ester	$C_{17}H_{34}O_2$	11.791	2.85	Fatty acid ester
23	Octadecane	$C_{18}H_{38}$	12.193	5.93	Alkane
24	3-(acetoxymethyl)biphenylene-2-carboxylic acid methyl ester	$C_{17}H_{14}O_4$	12.360	4.09	Fatty acid ester
25	1,2,4-Benzenetricarboxylic acid, 4-dodecyl dimethyl ester	$C_{23}H_{34}O_6$	14.766	5.20	Fatty acid ester
Total				**99.60%**	

Compounds are listed in order of retention time (Rt); N/A, not available in database.

The results described above indicate a two-fold effect of the drying process on the composition of the volatile oils. On the one hand, drying of the roots tends to reduce the content of some categories of compounds such as terpenes (e.g. 1R-α-pinene and β-selinene) and alkanes in the oils. On the other hand, moisture removal has resulted in the appearance of certain groups of compounds (fatty acid esters and aldehydes), probably as a result of an increase in their concentrations due to loss of water. These results are similar to those obtained by Alavi *et al.* (2010) who reported that drying of plants at temperatures up to 180°C resulted in a marked loss of terpenes, including α-pinene, β-pinene and sabinene, as well as an increase in some other compounds. The temperature-dependent nature of the stability of most terpenoids, coupled with the affinity of some terpenoids for the water fraction of plant parts enhances their loss with water during the drying process (Hamrouni-Sellami *et al.*, 2012; Rahimmalek and Goli, 2013). Rahimalek and Goli (2013) also noted the presence of essential oil components in dried plant samples that were not present in the essential oils of fresh samples.

Terpenes and their oxygenated derivatives, terpenoids, have a wide range of reported biological activities and therapeutic applications against malaria, cancer and inflammatory diseases. Limonene, one of the most abundant monoterpenes is well known for its chemopreventive properties against tumour cells (Crowell, 1999; Kris-Etherton *et al.*, 2002) while artemisinin is a sesquiterpene lactone containing an endoperoxide bridge that confers an antimalarial activity on the compound (Haynes, 2001; Abdin *et al.*, 2003).

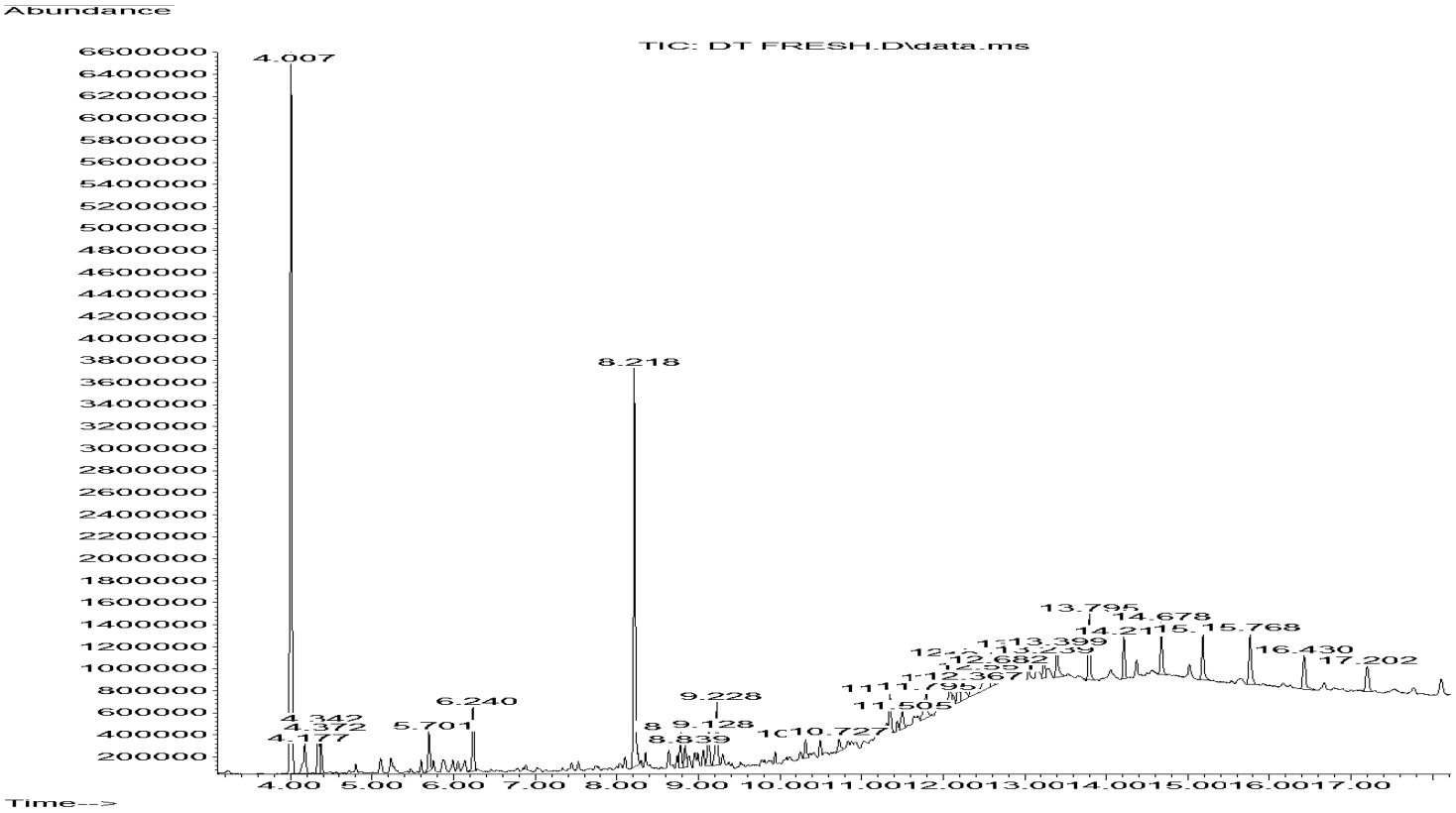


Figure 6.2: GC-MS total-ion chromatograms of the volatile oils from fresh roots of *D. thunbergii*

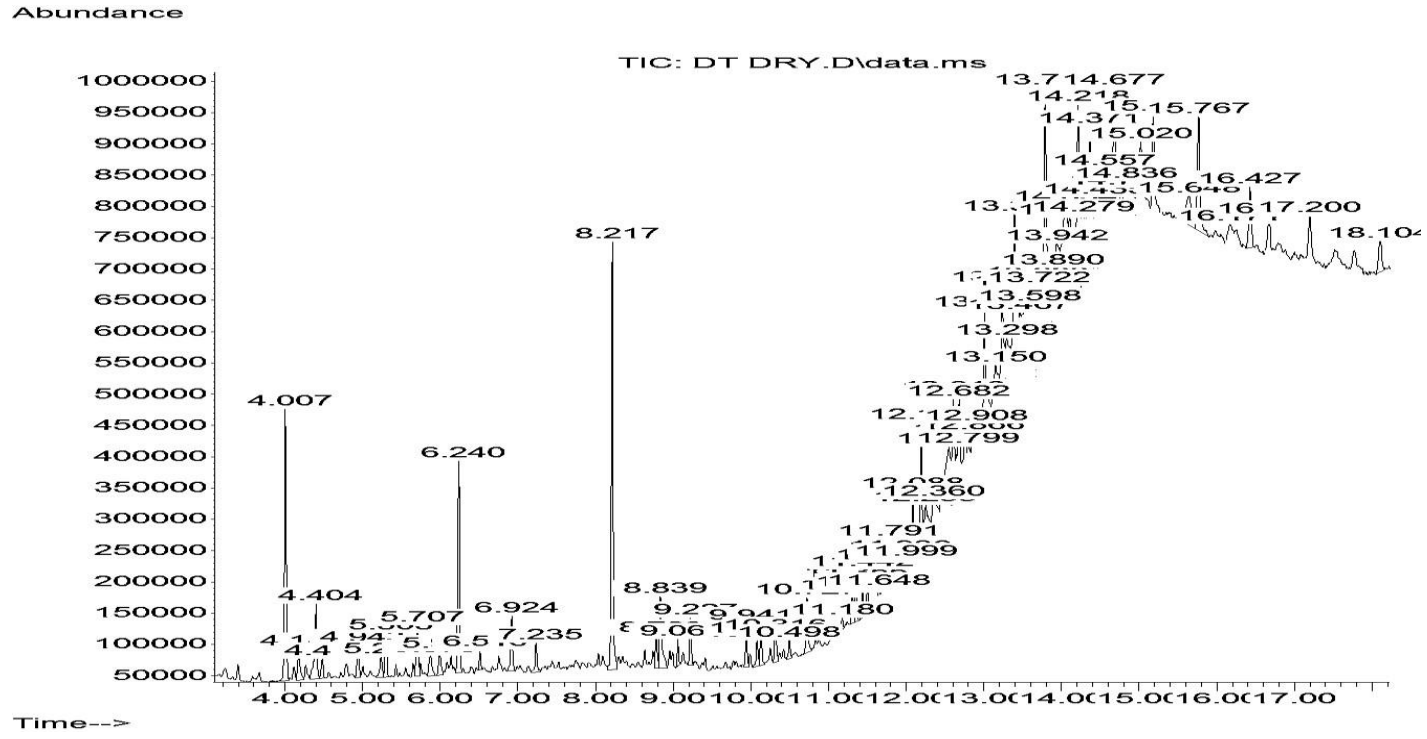
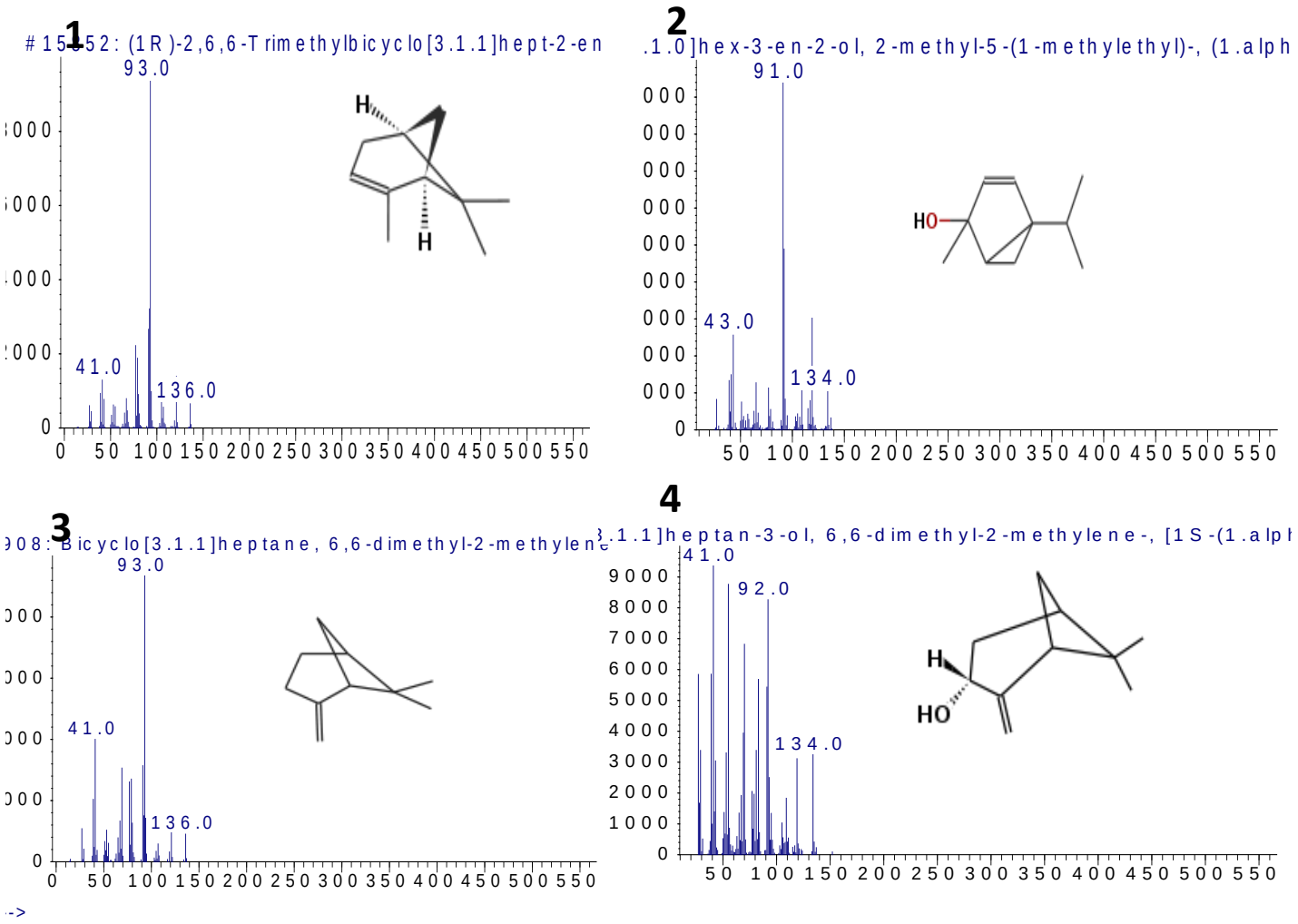


Figure 6.3: GC-MS total-ion chromatograms of the volatile oils from dried roots of *D. thunbergii.*

The mass spectra and structures of the major terpenoids identified in the oils from *D. thunbergii* roots are presented in Fig. 6.4. Compounds such as α–pinene, β–pinene, β–selinene identified in the oils from the roots of *D. thunbergii*, have been reported to be present in essential oils of some plants with reported anti–diabetic activities (Boukhris *et al.*, 2012; Sebai *et al.*, 2013). It can be suggested, therefore, that the significant loss of terpenes and terpenoids in the dried roots of *D. thunbergii* could impact negatively on their biological activities compared to oils from the fresh roots.



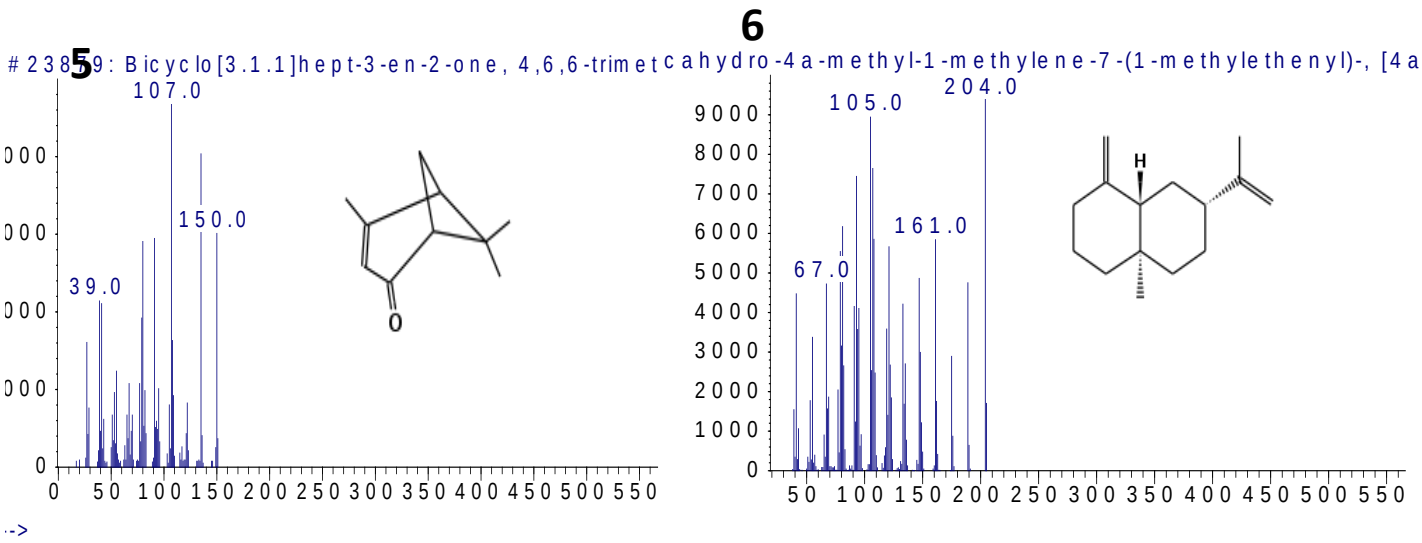


Figure 6.4: Mass spectra and structures of major terpenoids from the root oils of *Dianthus thunbergii*. X–axis: mass–to–charge ratio (m/z); y–axis: signal intensity (abundance). Note that appropriate synonyms of the compounds presented above have been used in the text: 1. 1R–α–pinene; 2. cis–2–Thujen–4–ol; 3. (–)–β–pinene; 4. L–Pinocarveol; 5. Verbenone; 6. β–selinene; 7. (–)–spathulenol; 8. γ–Muurolene; 9. Verbenol. MS spectra can be found in the NIST Chemistry Webbook.

Tables 6.4 and 6.5 respectively present the lists of compounds identified in the volatile oils of the fresh and dried corms of *Hypoxis argentea.* The respective total-ion chromatograms are shown in Figures 6.5 and 6.6. A total of 41 compounds, representing 96.01% of the total oil composition, were identified in the volatile oils of the fresh corms, while 65 different compounds, representing 98.12% of total oil composition, were found in the oil from the dried corms. The predominant categories of compounds identified in the oil from the fresh corms were amides (34.82%) and amines (12.42%), while terpenes and terpenoids made up 10.85% of the total oil composition. The content of these groups of compounds was considerably reduced with drying, with terpenoids and amides giving only 4.00% and 0.30% of the total oil composition, respectively, while amines were no longer detectable in the oil from the dried corms. The bulk of the oil from the dried corms was made up of alkanes (74.76%). As was the case for *D. thunbergii*, drying of the corms of *H. argentea* produced an overall increase in the total number of compounds identified, compared to the fresh corms.

This latter observation is in agreement with reports from other studies (Capecka *et al.*, 2005; Alavi *et al.*, 2010), suggesting that heat processing may indeed produce an increase in the content and/or biological activities of secondary metabolites in herbs and essential oils. A number of reasons have been adduced for such a heat-induced increase in the content of compounds in plant extract and essential oils. The application of heat or the drying of herbs, in some cases, results in the thermal destruction of cell walls and sub-cellular compartments, with the liberation of higher numbers of compounds that were otherwise bonded to structural components of the cell (Jimenez-Monreal *et al.*, 2009). In addition, an increase in the content

and/or biological activity of herbal constituents may be a result of the formation of novel compounds due to heat-induced bio-conversion of certain compounds which serve as precursors for the formation of other compounds (Tomaino *et al.*, 2005). For example, sun-drying of *Artemisia annua* leaves has been shown to increase the content of artemisinin, compared to fresh leaves, due to an improved bio-conversion of dihydro-artemisinic acid (precursor of artemisinin) (Ferreira and Luthra, 2010).

Table 6.4: Chemical composition of the essential oils of the fresh corms of *Hypoxis argentea*

Sr. No.	Compound	Chemical formula	Rt (min)	Area (%)	Class of compound
1	1,4-bis(3-phenyl ureido)-butane	$C_{18}H_{22}N_4O_2$	4.010	0.22	Alkane
2	Nonadecane	$C_{19}H_{40}$	5.046	0.15	Alkane
3	γ-gurjunene	$C_{15}H_{24}$	5.263	0.29	Sesquiterpenoid
4	Phytol	$C_{20}H_{40}O$	5.710	0.22	Diterpenoid
5	Benzoic acid, 4-ethoxy-ethyl ester	$C_{11}H_{14}O_3$	5.755	0.15	Acid ester
6	Citronellol	$C_{10}H_{20}O$	5.795	0.15	Monoterpenoid
7	Cedrene	$C_{15}H_{24}$	5.798	0.80	Sesquiterpenoid
8	Elixene	$C_{15}H_{24}$	6.010	0.07	Sesquiterpenoid
9	γ-Elemene	$C_{15}H_{24}$	6.131	0.29	Sesquiterpenoid
10	δ-cadinene	$C_{15}H_{24}$	6.677	0.44	Sesquiterpenoid
11	3,5-dimethyl phenyl Phthalic acid neopentyl ester	N/A	6.730	1.02	Acid ester
12	S-(neopentyl oxythiocarbonyl)thio hydroxylamine	N/A	6.830	0.37	Amine
13	Ledol	$C_{15}H_{26}O$	6.933	0.29	Sesquiterpenoid
14	Eremophilene	$C_{15}H_{24}$	6.980	0.29	Sesquiterpenoid
15	Octadecane	$C_{18}H_{38}$	7.784	2.57	Alkane
16	Tetrahydrofuran-2-carboxylic acid, dibenzo furan-3-ylamide	N/A	8.419	0.51	Amide
17	N-isobutyl-11-(3,4-methylene dioxyphenyl)-2E,4E,10E-undecatrienoic amide	$C_{22}H_{29}NO_3$	8.596	0.51	Amide
18	(-)-Spathulenol	$C_{15}H_{24}O$	8.789	5.36	Sesquiterpenoid
19	Alloaromadendrene	$C_{15}H_{24}$	8.842	2.65	Sesquiterpenoid
20	1-phenanthrene propane nitrile, 7α, diacetoxy-2-methylene-4b-methyl perhydro	N/A	8.893	1.03	N/A
21	1-Bromoeicosane	$C_{20}H_{41}Br$	8.950	1.77	Alkane
22	3-ethyl-5-(2-ethyl butyl)-Octadecane	$C_{26}H_{54}$	9.260	1.08	Alkane
23	17-hexadecyl tetratriacontane	$C_{50}H_{102}$	9.309	0.37	N/A
24	Cis-2-methyl cyclo hexylester, butanoic acid	$C_{11}H_{22}O_2$	9.420	0.97	Fatty acid ester

25	Eicosane	$C_{20}H_{42}$	9.616	1.87	Alkane
26	9-octyl Eicosane	$C_{28}H_{58}$	9.760	1.11	Alkane
27	2,2-dimethyl propanoic acid, 2,6-dimethyl non-1-en-3-yn-5-yl ester	$C_{16}H_{26}O_2$	10.061	0.59	Fatty acid ester
28	Oleyl alcohol, heptafluoro butyrate	$C_{22}H_{35}F_7O_2$	10.198	0.97	N/A
29	Z-14-Nonacosane	$C_{29}H_{60}$	10.259	1.69	Alkane
30	5H-Thiazolo[2,3-a]pyridine-8-carboxamide,3-(1-adammathyl)-7-methyl-5-oxo	N/A	10.578	1.18	Amide
31	1-Docosanethiol	$C_{22}H_{46}S$	10.597	4.26	Alkyl thiol
32	1,3-Dioxolane,4-ethyl-5-octyl-2,2-bis (trifluoromethyl)-,trans-	N/A	10.889	0.81	Heterocyclic acetal
33	Thiophen-2-methylamine, N-(2-fluorophenyl)-	$C_{11}H_{10}FNS$	10.950	12.05	Amines
34	2-methyl eicosane	$C_{21}H_{44}$	11.310	2.94	Alkane
35	2-ethylbutyric acid, 2,7-dimethyl oct-5-yn-7en-4-yl ester	N/A	11.367	1.91	Fatty acid ester
36	1H-indole-2-carboxylic aid, 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester	N/A	11.401	0.96	Fatty acid ester
37	1,1'-(1,3-propanediyl bis(oxy)bis-octadecane	N/A	11.491	1.62	Alkane
38	Di-n-decylsulfone	$C_{20}H_{42}O_2S$	11.764	5.01	Sulfone
39					
40	1-iodo Octadecane	$C_{18}H_{37}I$	11.333	4.85	Alkane
41	Acetamide, N-(4-fluorophenyl)-2,2,2-trifluro-	$C_8H_5F_4NO$	12.967	32.62	Amide
Total				**96.01%**	

Compounds are listed in order of retention time (Rt); N/A, not available in database.

Table 6.5: Chemical composition of the essential oils of the dried corms of *Hypoxis argentea*

Sr. No.	Compound	Chemical formula	Rt (min)	Area (%)	Class of compound
1	Furfural	$C_5H_4O_2$	3.199	0.57	Heterocyclic aldehyde
2	Furan carboxamide, N-(6-bromo-2H-[1,2,4] thiadiazolo [2,3-a] pyridine-2-ylidene)-	N/A	3.781	0.13	N/A
3	α-pinene	$C_{10}H_{16}$	4.008	1.74	Monoterpenoid
4	5-methyl Furan carboxaldehyde	$C_6H_6O_2$	4.195	0.35	Aldehyde
5	3-Bromoandrostane	$C_{19}H_{31}Br$	4.273	0.22	Steroid
6	Acetonitrile, 2-(2-hydroxyphenylazo)-2-(2-hydroxyphenylhydrazono)-	$C_{14}H_{11}N_5O_2$	4.374	0.17	Organonitrogen compounds
7	Furan, 2-pentyl	$C_9H_{14}O$	4.405	0.17	Aldehyde
8	Beta-alanine, N-(2-furoyl)-, heptyl ester	$C_{15}H_{23}NO_4$	4.565	0.10	Acid ester
9	Tetrahydropyran-4-carboxylic acid, 4-phenyl-, (2,4-dimethoxyphenyl) amide	N/A	4.878	0.17	Amide
10	4H-1,4-Benzothiazin-3 (2H)-one, 4-(4-fluorobenzyl)-6-trifluoromethyl	N/A	5.071	0.30	N/A
11	Benzamide, 3-methoxy-N-(4-(1-methylcyclopropyl) phenyl	N/A	5.133	0.08	Benzamide
12	Carbamic acid, ethyl phenyl-, 3-[[(1-methyl ethoxy) carbonyl] amino] phenyl ester	N/A	5.177	0.10	Fatty acid ester
13	3-Fluoro-o-xylene	C_9H_9NO	5.234	0.35	Benzenoid
14	Cholest-8-ene-3, 6-diol, 14-methyl-, (3β., 5α., 6α)-	$C_{27}H_{46}O_5$	5.292	0.15	Sterol lipid
15	Spiro [3.4] octan-5-one	$C_8H_{12}O$	5.346	0.22	N/A
16	N-cinnamoyl-N-(4-methoxyphenyl)-aminomalonic acid, diethyl ester	$C_{23}H_{25}NO_6$	5.432	0.10	Fatty acid ester
17	Cis-p-metha-1(7), 8-dien-2-ol	$C_{10}H_{16}O$	5.711	0.27	Alcohol
18	Androstan-17-one, 3,4,14-bis(acetyloxy)-5-hydroxy-(3α, 5α, 14β)	N/A	5.814	0.08	N/A
19	2-oxalidinone,4-phenyl-5-tolyl-, trans	$C_{16}H_{15}NO_2$	5.864	0.17	N/A
20	4-(2-Methylcyclohex-1-enyl)-but-2-enal	$C_{11}H_{16}O$	5.911	0.27	Aldehyde
21	1H-Pyrrole, 1-(2-furanylmethyl)-	C_9H_9NO	5.965	0.17	
22	Elixene	$C_{15}H_{24}$	6.012	0.37	Monoterpenoid
23	Verbenone	$C_{10}H_{14}O$	6.243	0.27	Monoterpenoid
24	O-Methoxy-.alpha.-methylbenzyl alcohol	$C_9H_{12}O_2$	6.685	0.15	Alcohol
25	Ethanamine, 2-phenoxy-	$C_8H_{11}NO$	6.826	0.05	N/A
26	3-[p-Methoxyphenyl]-5-methylrhodanine	$C_{11}H_{11}NO_2S_2$	6.920	0.28	N/A
27	2,4-Decadienal, (E,E)	$C_{10}H_{16}O$	6.925	0.37	Aldehyde
28	Indole, 3-(4-nitrophenyl amino)-	$C_{14}H_{11}N_3O_2$	7.066	0.23	Indole
29	2-[p-Cyanophenyl]-5-chlorobenzimidazole	$C_{14}H_8CIN_3$	7.144	0.05	Benzimidazole
30	3-(4-Methoxy-phenylimino)-1-phenyl-propen-1-ol	$C_{16}H_{15}NO_2$	7.439	0.08	Alcohol

31	Benzene sulfonamide, 3-(4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-1-yl)-	$C_{10}H_{11}N_3O_3S$	7.871	0.13	Sulfonamide
32	1,2-Dimethoxy-3,4-dichloro-benzene	$C_8H_8Cl_2O_2$	8.038	0.17	N/A
33	β-Selinene	$C_{15}H_{24}$	8.219	0.80	Sesquiterpenoid
34	Propanephosphonic acid, bis(trimethylsilyl) ester	$C_9H_{25}O_3\psi_2$	8.456	0.13	Acid ester
35	3-Pyridinecarbonitrile, 2-[(1,3-benzodioxol-5-ylmethyl)amino]	N/A	8.638	0.08	N/A
36	Benzene, 1,2-dichloro-4,5-dimethoxy	$C_8H_8Cl_2O_2$	8.791	1.07	N/A
37	(-)-Isolongifolol, heptafluorobutyrate	$C_{19}H_{25}F_7O_2$	8.841	0.55	Sesquiterpenoid
38	N'-[2-amino-6-[2-naphthylsulfonyl]-4-quinazolinyl]-N, N-dimethyl formamidine	N/A	8.960	0.08	N/A
39	Benzamide, 4-methoxy-N-(2-(1-methylcyclopropyl) phenyl	N/A	8.995	0.05	Benzamide
40	Dibenzo[b,k]-18-crown-6,2-nitro-	$C_{20}H_{23}NO_8$	9.130	0.08	
41	Chromone, 5-bromo-6-hydroxy-2-methyl-	$C_{10}H_7BrO_3$	9.233	0.30	Chromone
42	(7E))-2-amino-4-hydroxy-7-[2-(4-methoxyphenyl)-2-oxoethylidene]-7,8-dihydro-6(5H)-pteridinone	N/A	9.304	0.13	N/A
43	Phenol, 2,4,6-trichloro-3-methoxy-5-methyl	$C_8H_7Cl_3O_2$	9.350	2.47	Phenol
44	1,3,5-Trimethyl-2-(2,2,2-trifluoro-ethoxy)-benzene	$C_{11}H_{13}F_3O$	9.414	0.13	
45	11,15-Dimethylheptatriacontane	$C_{39}H_{80}$	9.515	0.05	Alkane
46	3-Bromomethyl benzoic acid	$C_7H_6O_4$	9.726	0.08	Benzenoid
47	Hexadecane,-2-methyl-	$C_{17}H_{36}$	9.814	0.15	Alkane
48	1-Bromoeicosane	$C_{20}H_{41}Br$	9.946	0.28	Alkane
49	Ethanone, 2-(5,7-bisethylamino-[1,2,4]triazolo[4,3-a][1,3,5]triazin 3-ylsulfanyl)-1-(4-methoxyphenyl)	N/A	10.000	0.08	N/A
50	Beta-Phellandrene	$C_{10}H_{16}$	10.134	0.27	Monoterpenoid
51	1-(1-Adamantylcarbonyl)-4-(2-furyl carbonyl) piperazine	$C_{20}H_{26}N_2O_3$	10.195	0.10	N/A
52	1-Decanol, 2-hexyl	$C_{16}H_{34}O$	10.567	0.32	Alcohol
53	Heptacosyl acetate	$C_{29}H_{58}O_2$	10.637	0.42	Acid ester
54	1,3-Dioxolane, 4-ethyl-5-octyl-2,2-bis (trifluoromethyl)-, trans	N/A	10.770	0.27	Alkane
55	Docosane, 1,22-dibromo-	$C_{22}H_{44}Br_2$	10.865	2.12	Alkane
56	Eicosane, 9-octyl	$C_{28}H_{58}$	10.968	7.95	Alkane
57	Eicosane	$C_{20}H_{42}$	10.978	6.13	Alkane
58	Octadecane, 1-iodo	$C_{18}H_{37}I$	12.017	11.49	Alkane
59	Octadecane	$C_{18}H_{38}$	13.537	20.11	Alkane
60	Hexatriacontyl pentafluoropropionate	$C_{39}H_{73}F_5O_2$	11.226	2.09	
61	Heptadecane,2,6,10,15-tetramethyl	$C_{21}H_{44}$	11.310	1.52	Alkane
62	Nonadecane,1-chloro	$C_{19}H_{39}Cl$	11.358	1.72	Alkane
63	Z-14-Nonacosane	$C_{29}H_{60}$	11.922	16.62	Alkane
64	Heneicosane, 3-methyl-	$C_{22}H_{46}$	12.509	6.35	Alkane
65	1-Docosanethiol	$C_{22}H_{46}S$	12.762	6.72	Alkyl thiol
Total				**98.12%**	

Compounds are listed in order of retention time (Rt); N/A, not available in database.

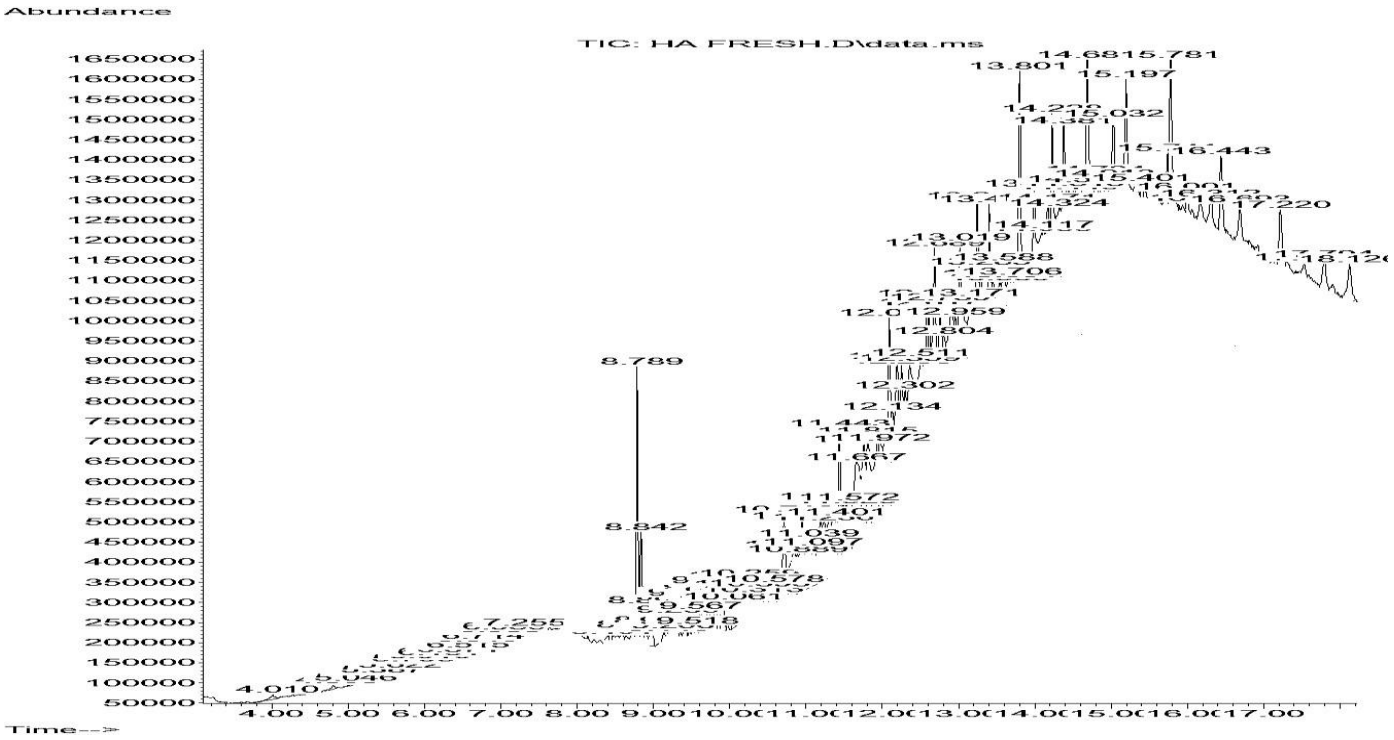


Figure 6.5: GC-MS total-ion chromatograms of the volatile oils from fresh corms of *H. argentea*

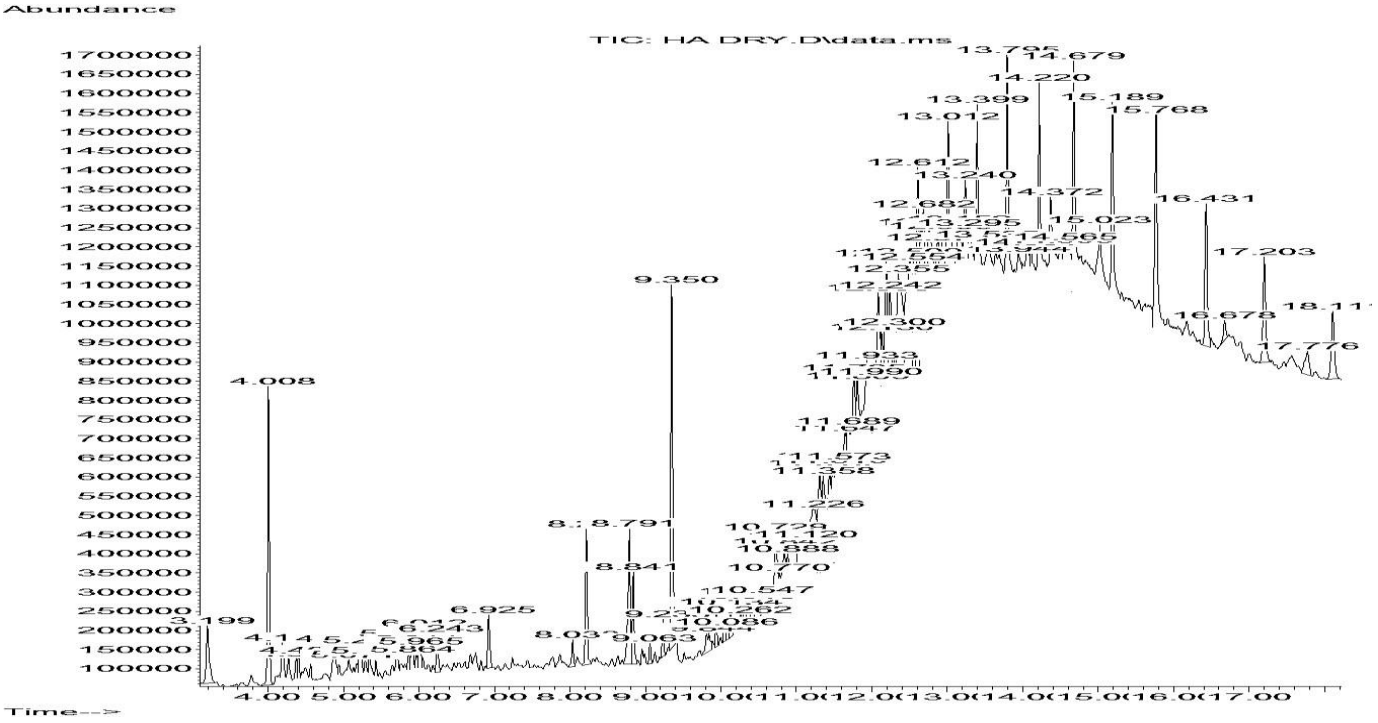
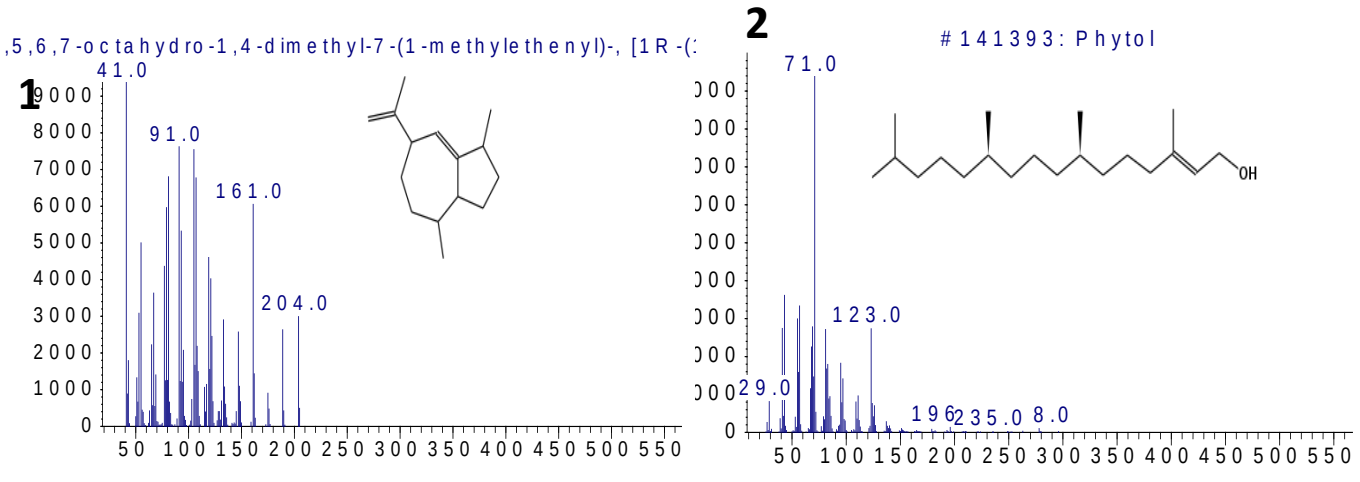


Figure 6.6: GC-MS total-ion chromatogram of the volatile oils from dried corms of *H. argentea*

The major terpenoids identified in *H. argentea* oils included: γ-gurjunene, Phytol, Citronellol, Cedrene, Elixene, γ-Elemene, δ-cadinene, Ledol, Eremophilene, Alloaromadendrene, Spathulenol, α-pinene, Verbenone and β-phellandrene. The mass spectra and structures of these compounds are presented in Fig. 6.7. Scientific literature has revealed very little information regarding the chemical composition of essential oils from other *Hypoxis* species. However, comparisons can be made with the composition of oils from other plant species. A search through literature revealed studies reporting the anti-diabetic activities of some of the compounds identified from the volatile oils from the corms of *Hypoxis argentea*. For example, Sebai *et al*. (2013) reported the contribution of δ-cadinene, γ-gurjunene and β-selinene, among other compounds, to the attenuation of hyperglycemia in alloxan-induced diabetic rats using essential oils from *Lavandula stoechas* (Sebai *et al.*, 2013). Phytol is also a component of essential oils from various plants. It has been reported to contribute to the anti-hyperglycemic activity of *Cynodon dactylon* on streptozotocin-induced diabetes in rats (Jananie *et al.*, 2011).


,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(
1
41.0
91.0
161.0
204.0
2
#141393: Phytol
71.0
123.0
OH
3
#28227: Citronellol
71.0
HO
123.0
4
alene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-met
119.0
41.0
81.0
204.0
H
5
#79361: Ledol
43.0
HO
109.0
161.0
204.0
6
,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-
161.0
41.0
93.0
204.0

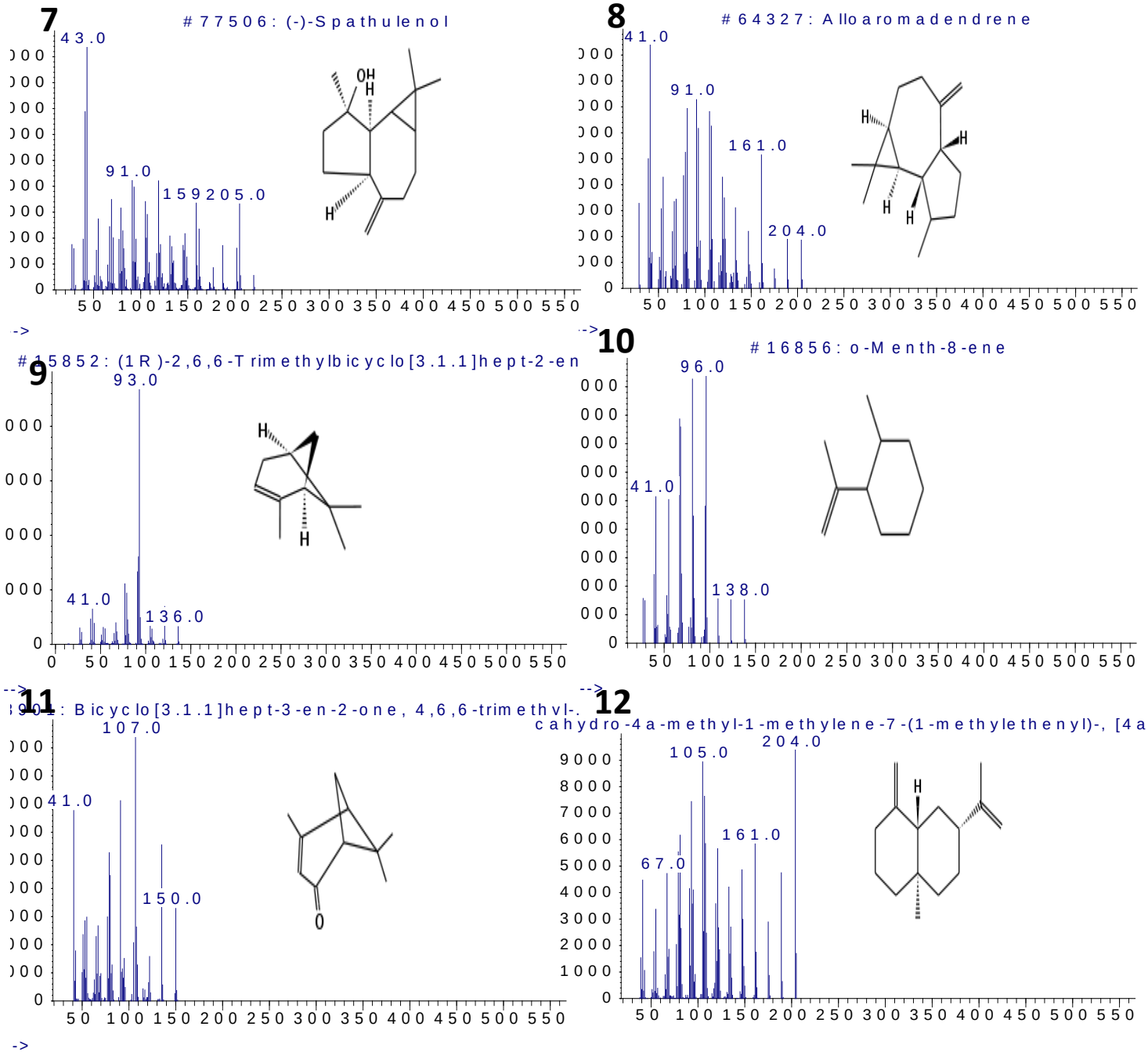


Figure 6.7: Mass spectra and structures of major terpenoids identified from the oils of *Hypoxis argentea* corms. X–axis: mass–to–charge ratio (m/z); y–axis: signal intensity (abundance).). Note that appropriate synonyms of the compounds presented above have been used in the text: 1. γ–gurjunene; 2. Phytol; 3. Citronellol; 4. δ–cadinene; 5. Ledol; 6. Eremophilene; 7. (–)–Spathulenol; 8. Alloaromadendrene; 9. α–pinene; 10. Elixene; 11. Verbenone; 12. β–selinene. MS spectra can be found in the NIST Chemistry Webbook.

6.4 Conclusions

GC-MS analysis in the present study has revealed, for the first time, the presence of several bioactive compounds in the essential oils of the roots of *Dianthus thunbergii* and corms of *Hypoxis argentea*. A variety of terpenes and terpenoids with reported biological activities as antioxidants (Basak and Candan, 2010), anti-bacterial (Leite *et al.*, 2007), antidiabetic (Boukhris *et al.*, 2012; Sebai *et al.*, 2013) and anti-inflammatory (Rufino *et al.*, 2014) were identified in these oils. Further studies will be required to evaluate these biological activities, as well as the potential medicinal applications of the oils from these plants. *Dianthus thunbergii* oils contain large amounts of terpenoids such as α-pinene and β-selinene and may thus represent a good source for specific isolation of these compounds.

An important finding from the present studies relates to the variations observed in the quantity and quality of essential oil components as a result of moisture removal (drying) of plant material. Traditional utilization of these plants usually involves preservation by drying of plant materials that are not meant for immediate use. The data generated here suggests the possibility of considerable loss of inherent bioactive properties of the plant materials after drying. This information must be considered during preparation of these plants for medicinal use. Furthermore, future efforts to isolate specific compounds from the essential oils of these plants will benefit from utilizing the fresh, rather than dried plant material.

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CHAPTER 7

Identification of bioactive compounds in extracts of Dianthus thunbergii and Hypoxis argentea using high performance liquid chromatography and mass spectrometry (HPLC-MS)

Results from this chapter are part of data being submitted for publication in Biomed Research International.

Chapter 7

IDENTIFICATION OF BIOACTIVE COMPOUNDS IN EXTRACTS OF *DIANTHUS THUNBERGII* AND *HYPOXIS ARGENTEA* USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY (HPLC-MS)

7.1 Background

Plant secondary metabolites include a wide variety of phytochemicals (e.g., hydrocarbons, terpenoids, phenolics, alkaloids and their derivatives) present in extracts and essential oils prepared from different plant parts. In contrast to primary metabolites (mainly nutrients), secondary metabolites are generally considered to be metabolic by-products, are not universal across the plant kingdom and many are without recognized nutritional value (Wink, 2003) Secondary metabolites are believed to provide an evolutionary advantage for the plant species, which may include protection against herbivores and pathogen attacks such as fungi and insects. The evolution of defense compounds in plants has given rise to bioactive compounds that may have beneficial effects in pharmacy, biotechnology and medicine. Increasing numbers of novel by-products from different plant species are being identified for their potential pharmacological use. The term ‘bioactive’ compound, therefore, refers to secondary plant metabolites eliciting pharmacological or toxicological effects in human and animals (Bernhoft, 2010)

Hyphenated HPLC-MS technique is an effective method used for identifying compounds in complex mixtures, such as phenolics from crude extract or the fractions (Xing et al., 2007; Zhou et al., 2009; Steinmann and Ganzera, 2010). This can be achieved either by using standard compounds (target identification) or by comparing mass spectrum obtained from literatures and databases (tentative or putative identification). Classical techniques for phytochemical isolation

and structural elucidation in plant extracts, when applied directly, are usually time and solvent-consuming and normally result in the isolation and identification of only a few compounds. Similarly, bioactivity-guided isolation is a time-consuming process and often results in the isolation of already known bioactive compounds. As a result, preliminary untargeted metabolite analysis, using techniques such as LC-MS for identification and characterization of the diverse array of chemical constituents in plant extracts is considered a valuable step for natural product isolation (Sashidhara and Rosaiah, 2007). It will avoid replication (de-replication) as well as save time and money used in the isolation and identification of known compounds (Wu *et al*., 2013).

7.1.1 Principle and instrumentation of LC-MS

In liquid chromatography, the components (analytes) in a sample are selectively distributed between two phases: a mobile (liquid) phase and a stationary phase. The liquid flows through the stationary phase (typically made up of small porous particles with a large surface area) under high pressure supplied by a pump. Separation occurs as a result of differences in the distribution coefficients of the components of the sample, causing variations in their mobilities as they interact with the surface of the stationary phase. Sample molecules that interact strongly with the stationary phase require a longer time to travel through the chromatographic system and thus, have longer retention times. Molecules eluted from the chromatographic column enter a detector which produces a signal, the intensity and duration of which corresponds to the amount or nature of the analyte.

The basic components of a LC-MS system are depicted schematically in Figure 7.1. They include a pump, injector system, thermostated column compartment (TCC), chromatographic column, detector and data acquisition system. Detectors usually coupled with liquid chromatography are of different types and include Mass spectrometers (MS), fluorescence

detectors (FLD), nuclear magnetic resonance spectrometers (NMR), Fourier transform infrared spectrometers (FTIR) and Diode array UV/vis spectrometers (DAD). Mass spectrometry detectors are the most popular detector systems in use today. The mass spectrometer consists of three major parts: the ion source, mass analyzer and detector. The ion source is responsible for the conversion of electrically neutral compounds in a sample into charged ions. Ionization can be produced via different techniques including electrospray ionization (ESI), fast atomic bombardment (FAB), matrix-assisted laser-desorption ionization (MALDI) and atmospheric pressure chemical ionization. ESI is the most commonly used method in most LC-MS procedures (Fenn *et al.*, 1989). Ionization may be performed in either the positive (+ve) or negative (-ve) modes or both modes may be used, in order to provide a more comprehensive coverage of metabolites

The motion of the ions formed is determined by the mass-to-charge ratio (m/z) under the influence of the electric field, the magnetic field and the condition of the ion. The mass analyzer sorts out the ions produced from the ion source according to their m/z ratios. Examples of commonly used mass analyzers are quadrupole, time-of-flight (TOF), Orbitrap, ion-trap and Fourier transform ion cyclotron (FT-ICR) (Williamson and Bartlett, 2007). The detector component of the mass spectrometer then converts the abundances of the ions sorted in the mass analyzer into electrical signals. The information is recorded in an electronic device such as a computer connected to the detector, and may include information on the retention times for each metabolite, the m/z value corresponding to the molecular weight, as well as a quantitation of the abundance of the metabolites (Wu *et al*., 2013).

Precise identification of metabolites with LC-MS is often complicated by situations in which different metabolites possess similar molecular weights despite different molecular

formulae. Furthermore, certain metabolites are isomers, which possess similar molecular formula exhibit different structures. To prevent ambiguity, current research efforts adopt the use of tandem mass spectrometry (MS/MS), which requires that more than one stage of mass spectrometry analysis be performed, leading to molecular fragmentation between the stages. Thus multiple mass analyzers can be separated in space or time in order to achieve specific identification of metabolites even in cases where they have similar molecular formula or structures (Pitt, 2009).

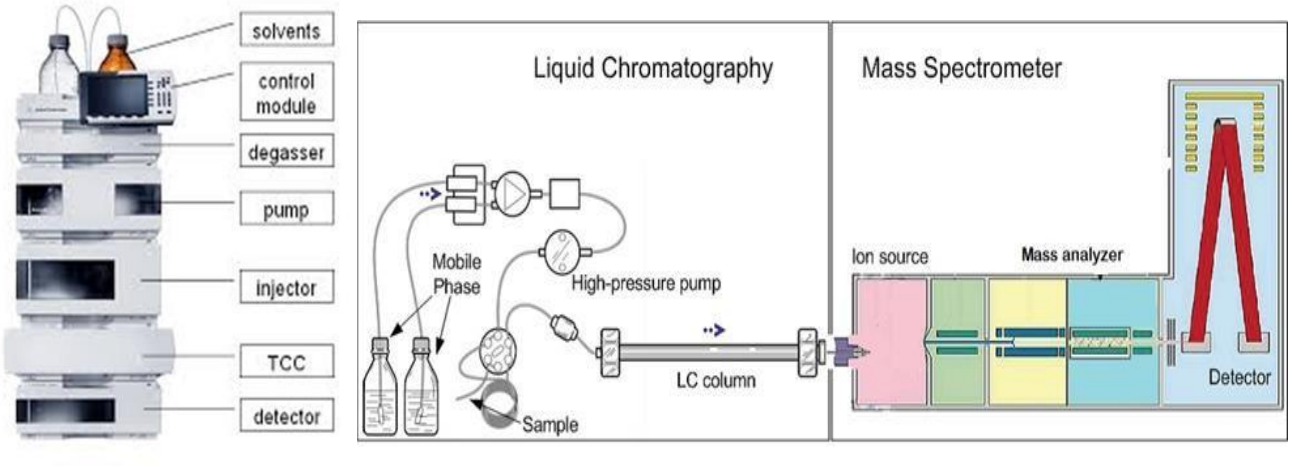

Figure 7.1: Basic components of LC-MS instrumentation

7.1.2 Plant Bioactive compounds

Bioactive compounds isolated from plants constitute a vast range of non-nutritive plant components that typically occur in small quantities, many of which are known to offer benefits in the promotion of health and prevent the risk of chronic diseases such as diabetes, cancer, heart disease and neurodegenerative diseases. Most bioactive compounds from plants belong to one of three main classes of structurally diverse groups of compounds: Phenolic compounds

(polyphenols), isoprenoids (terpenoids) or other lipidic compounds and alkaloids (De La Rosa *et al.*, 2009).

Polyphenols refer to "plant secondary metabolites exclusively derived from the shikimate-derived phenylpropanoid and/or polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression" (Quideau *et al.*, 2011). On the basis of the complexity of their carbon skeletons (Pietta *et al.*, 2003), they can be categorized as follows:

i. Simple phenols and benzoquinones (C_6)

ii. Phenolic acids (C_6-C_1)

iii. Phenylacetic acids (C_6-C_2)

iv. Hydroxycinnamic acids, coumarins, phenylpropanes and chromones (C_6-C_3)

v. Naphthoquinones (C_6-C_4)

vi. Xanthones (C_6-C_1-C_6)

vii. Stilbenes and anthraquinones (C_6-C_2-C_6)

viii. Flavonoids, isoflavonoids and neoflavonoids (C_6-C_3-C_6)

ix. Bi- and tri-flavonoids $(C_6\text{-}C_3\text{-}C_6)_{2,3}$

x. Lignans and neolignans $(C_6\text{-}C_3)_2$

xi. Lignins $(C_6\text{-}C_3)_n$

xii. Catechol melanins $(C_6)_n$

xiii. Condensed tannins $(C_6\text{-}C_3\text{-}C_6)_n$

Phenolic compounds have attracted significant interest as they exhibit a wide range of medicinal properties, mostly related to their antioxidant and redox properties (Duthie *et al.*, 2000). They act as reducing agents, hydrogen-donating agents, metal chelators or singlet-oxygen

quenchers and have thus been found to demonstrate anti-inflammatory, anti-microbial, anti-cancer, anti-hepatotoxic and cardio-protective properties, among others (Balasundram *et al.*, 2006). Several sources of evidence also exist on the potential efficacy of polyphenolic compounds against diabetes and metabolic disorders (Asgar, 2011; Bahadoran *et al.*, 2013; Pandita and Vadiya, 2014).

Alkaloids are a group of nitrogen-containing, alkali-like (basic) compounds possessing characteristic heterocyclic rings and produced by many plant families (and sometimes bacteria, fungi and animals) as secondary metabolites. They are synthesized by decarboxylation of amino acids via long, intricate and stereo-chemically precise biochemical pathways (Wooley, 2001). Alkaloids possess considerable structural diversity which presents difficulty in achieving a uniform classification for this group of compounds. However, certain classifications are based on their heterocyclic ring systems, while other classifications are based on the precursor amino acids involved in their biosynthesis. Alkaloids exhibit diverse biological activities in mammalian systems. Many alkaloids are pharmacologically active in the central nervous system as stimulants, e.g., caffeine, nicotine (Ramawat *et al.*, 2009); some have analgesic effects, e.g., morphine, codeine (Kutchan, 1995 ; Pan *et al.*, 2011;); some function as anti-cancer agents, e.g., vinblastine, vincristine (Chikezie *et al.*, 2015). The potential anti-diabetic activities of alkaloids have also been reported (Agrawal *et al.*, 2013; Tiong *et al.*, 2013).

The studies in this chapter are focused on the analyses of the aqueous and ethanol extracts of *Dianthus thunbergii* and *Hypoxis argentea* by LC-MS for identification of bioactive compounds within these plants.

7.2 Materials and Methods

7.2.1 Sample preparation

Water and ethanol extracts were dissolved in distilled water and HPLC grade ethanol respectively, at a concentration of 10 mg/ml. They were then filtered through a syringe-filter membrane, before injection into the HPLC equipment

7.2.2 HPLC-MS conditions

Liquid chromatography with mass spectrometry analysis was carried out using a high performance Agilent 1260 Infinity Liquid chromatography system, equipped with an AbSciex 5600 Triple TOF hybrid mass spectrometer (Applied Biosystems Sciex, USA) and operated in the negative electrospray ionization (ESI) mode. The LC system consisted of a 4.6 x 50 mm reverse phase column (ProShell 120; EC-C18) with diameter 7 µm. The mobile phase for gradient elution consisted of two solvents: Solvent A: Water with 0.1% formic acid and Solvent B: Acetonitrile with 0.1% formic acid. The linear elution gradient used for the elution of bound compounds was as follows: 75% Solvent A (2 mins.), 75% Solvent A (6 mins.), 25% Solvent A (4 mins), 25% Solvent A (3 mins.) and 75% Solvent A (2 mins.), giving a total run time of 17 minutes. The injection volume was 5.0 µl and the flow rate was 1 ml/minute.

TOF-MS parameters were as follows: The Declustering potential (DP) was 60 V, while collision energy (CE) was 35 V. Product ion parameters were as follows: Ion Spray voltage floating (ISVF): 4500; Ion Source Gas 1 (GS 1): 45 psi; Ion Source Gas 2 (GS 2): 45 psi and Temperature (TEM): 450. TOF masses were acquired using the Analyst Software for masses ranging from 100–1000 Da, while spectra were recorded in the ESI negative mode between m/z 50 and 1000.

7.2.3 Data analysis

To provide identification or tentative identification of the compounds present in the various extracts, the mass spectra of the extracted ions were subjected to data analysis using Formula Finder plug-in feature of the PeakView® qualitative processing, review and comparison software Version 2.2 (AbSciex Pte. Ltd., MA, USA). The chemical formulae generated were screened against a Natural product library (TCM HR-MS/MS Spectral library) containing data for 22, 938 natural products. Compounds with Formula finder scores ≥ 70% were taken as major compounds in the different extracts.

7.3 Results and Discussion

This study was focused on the phytochemical analysis of the ethanol and aqueous extracts of *D. thunbergii* and *H. argentea* using high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS). Figures 7.2–7.5 show the overlaid traces of total ion chromatograms obtained from LC-MS, as well as heat map chromatograms showing the relative intensities as a reflection of the abundance of the compounds detected in the aqueous and ethanol extracts of each plant. The MS and MS/MS spectra obtained by TOF-MS from each chromatogram were investigated by comparison with data in literature to identify or tentatively identify the major components of each sample. Total numbers of MS/MS spectra generated for the extracts were 3361 (*D. thunbergii* aqueous extract); 3621 (*D. thunbergii* ethanol extract); 2002 (*H. argentea* aqueous extract) and 4011 (*H. argentea* ethanol extract). Due to the large numbers of data generated for each extracts, compounds with 70% quality or greater were considered.

In this study, the masses were acquired with ESI at the negative ionization mode as it is reported to be more sensitive in detecting polyphenols and also has lower limits of detection, compared to the positive mode of analysis. Negative ionization is especially sensitive to

detection of flavonoids (Fabre *et al.*, 2001). It is believed that polyphenols possess the tendency to form phenolate ions and therefore, the formation of a deprotonated molecular ion is more favorable than a protonated one in the detection of polyphenols (De Rijke *et al.*, 2003).

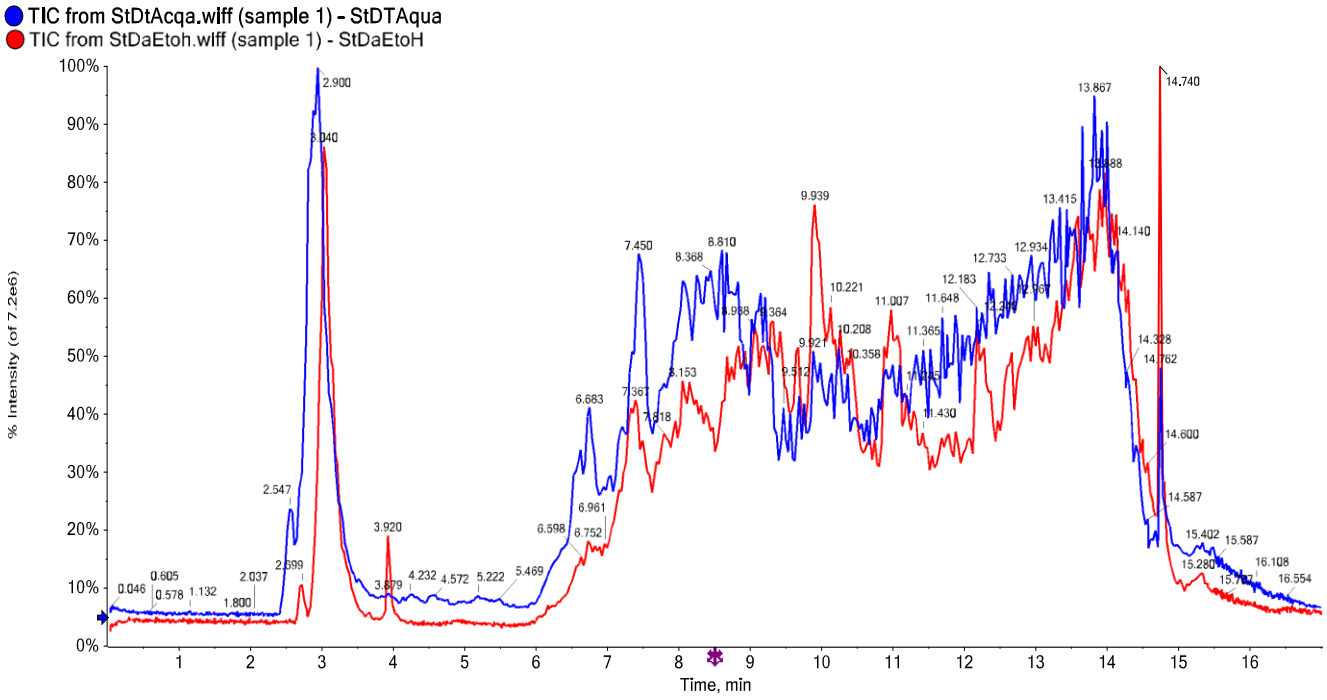

Figure 7.2: Overlaid traces of the total ion chromatograms of the aqueous (StDtAqua; blue) and ethanol (StDaEtoH; red) extracts of Dianthus thunbergii. Peaks are identified by their retention times.

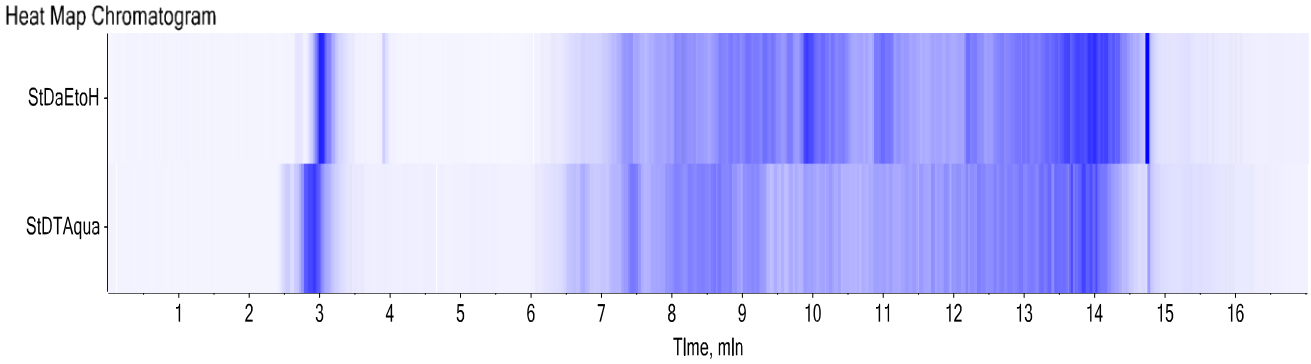

Figure 7.3: Overlaid traces as heat map of the relative intensities of total ion chromatograms of Dianthus thunbergii aqueous (StDTAqua) and ethanol (StDaEtoH) extracts.

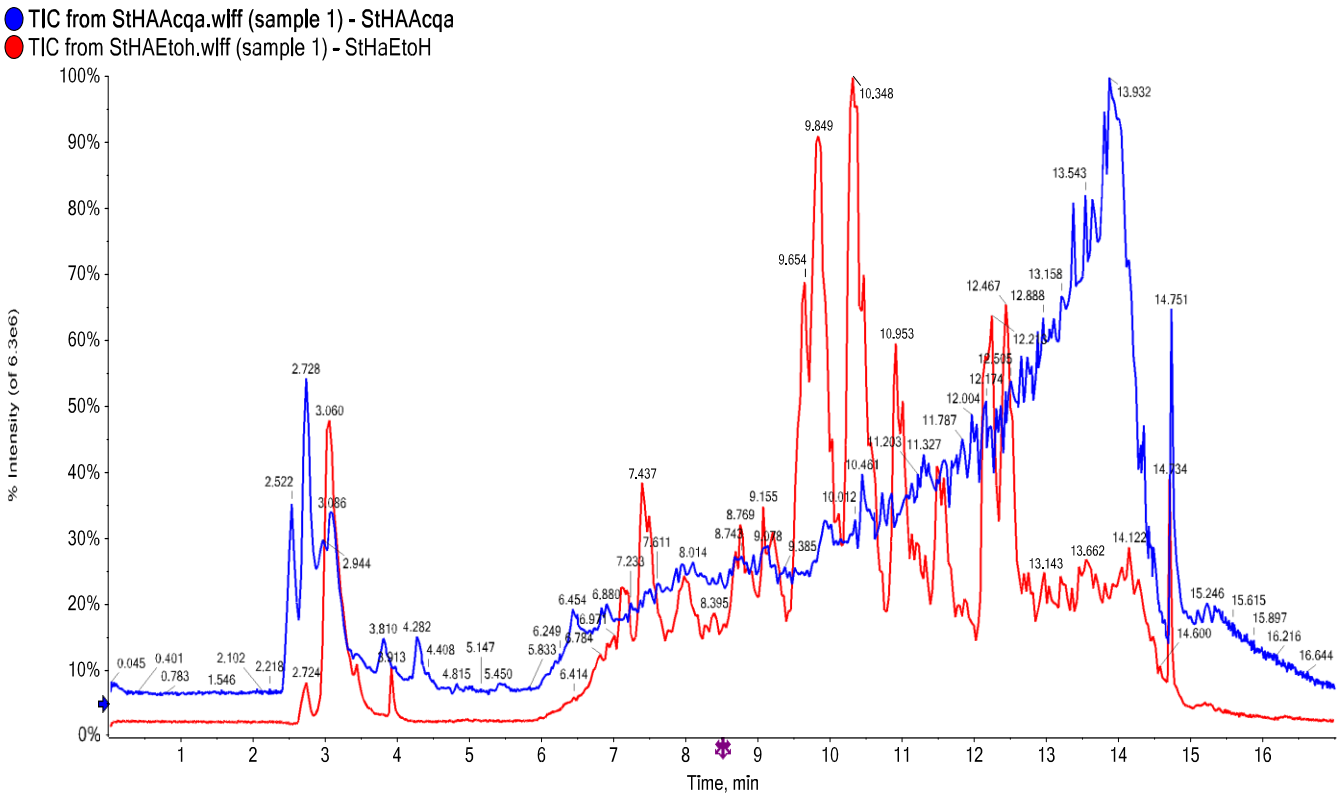

Figure 7.4: Overlaid traces of the total ion chromatograms of the aqueous (StHAAqua; blue) and ethanol (StHaEtoH; red) extracts of Hypoxis argentea. Peaks are identified by their retention times.

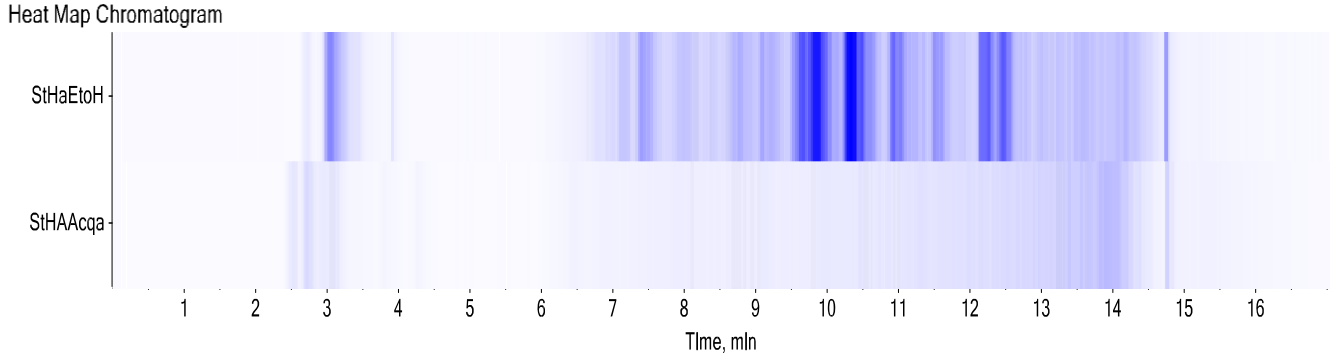

Figure 7.5: Overlaid traces as heat map of the relative intensities of total ion chromatograms of Hypoxis argentea aqueous (StHAAqua) and ethanol (StHaEtoH) extracts.

Tables 7.1 and 7.2 summarize the MS data, including theoretical and measured masses for the molecular formulae generated by formula finder, error, formula finder score and the names of the proposed compound(s) tentatively identified for the major peaks in *D. thunbergii* extracts from the Natural product library utilized. The work flow settings adopted for processing the data using PeakView® and MasterView® software are presented in Appendices 3a-c. It should be noted that the software provided, in some cases, more than one tentative identification at certain peaks for which it was impossible to assign a specific identification due to their similar molecular formula. The results in Tables 7.1 and 7.2 show a variety of compounds present in *D. thunbergii* extracts in different classes, including phenols, alkaloids and terpenoids, some of which have been previously reported to exhibit bioactivities that are relevant to those obtained in the present studies.

The aqueous extracts of *D. thunbergii* appeared to be dominated by compounds associated with cytotoxicity. Vanicoside B, a phenylpropanoid ester of sucrose, tentatively identified as the compound eluting at 2.82 minutes has been reported to demonstrate cancer chemopreventive activities (Takasaki *et al.*, 2001). Lappaol, a lignin eluting at 8.89 minutes was identified as an anticancer agent from the plant *Arctium lappa* (Sun *et al.*, 2014), although it has also been reported to have antihyperglycemic effects (Xu *et al.*, 2006). Furthermore, the β-carboline alkaloid, Canthin-6-one 9-O-β-glucopyranoside eluting at 10.25 minutes has been associated with cytotoxic and antimalarial activities (Kuo *et al.*, 2003), while Caseamembrin, a diterpenoid found at 13.86 minutes, has been associated with cytotoxic activities (Shen *et al.*, 2005). Ginsenoside Rh4 (retention time, RT: 12.96 minutes), like other ginsenosides is a steroid glycoside isolated from ginseng and most of these compounds have been associated with both anti-diabetic and anti-cancer properties (Lee *et al.*, 2006). The phenolic glycoside, Lobetyolin

(RT: 6.73 minutes) is a major contributor to the antioxidant activity of *Campanula alliariifolia* (Dumlu *et al.*, 2008).

A number of compounds identified in the ethanol extracts of *D. thunbergii* have been reported to possess anti-diabetic properties. Quinic acid (RT: 2.97 minutes) is a crystalline acid obtained from many plant products which can also be synthesized by the hydrolysis of chlorogenic acid. Arya *et al*. (2014) reported an anti-diabetic effect of this compound, acting synergistically with quercetin to alleviate tissue degeneration in streptozotocin-induced diabetic rats. 4-O-caffeoylquinic acid methyl ester, detected at RT 7.32 minutes has also been associated with anti-diabetic properties, mediated via its inhibitory activities against α-glucosidase and PTP-1β (Chen *et al*., 2014). In addition, Macrophylloside, a flavone glucoside, reportedly inhibits aldose reductase and sorbitol accumulation as a mechanism for its improvement of diabetic complications.

Table 7.1: Tentative identification of major compounds in Dianthus thunbergii aqueous extract

#	*RT (min)*	*Theoretical mass (Da)*	*Found at mass (Da)*	*Error (ppm)*	*Formula finder result*	*Formula finder score*	*Proposed compound from library*
1	2.82	956.27386	957.28178	0.7	$C_{49}H_{48}O_{20}$	70.2	Vanicoside B
2	6.73	396.17841	397.18652	2.1	$C_{20}H_{28}O_8$	93	Lobetyolin
3	7.15	414.15259	415.16066	1.9	$C_{19}H_{26}O_{10}$	92.6	Hymenoside
4	7.55	552.18427	553.19148	-0.1	$C_{26}H_{32}O_{13}$	73.4	Cucurbitoside B
5	8.07	246.11034	247.11881	4.8	$C_{11}H_{18}O_6$	96	Jioglutin D
6	8.89	554.21521	555.22305	1	$C_{30}H_{34}O_{10}$	76.6	Lappaol
7	10.25	398.11139	399.11914	1.2	$C_{20}H_{18}N_2O_7$	84	Canthin-6-one 9-O-β-glucopyranoside
8	12.96	620.42883	621.43634	0.4	$C_{36}H_{60}O_8$	86.2	Ginsenoside Rh4
9	13.4	602.41827	603.42614	1	$C_{36}H_{58}O_7$	92	$C_{36}H_{58}O_7$
10	13.6	516.34509	517.35252	0.3	$C_{31}H_{48}O_6$	81.7	Phytolaccagenic acid
11	13.77	486.37091	487.37831	0.2	$C_{31}H_{50}O_4$	80	Regelindiol; Hederagenin methyl ester; Tumulosic acid; Sulfurenic acid
12	13.78	270.21948	271.22754	2.9	$C_{16}H_{30}O_3$	87.8	2-Oxohexadecanoic acid
13	13.79	444.32397	445.3316	0.8	$C28H_{44}O_4$	75.2	5α,9α-Epidioxy-(22E)-ergosta-7,22-diene-3β,6α-diol; Bethogenin
14	13.82	414.31339	415.32137	1.7	$C_{27}H_{42}O_3$	90.5	22-Cyclopentyloxil-22-deisopenty-3β-hydroxyl-furostanol
15	13.86	416.25629	417.26397	1	$C_{25}H_{36}O_5$	83.7	Caseamembrin; Visbanin

RT = Retention time.

Table 7.2: Tentative identification of major compounds in Dianthus thunbergii ethanol extract

#	RT (min)	Theoretical mass (Da)	Found at mass (Da)	Error (ppm)	Formula finder result	Formula finder score	Proposed compounds from library
1	2.86	873.26917	874.27814	1.9	$C_{41}H_{47}NO_{20}$	84.4	Isowilfortrine; Wilfotrine; Hypoglaunine
2	2.97	192.06339	193.0716	4.8	$C_7H_{12}O_6$	91.4	Quinic acid
3	3.12	504.16904	505.17659	0.5	$C_{18}H_{32}O_{16}$	83.7	Raffinose; 1-Ketose; Panose; Neokestose; Manninotriose; Nigellamose ; Gentianose; Planteose
4	7.16	746.26331	747.27004	-0.7	$C_{33}H_{46}O_{19}$	73.4	6'-O-(7α-Hydroxy swerosyloxy) loganin
5	7.32	368.11072	369.11856	1.5	$C_{17}H_{20}O_9$	81.7	4-O-Caffeoylquinic acid methyl ester; 3-O-Feruloylquinic acid; 6-Methoxy-7-O-β-D-(4'-methoxy) glucopyranosyl coumarin; Phellodenol E; Methyl chlorogenate; Cnidioside A
6	7.59	558.19489	559.20295	1.4	$C_{25}H_{34}O_{14}$	75.9	Macrophylloside D
7	7.6	388.17334	389.18102	1	$C_{18}H_{28}O_9$	82.4	(1R,2R)-5'-Hydroxyjasmonic acid 5'-O-β-D-glucopyranoside; Nuezhengalaside; Tuberonic acid glucoside; Qingjueine I
8	8.08	534.17371	535.18109	0.2	$C_{26}H_{30}O_{12}$	81.1	Phellatin; 6-γ,γ-Dimethylallyltaxifolin 7-O-β-D-glucoside; Noricariside; Amurensin
9	8.5	672.26294	673.26936	-1.3	$C_{31}H_{44}O_{16}$	71	$C_{31}H_{44}O_{16}$
10	8.83	926.45111	927.45998	1.7	$C_{46}H_{70}O_{19}$	94.9	Spinacoside C
11	9.17	242.11542	243.12343	3	$C_{12}H_{18}O_5$	84.7	Drummondol; 3-n-Butyl-3-hydroxy-4,5,6,7-tetrahydro-6,7-dihydroxy phthalide
12	9.54	540.25708	541.26453	0.3	$C_{27}H_{40}O_{11}$	79.1	Isovaleroxy-hydroxy dihydrovaltrate
13	10.37	464.20462	465.21216	0.6	$C_{24}H_{32}O_9$	81.9	Shikokianoic acid; Enanderianin A; Caesalpinin MH; Teucrolivin B
14	11.46	242.15181	243.15957	2	$C_{13}H_{22}O_4$	74.8	Annuionone F
15	12.28	472.31888	473.32662	1	$C_{29}H_{44}O_5$	78.1	Siraitic acid A
16	14	372.19366	373.20199	2.8	$C_{22}H_{28}O_5$	98.5	Galbelgin; Galgravin; Acetoxy-7-oxo-dehydroabietic acid; Saucernetin; Recedensolide
17	14.29	442.30832	443.31603	1	$C_{28}H_{42}O_4$	85.2	Triptocalline A
18	14.74	430.30832	431.31696	3.2	$C_{27}H_{42}O_4$	93.5	Cryptogenin; Torvogenin; Hecogenin;
19	14.74	486.37091	487.37893	1.5	$C_{31}H_{50}O_4$	92.8	Regelindiol

RT = Retention time

The MS data for compounds tentatively identified in *Hypoxis argentea* extracts are presented in Tables 7.3 and 7.4. A few of these compounds have been previously reported to possess anti-diabetic properties as well as tissue-protective properties in experimentally-induced diabetes. Ampelopsin identified in the aqueous extract of *H. argentea* (RT 8.54 minutes) has been reported to offer protection to endothelial cells during hyperglycemia-induced oxidative damage (Liang *et al*., 2015). The same compound has recently been shown to improve insulin resistance via activation of PPARγ (Zhou *et al*., 2016). Yamogenin, also detected in the aqueous extract (RT 13.73 minutes) has originally been purified from fenugreek extract and it has been shown to cause inhibition of lipid accumulation in liver tissues via suppression of gene expression in fatty acid synthesis. In addition, Calycosin, tentatively identified in the ethanol extract has been reported to reduce hyperglycemia and improve hypoinsulinemia in streptozotocin-diabetic mice (Ma *et al.*, 2007). Another recent study suggested its effectiveness in ameliorating cognitive impairments during diabetes induced in rats via a reduction in oxidative stress (Wang and Zhao, 2016).

Table 7.3: Tentative identification of major compounds in aqueous extracts of Hypoxis argentea

#	*RT (min)*	*Theoretical mass (Da)*	*Found at mass (Da)*	*Error (ppm)*	*Formula finder result*	*Formula finder score*	*Proposed compound from library*
1	2.97	582.13733	583.14499	0.7	$C_{29}H_{26}O_{13}$	87.5	2''-O-Vanilloylvitexin
2	6.52	324.13214	325.14069	3.9	$C_{15}H_{20}N_2O_6$	94.4	$C_{15}H_{20}N_2O_6$
3	6.7	638.18469	639.19346	2.3	$C_{29}H_{34}O_{16}$	92.3	Ombuoside
4	7.05	442.1839	443.19145	0.6	$C_{21}H_{30}O_{10}$	81.5	(1'S,6'R)-8'-Hydroxyabscisic acid β-D-glucoside; 15-O-β-D-Glucopyranosyl-11β,13-dihydrourospermal A 15-O-β-D-Glucopyranosyl-11β,13-dihydrourospermal
5	7.9	462.24649	463.25395	0.4	$C_{22}H_{38}O_{10}$	80.1	Shionoside B
6	8.54	320.05322	321.06197	4.6	$C_{15}H_{12}O_8$	88.1	Ampelopsin
7	8.59	286.14163	287.15015	4.3	$C_{14}H_{22}O_6$	95.1	Bursephenylpropane
8	8.71	530.10602	531.11416	1.6	$C_{25}H_{22}O_{13}$	94.1	6,7-Dihydroxy-3-methoxy-8-[2-oxo
9	8.72	405.15762	406.16655	4.1	$C_{24}H_{23}NO_5$	79.1	6-Acetonyldihydrochelerythrine
10	9.7	954.44604	955.45708	3.9	$C_{47}H_{70}O_{20}$	72.1	Bidentatoside; Betavulgaroside
11	9.79	402.189	403.19702	1.9	$C_{19}H_{30}O_9$	91.6	(1R,2R)-Methyl-5'-hydroxyjasmonate 5'-O-β-D-glucopyranoside; 6-Hydroxy-junipeionoloside; Sauroposide; Glochidionionoside; Apocynoside; Spionoside; Saussureoside; Cannabiside
12	13.41	430.30832	431.31646	2	$C_{27}H_{42}O_4$	94.7	Yuccagenin; Gloriogenin; Sisalagenin; Isonarthogenin; Laxogenin; Torvogenin; Hecogenin; Lilagenin; Nuatigenin; Ruscogenin; Heloniogenin; Cryptogenin
13	13.73	414.31339	415.32106	1	$C_{27}H_{42}O_3$	82.9	Smilagenone; Trigonegenin; Yamogenin; Sarsasapogenone; Neotigogenone; Tigogenone; 22-Cyclopentyloxil-22-deisopenty-3β-hydroxyl-furostanol
14	13.79	203.05824	204.06626	3.6	$C_{11}H_9NO_3$	82.3	Doryanine
15	13.92	366.18311	367.19112	2	$C_{23}H_{26}O_4$	90.1	Clusiaphenone; Cudraphenone
16	14.01	383.35426	383.35287	-3.6	$C_{24}H_{48}O_3$	94.8	$C_{24}H_{48}O_3$

RT = Retention time.

Table 7.4: Tentative identification of major compounds in ethanol extracts of Hypoxis argentea.

#	*RT (min)*	*Theoretical mass (Da)*	*Found at mass (Da)*	*Error (ppm)*	*Formula finder result*	*Formula finder score*	*Proposed compound from library*
1	3.91	372.09976	373.10852	4	$C_{23}H_{16}O_5$	86.6	Ohioensin
2	3.91	464.15298	465.16173	3.2	$C_{19}H_{28}O_{13}$	92.1	Canthoside B
3	7.01	656.19525	657.20363	1.7	$C_{29}H_{36}O_{17}$	100	Hellicoside
4	7.17	494.14243	495.15142	3.5	$C_{23}H_{26}O_{12}$	87.4	Peruvianoside; 2'-(o,m-Dihydroxybenzyl) sweroside;
5	7.23	326.10016	327.10821	2.4	$C_{15}H_{18}O_8$	89.7	(Z)-8-β-D-Glucopyranosyl oxycinnamic acid; 2-Oxoneomajucin; cis-p-Coumaric acid 4-O-β-D-glucopyranoside; Bilobalide
6	7.76	510.19485	511.20394	3.5	$C_{21}H_{34}O_{14}$	86.1	Rehmannioside C
7	7.81	402.15259	403.16062	1.9	$C_{18}H_{26}O_{10}$	91.4	Benzyl alcohol O-β-D-primveroside
8	7.96	382.09	383.09754	0.7	$C_{17}H_{18}O_{10}$	78.3	5-Carboxy-7-glucosyloxy-2-methyl-benzopyran-γ-one
9	8.17	428.16824	429.17593	0.9	$C_{20}H_{28}O_{10}$	80.1	Hyuganoside ; trans-p-Ferulylalcohol-4-O-[6-(2-methyl-3-hydroxypropionyl)]glucopyranoside; p-β-Rutinosyloxy styrene
10	8.32	284.06848	285.07702	4.4	$C_{16}H_{12}O_5$	93.9	Maackiain; 3'-Methoxydaidzein; Calycosin; Geraldone; Acacetin; Physcion; Questin
11	8.41	372.17841	373.18688	3.2	$CH_{28}O_8$	93.4	Celephtalide C
12	8.45	620.21051	621.21947	2.7	$C_{30}H_{36}O_{14}$	90.5	Dracunculifoside N; Masutakeside
13	8.8	362.12665	363.13511	3.3	$C_{21}H_{18}N_2O_4$	97.8	Zanthobisquinolone
14	12.79	264.17255	265.18097	4.3	$C_{16}H_{24}O_3$	98.2	Actinolide A; 10β-Hydroxy-6β-methoxy-furanoeremophilane; Dehydrojuvabione
15	13.96	915.51917	916.52539	-1.1	$C_{46}H_{77}NO_{17}$	71.4	Abutiloside B
16	14.73	532.34003	533.34982	4.7	$C_{31}H_{48}O_7$	71.6	Phytolaccagenin

RT = Retention time.

7.4 Conclusion

The LC-MS analysis of the aqueous and ethanol extracts of *D. thunbergii* and *H. argentea* has revealed the tentative identities of compounds with reported bioactivities in the literature, which are relevant to those explored in this project. It must be pointed out that while many of the compounds identified in the extracts may not have been associated with direct anti-diabetic activities, many of them act as antioxidants neutralizing the reactive oxygen species generated as a result of hyperglycemic conditions that occur in diabetes. Thus, from an antidiabetic standpoint, several other compounds identified in this study potentially possess bioactivities that may be of importance, although many are yet to be studied in this regard. More detailed targeted studies and structural elucidation by nuclear magnetic resonance spectroscopy techniques are required to fully characterize the specific identity of the compounds present in these extracts.

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CHAPTER 8

General Discussions, Conclusions and Future Directions

Chapter 8

GENERAL DISCUSSIONS, CONCLUSIONS AND FUTURE DIRECTIONS

8.1. Discussions

The main goal of anti-diabetic therapy is the restoration of blood glucose from hyperglycemic to normal physiological levels, leading to the relief of symptoms and the alleviation of associated health complications. Conventional antidiabetic agents act via several mechanisms to achieve these therapeutic targets. These mechanisms may include the inhibition of carbohydrate and fat digesting enzymes (e.g. α-amylase, α-glucosidase, and lipase), stimulation of glucose utilization and insulin sensitivity in peripheral tissues (adipose tissue, muscle), reduction of oxidative stress and the inhibition of processes involved in development of diabetic complications. These medications, however, present with adverse side effects and are usually very costly. Therefore, the development of safe, cost-effective and pharmacologically-effective alternative medicines for the treatment of diabetes mellitus continues to be a challenge.

Many communities in Africa have sought alternatives to conventional antidiabetic therapy from natural sources, especially plants. In the Eastern Cape Province of South Africa, traditional healers have utilized plants for the treatment of diabetes, and a number of these are documented in previously conducted ethnobotanical surveys (Erasto *et al.*, 2005; Oyedemi *et al.*, 2009). *Dianthus thunbergii* and *H. argentea* are among the most frequently mentioned plants and were chosen for this research as there are no available scientific reports on their chemical composition, toxicology or the nature of their acclaimed anti-diabetic actions. Thus the studies in this research were devoted to providing information that may contribute to validating or nullifying the ethno-medicinal claims for the use of the two plants in the treatment of diabetes. The underground parts of the plants were employed as they are the parts mostly utilized

traditionally and this is backed by the understanding that these portions generally have the capacity to accumulate the highest levels of secondary metabolites with medicinal values.

Quantitatively, the analysis of crude extracts from both plants revealed the presence of important groups of phytochemicals in amounts that suggest their potential to offer benefits for medicinal applications. The relative contents of phenols, flavonoids, alkaloids, tannins and proanthocyanidins were found to have a direct correlation to the antioxidant activities as measured by different *in –vitro* assays. The pathogenesis of both Type 1 and Type 2 diabetes has been linked with an underlying oxidative stress component due to the formation of reactive oxygen species. Among other mechanisms promoting oxidative stress, hyperglycemia is a major factor which induces mitochondrial dysfunction and endoplasmic reticulum stress, thereby promoting ROS accumulation that, in turn, leads to cellular damage and contributes to the development of diabetic complications (Fiorentino *et al.*, 2013).

Antioxidants, whether endogenously derived or exogenously supplied (consumed), are species or molecules that inhibit or delay the oxidation of a substrate (Asmat *et al*., 2016). Many phytochemicals are known to possess strong antioxidant properties via mechanisms that may involve direct free-radical scavenging activities, chelation of metal ions involved in free-radical reactions or an upregulation of the activities of antioxidant enzymes. The results of the present research indicate the presence of higher amounts of phenols, flavonoids, tannins, proanthocyanidins and alkaloids in the ethanol extracts of both *D. thunbergii* and *H. argentea*, compared to the aqueous extracts. Similarly, the ethanol extracts showed better ferric reducing potentials, while also demonstrating higher scavenging capacities against hydrogen peroxide, nitric oxide and ABTS. It was also observed that the extracts may offer better antioxidant activities compared to established antioxidants such as ascorbic acid, butylated hydroxytoluene

and Rutin that were employed as standards in this study. It seems reasonable to suggest, therefore, that ethanol may represent a more suitable solvent for the extraction of compounds with potent antioxidant properties from these plants. This finding has been corroborated by the observations from other studies (Sun *et al.*, 2015), which suggest that ethanol or ethanol and water mixtures are suitable solvents for extracting many bioactive compounds due to the broad range of polarity available.

Traditionally, medicinal plants are assumed to be safe due to their natural origin. However, adverse reactions due to medicinal herbs are frequently under-reported and information on potential toxicities is either lacking or incomplete. Scientific evidence suggests that a variety of adverse reactions are attributable to medicinal plants themselves, due to their inherent poisonous chemical constituents, producing cytotoxicity or herb-drug interactions that may compromise therapeutic end-points, especially when herbs are used in combination with conventional medications (Jordan *et al.*, 2010). To assess possible cytotoxicity and herb-drug interactions in this research, the extracts of *D. thunbergii* and *H. argentea* were screened against HepG2 cells and INS-1 cells.

The results showed that the aqueous extracts of *D. thunbergii* exhibited considerable toxicity to both cell lines, a finding that raises significant concerns over its suitability for antidiabetic therapy. Although, water is commonly regarded as the ideal solvent for preparation of plant extracts as it is safe for human consumption, the consistency of cytotoxicity obtained with *D. thunbergii* aqueous extracts suggests that more studies are necessary to fully characterize the toxic components therein. The fact that the cytotoxic effect has been established in this study with different approaches including MTT and crystal violet assays, as well as live cell fluorescence imaging, lends support to the credibility of the results obtained.

In contrast, the different assays employed in this research suggest that the extracts of *H. argentea* were largely non-toxic at the concentrations tested. More importantly, this plant showed a tendency to produce stimulation of a proliferation of INS-1 cells, a property that could be of significant application in preserving pancreatic β cell populations *in vivo*. As a result, the plant may offer beneficial applications in restoring or, at least, augmenting insulin levels in diabetic patients. Compounds that show potential to preserve β cell proliferation are currently attracting interest as promising candidates for the management of diabetes (Oh, 2015). Further studies are required to elucidate the precise mechanism(s) involved in stimulation of cell proliferation by this plant, as well as identifying compounds that may be responsible for this effect.

The present research has employed a vast array of biochemical and cell-based assays to identify potential mechanism(s) of probable antidiabetic actions of the two plants. Using concentrations ranging from 12.5 to 200 µg/ml, it was observed that the extracts of both plans exhibited very weak inhibitory activities against relevant enzymes, including α-amylase, α-glucosidase, lipase, collagenase and DPP-IV, as well as very low inhibition of protein glycation. This suggests that the inhibition of carbohydrate and fat metabolism via the measured enzyme activities may not represent viable mechanisms of action for these two plants.

However, the effects produced by these extracts on cell-based parameters of anti-diabetic activity suggest that the plants may indeed possess properties that support their claims for antidiabetic therapy. In this regard, *H. argentea* holds the better promise, with its aqueous extract producing some degree of stimulation of glucose uptake via a probable insulin-dependent mechanism in L6 myotubes, and also a likely insulin-independent mechanism in HepG2 cells. The same extract produced no increases in triglyceride accumulation in 3T3-L1 pre-adipocytes,

suggesting a non-adipogenic effect that may be useful in improving insulin resistance. In fact, the ideal anti-diabetic agent has been proposed to be one that neither has a pro-adipogenic nor an anti-adipogenic effect (Alonso-Castro and Salazar-Olivo, 2008), a theory that has been well mirrored by the observations with *H. argentea.* In addition, a concentration-dependent lowering of nitric oxide production in RAW 264.7 macrophages by the aqueous extract of *H. argentea* also supports its ability to ameliorate inflammatory conditions which sometimes occur in insulin-resistant tissues in Type 2 diabetes.

The results of the present study showed that although *D. thunbergii* demonstrated a stimulation of glucose uptake in L6 myotubes, the effect was largely overshadowed by toxicities demonstrated towards other cells including HepG2 cells, INS-1 cells and RAW macrophages. Interestingly, L6 myotubes were resistant to the toxic effect of the aqueous extract of *D. thunbergii*. This extract exhibited some degree of stimulation of adipogenesis in 3T3-L1 adipocytes, and suggests that it may be unsuitable for its use in managing insulin resistance, due to a tendency to increase lipid accumulation in peripheral tissues (Weyer *et al.*, 2000). Both plants appeared not to influence glucose metabolism in INS-cells, as reflected by the extent of MTT reduction. It does not appear that they may stimulate increased insulin secretion via an increase in β cell glucose metabolism, although this may be possible by an increase in the proliferation of β cells themselves, as obtained with *H. argentea.*

The observed antidiabetic and/or cytotoxic activities of the extracts of both *D. thunbergii* and *H. argentea* could be attributed to the presence of bioactive compounds, some of which were tentatively identified by LC-MS analysis. These compounds are yet to be fully characterized, thereby necessitating further studies to elucidate their structural details. Nevertheless, the most likely compounds identified in each extract have been reported in literature to produce

bioactivities similar to those obtained in the present studies. The findings from GC-MS analysis of volatile oils extracted from parts of the two plants suggest that the plants are most suitable for use in their fresh, rather than the dried forms. Drying of the plant parts at 40ºC resulted in considerable reduction in the quantity and quality of important compounds known to possess medicinal values. This information is considered useful, not only for traditional healers who employ these plants for therapy, but also for future studies in isolating bioactive components from the plants.

8.2 Conclusions

The studies conducted in the present research demonstrated that:

i. The antioxidant properties of *D. thunbergii* and *H. argentea* are more pronounced in the ethanol extracts, as demonstrated by their in-vitro free radical scavenging activities. This observation concurs with the relative contents of phenols, flavonoids, proanthocyanidins, alkaloids and tannins, all of which occurred in larger quantities in the ethanol extracts.

ii. LC-MS analysis of the aqueous and ethanol extracts of *D. thunbergii* and *H. argentea* revealed the presence of important bioactive compounds with reported anti-diabetic and/or cytotoxic activities.

iii. *D. thunbergii* exhibited significant toxicity to most cell lines used in this study, namely HepG2 cells, INS-1 cells and RAW macrophages, while *H. argentea* was relatively non-toxic, and induced proliferation of INS-1 cells.

iv. At concentration ranging from 12.5 to 200 µg/ml, the aqueous extracts of both *D. thunbergii* and *H. argentea* exhibited weak inhibitory activities against α-amylase, α- glucosidase, lipase, DPP-IV and collagenase, as well as weak

inhibition of protein glycation. It therefore appears that the inhibition of these enzymes may not represent a viable antidiabetic mode of action for the plants.

v. The antidiabetic properties of *H. argentea* included its ability to stimulate glucose uptake in L6 myotubes and HepG2 cells, without an increase in triglyceride accumulation in 3T3-L1 pre-adipocytes. It also produced a concentration-dependent reduction in nitric oxide production, and as well stimulated INS-1 cell proliferation.

vi. Although, *D. thunbergii* showed a tendency to stimulate glucose utilization in L6 myotubes, its toxicity to other cell lines is a significant worry for its acclaimed antidiabetic use.

vii. Drying of plant materials prior to extraction of volatile oils by hydrodistillation led to considerable alterations in the chemical composition of the oils, most notably a reduction in the terpenoid contents of the oils from dried plants compared to the fresh parts.

viii. Both plants did not show significant inhibition of CYP 3A4, compared to the standard drug, Ketoconazole. Therefore, they may not be expected to exhibit significant interactions with drugs metabolized by this isoform of CYP450.

Overall, the results of the present studies support the traditional medicinal use of *Hypoxis argentea*, with its probable mechanisms of action including stimulation of glucose uptake via insulin-dependent pathways in skeletal muscle or insulin-independent pathways in hepatocytes, as well as the probable stimulation of pancreatic β cell proliferation. In contrast, the toxicity of *Dianthus thunbergii* to hepatic and β cell lines, as well as RAW macrophages suggests that its use as an antidiabetic remedy must be with caution.

8.3. Future directions

The results obtained in the studies conducted in this PhD research have led us to encourage the following recommendations for future studies:

i. Bioassay-guided fractionation of crude extracts of *D. thunbergii* and *H. argentea* in order to purify fractions and/or isolate compounds responsible for the activities observed in the present studies. This will be useful to assess whether individual isolated compounds retain or lose their activity or whether they are more active than the parent crude extracts.

ii. Comprehensive toxicity studies using *in-vivo* models are necessary to establish a full toxicity profile for the two plants and to confirm the consistent cytotoxicity observed with *D. thunbergii*

iii. Assessment of the antidiabetic properties of *D. thunbergii* and *H. argentea* in *in vivo* models to confirm the activities observed *in vitro*.

iv. Evaluation of molecular mechanisms involved in the stimulation of glucose uptake and cellular proliferation by *H. argentea*

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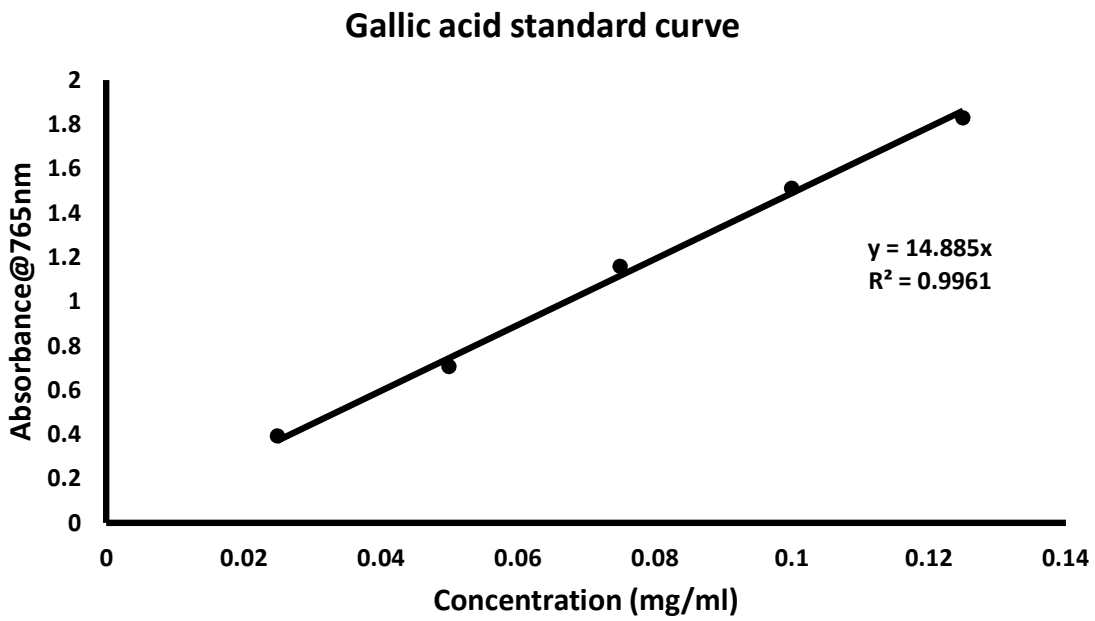
Appendix I

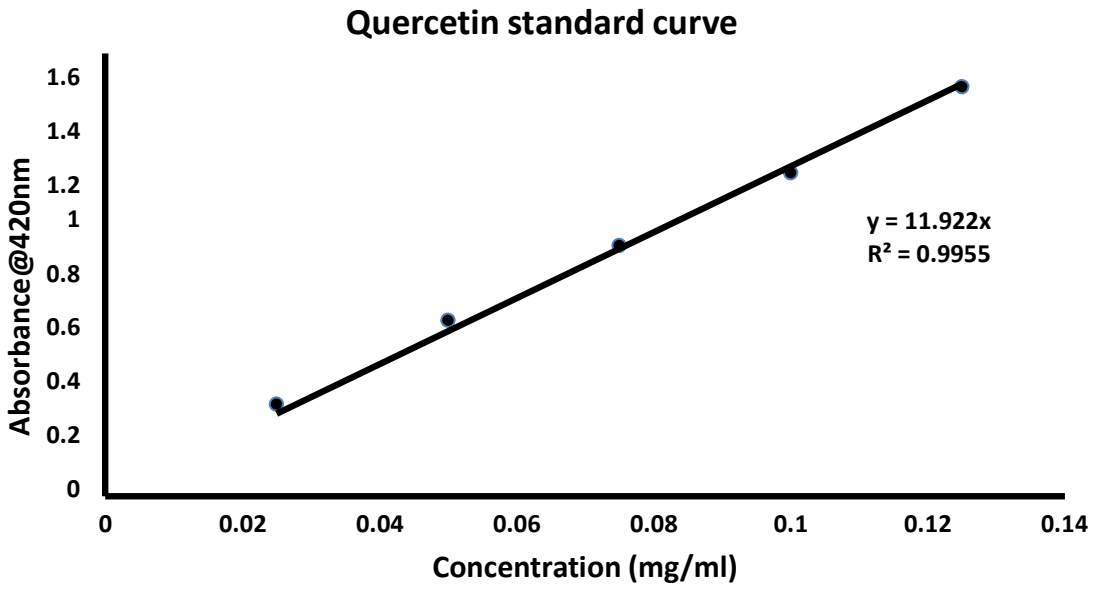
Appendix 1: Standard Curves For determination of Phytochemical Composition of *Dianthus thunbergii* and *Hypoxis argentea*

1. **GALLIC ACID STANDARD CURVE (Total Phenolic content)**



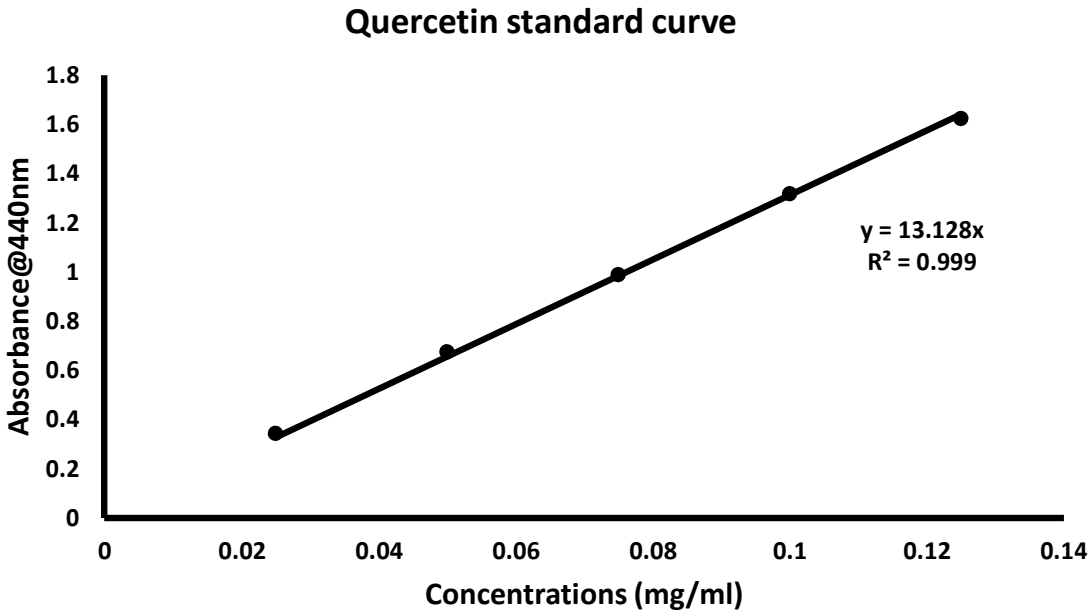
Concentrations (mg/ml)	Stock concentration (mg/ml)	Volume of Stock (ml)	Volume of diluent (ml)	Final volume (ml)	Absorbances@765nm (average of three replicates)
0.025	0.25	0.10	0.90	1.00	0.3927±0.011
0.050	0.25	0.20	0.80	1.00	0.7060±0.065
0.075	0.25	0.30	0.70	1.00	1.1590±0.061
0.100	0.25	0.40	0.60	1.00	1.5110±0.042
0.125	0.25	0.50	0.50	1.00	1.8283±0.012

2. QUERCETIN STANDARD CURVE (Total flavonoid content)



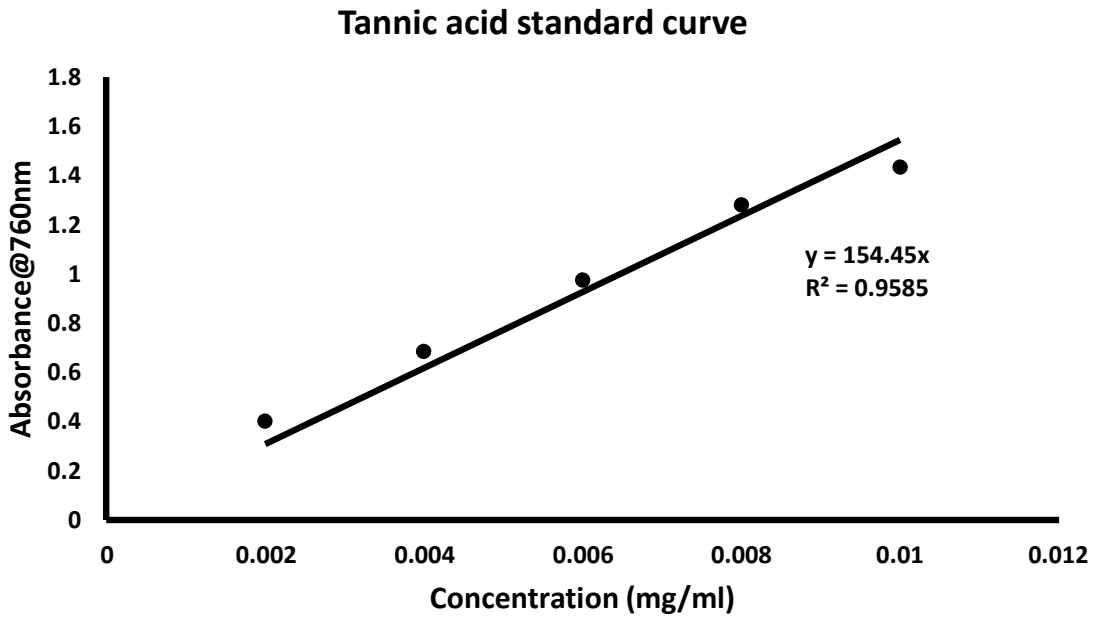
Concentrations (mg/ml)	Stock concentration (mg/ml)	Volume of Stock (ml)	Volume of diluent (ml)	Final volume (ml)	Absorbances@420nm (average of three replicates)
0.025	0.25	0.10	0.90	1.00	0.3323±0.022
0.050	0.25	0.20	0.80	1.00	0.6357±0.045
0.075	0.25	0.30	0.70	1.00	0.9063±0.011
0.100	0.25	0.40	0.60	1.00	1.1683±0.051
0.125	0.25	0.50	0.50	1.00	1.4793±0.018

3. QUERCETIN STANDARD CURVE (Total Flavonol content)



Concentrations (mg/ml)	Stock concentration (mg/ml)	Volume of Stock (ml)	Volume of diluent (ml)	Final volume (ml)	Absorbances@440nm (average of three replicates)
0.025	0.25	0.10	0.90	1.00	0.3447±0.016
0.050	0.25	0.20	0.80	1.00	0.6757±0.034
0.075	0.25	0.30	0.70	1.00	0.9890±0.004
0.100	0.25	0.40	0.60	1.00	1.3180±0.006
0.125	0.25	0.50	0.50	1.00	1.6233±0.021

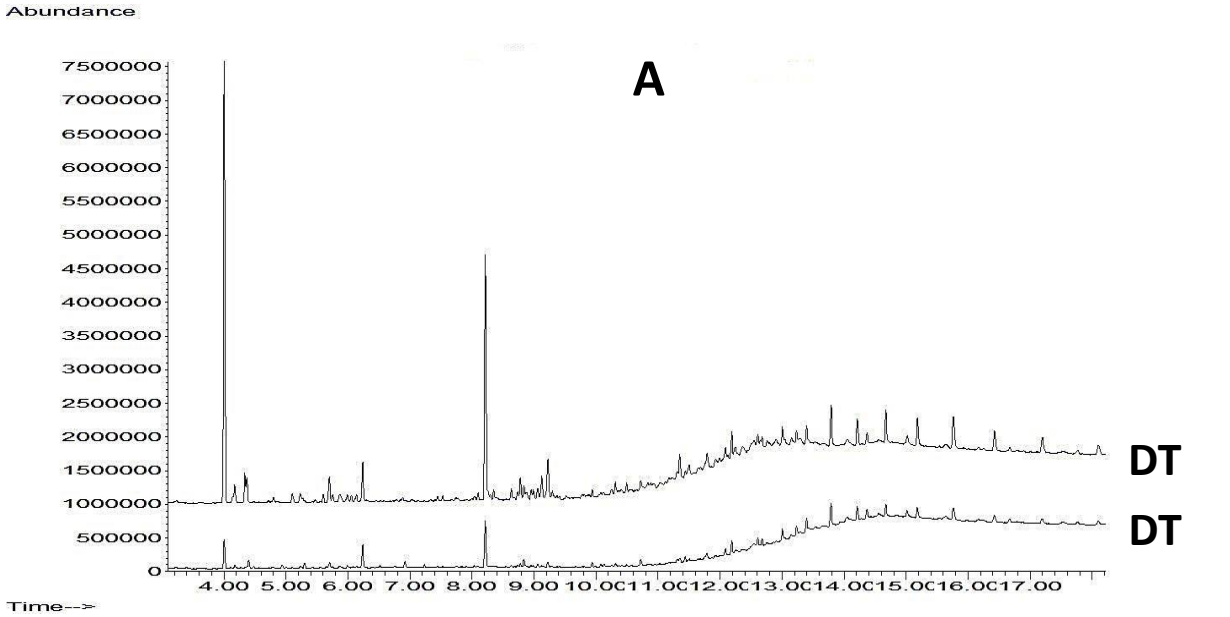
4. TANNIC ACID STANDARD CURVE (Tannic acid content)

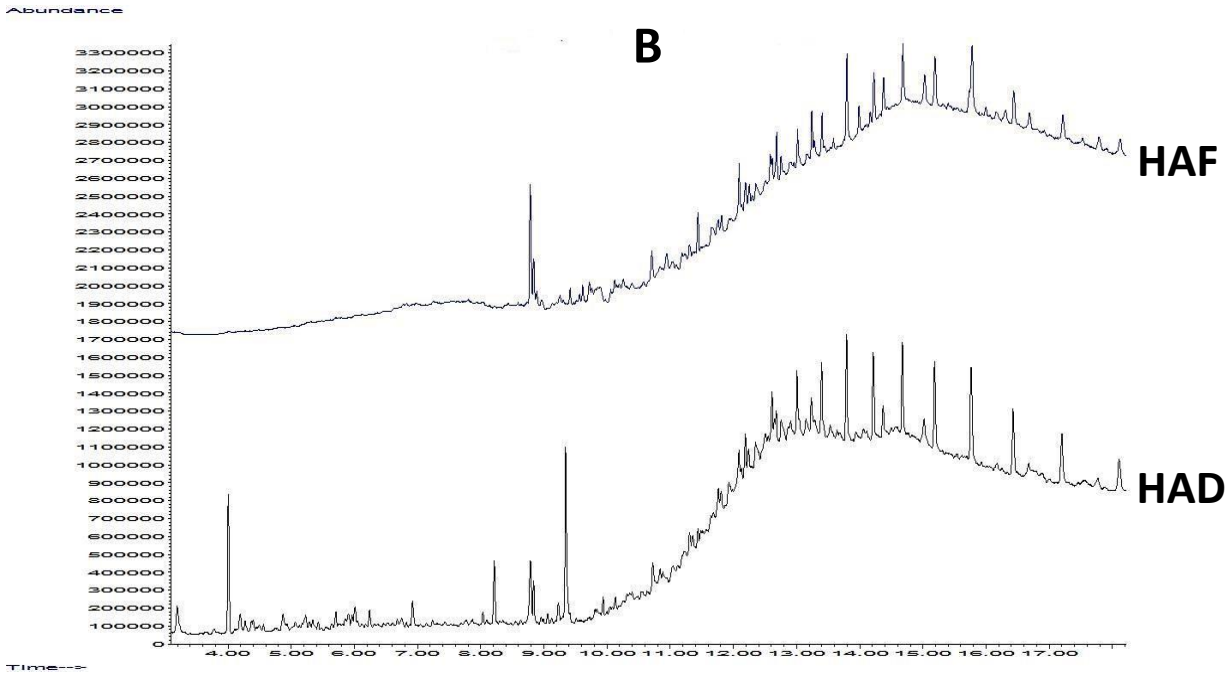


Concentrations (mg/ml)	Stock concentration (mg/ml)	Volume of Stock (ml)	Volume of diluent (ml)	Final volume (ml)	Absorbances@760nm (average of three replicates)
0.002	2.00	0.20	0.80	1.00	0.401±0.0180
0.004	2.00	0.40	0.60	1.00	0.685±0.0140
0.006	2.00	0.60	0.40	1.00	0.9753±0.009
0.008	2.00	0.80	0.20	1.00	1.281±0.015
0.010	2.00	1.00	0.00	1.00	1.4337±0.032

Appendix 2

Overlay of GC-MS Total ion chromatograms of essential oils from *Dianthus thunbergii* roots and *Hypoxis argentea* corms

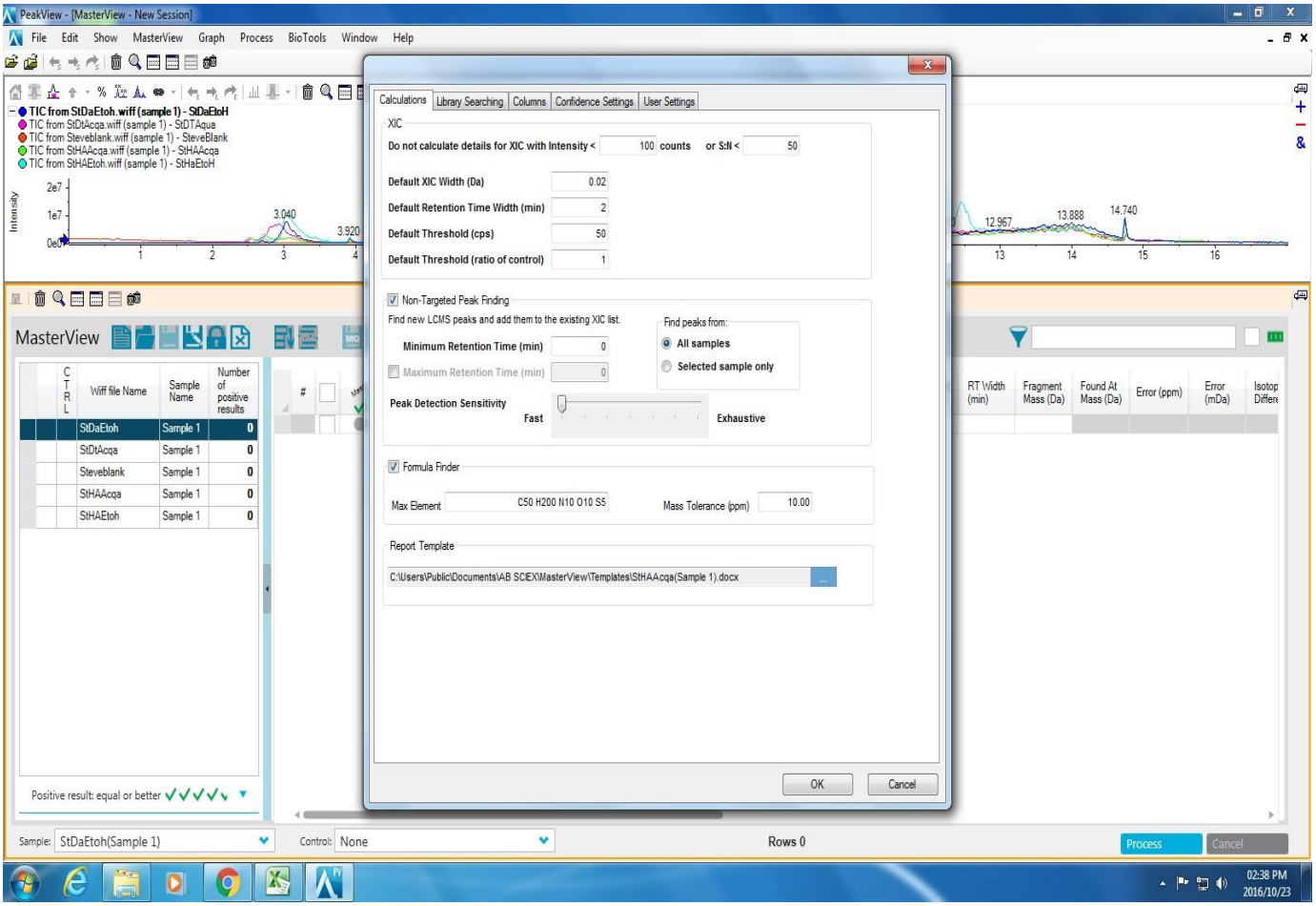


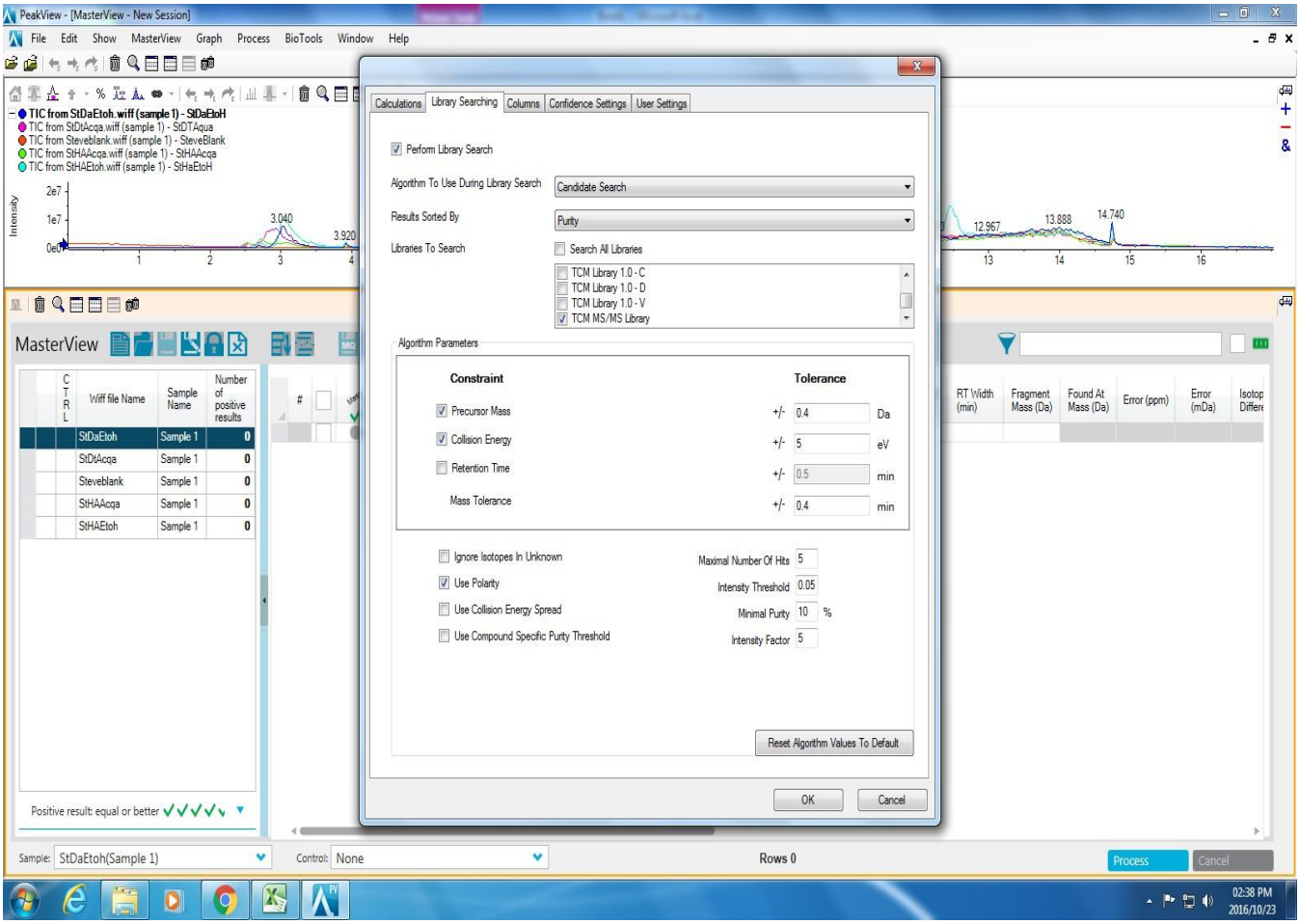


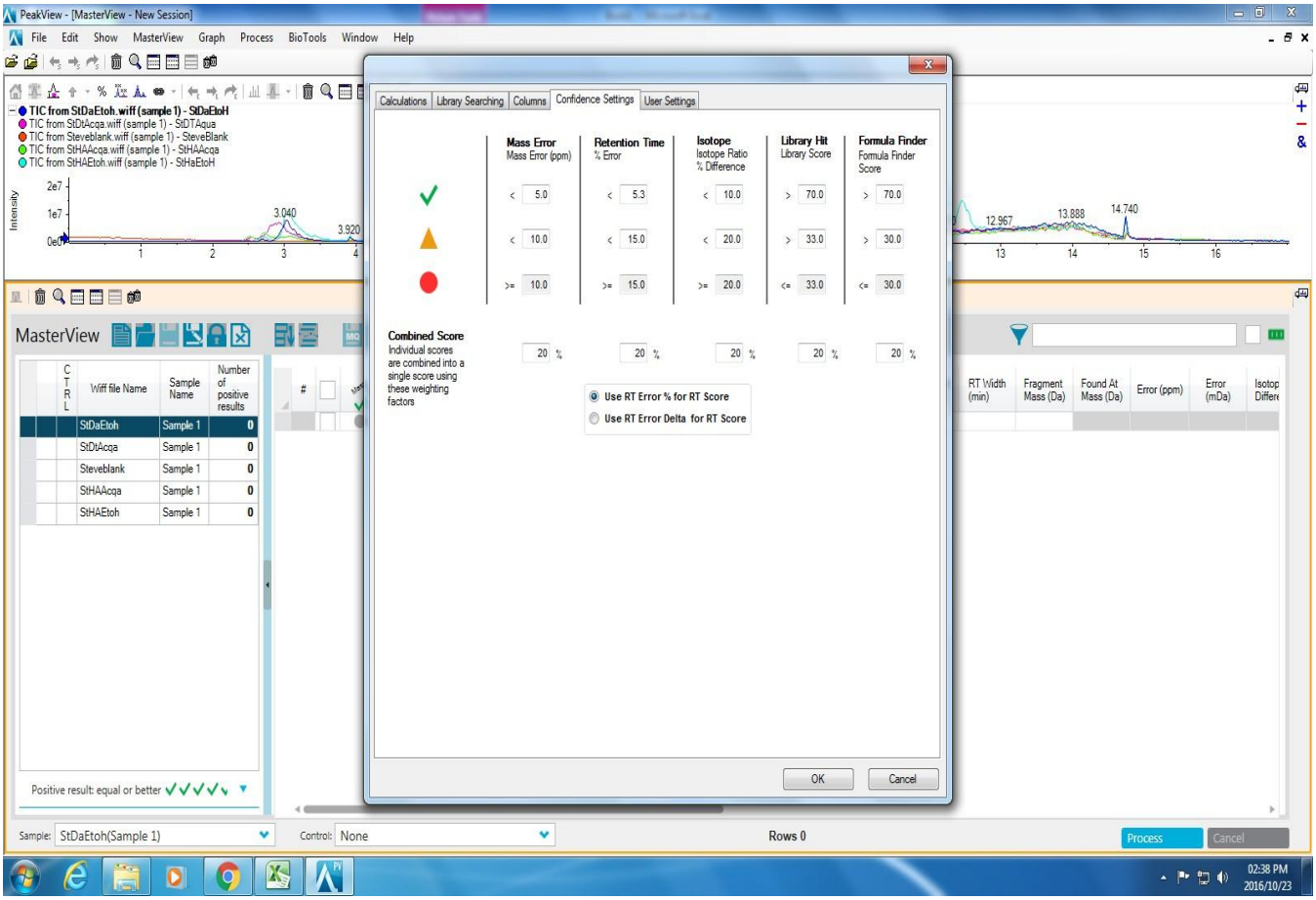
Overlay of Total ion chromatograms of the essential oils from (A) D. thunbergii roots, and (B) Hypoxis argentea corms. DTF = D. thunbergii fresh roots; DTD = D. thunbergii dried roots; HAF = H. argentea fresh roots; HAD = H. argentea dried roots.

Appendix 3

PeakView® and Master View® Interface and settings for data analysis of LC-MS data.




PeakView - [MasterView - New Session]
File Edit Show MasterView Graph Process BioTools Window Help
TIC from StDaEtoh.wiff (sample 1) - StDaEtoH
TIC from StDtAcqa.wiff (sample 1) - StDTAqua
TIC from Steveblank.wiff (sample 1) - SteveBlank
TIC from StHAAcqa.wiff (sample 1) - StHAAcqa
TIC from StHAEtoh.wiff (sample 1) - StHaEtoH
Intensity
3.040
3.920
12.967
13.888
14.740
MasterView
CTRL
Wiff file Name
Sample Name
Number of positive results
StDaEtoh Sample 1 0
StDtAcqa Sample 1 0
Steveblank Sample 1 0
StHAAcqa Sample 1 0
StHAEtoh Sample 1 0
Positive result: equal or better
RT Width (min)
Fragment Mass (Da)
Found At Mass (Da)
Error (ppm)
Error (mDa)
Isotop Differ
Calculations
Library Searching
Columns
Confidence Settings
User Settings
Perform Library Search
Algorithm To Use During Library Search
Candidate Search
Results Sorted By
Purity
Libraries To Search
Search All Libraries
TCM Library 1.0 - C
TCM Library 1.0 - D
TCM Library 1.0 - V
TCM MS/MS Library
Algorithm Parameters
Constraint
Tolerance
Precursor Mass +/- 0.4 Da
Collision Energy +/- 5 eV
Retention Time +/- 0.5 min
Mass Tolerance +/- 0.4 min
Ignore Isotopes In Unknown
Use Polarity
Use Collision Energy Spread
Use Compound Specific Purity Threshold
Maximal Number Of Hits 5
Intensity Threshold 0.05
Minimal Purity 10 %
Intensity Factor 5
Reset Algorithm Values To Default
OK
Cancel
Sample: StDaEtoh(Sample 1)
Control: None
Rows 0
Process
Cancel
02:38 PM
2016/10/23


PeakView - [MasterView - New Session]
File Edit Show MasterView Graph Process BioTools Window Help
TIC from StDaEtoh.wiff (sample 1) - StDaEtoH
TIC from StDtAcqa.wiff (sample 1) - StDTAqua
TIC from Steveblank.wiff (sample 1) - SteveBlank
TIC from StHAAcqa.wiff (sample 1) - StHAAcqa
TIC from StHAEtoh.wiff (sample 1) - StHaEtoH
Intensity
3.040
3.920
12.967
13.888
14.740
Calculations
Library Searching
Columns
Confidence Settings
User Settings
Mass Error
Mass Error (ppm)
Retention Time
% Error
Isotope
Isotope Ratio % Difference
Library Hit
Library Score
Formula Finder
Formula Finder Score
< 5.0
< 5.3
< 10.0
> 70.0
> 70.0
< 10.0
< 15.0
< 20.0
> 33.0
> 30.0
>= 10.0
>= 15.0
>= 20.0
<= 33.0
<= 30.0
Combined Score
Individual scores are combined into a single score using these weighting factors
20 %
20 %
20 %
20 %
20 %
Use RT Error % for RT Score
Use RT Error Delta for RT Score
OK
Cancel
MasterView
CTRL
Wiff file Name
Sample Name
Number of positive results
StDaEtoh Sample 1 0
StDtAcqa Sample 1 0
Steveblank Sample 1 0
StHAAcqa Sample 1 0
StHAEtoh Sample 1 0
Positive result: equal or better
RT Width (min)
Fragment Mass (Da)
Found At Mass (Da)
Error (ppm)
Error (mDa)
Isotop Differ
Sample: StDaEtoh(Sample 1)
Control: None
Rows 0
Process
Cancel
02:38 PM
2016/10/23

Appendix 4

Publications/ Conferences attended and papers presented

Conferences attended and papers presented:

1. 25th South African Society of Biochemistry and Molecular Biology (SASBMB) conference, International Convention Centre, East London, South Africa. July 10-13, 2016.

   Paper presented: Phytochemical composition, cytotoxicity screening and antioxidant activities of the essential oils and extracts of *Hypoxis argentea* L. corms (Poster)

2. 1st Society of Medicinal plants for Economic Development (SOMPED) conference, Kopanong Hotel and Conference Centre, August 14-17, 2016.

   Paper presented: *In-vitro* anti-diabetic properties and cytotoxicity of *Dianthus thunbergii* and *Hypoxis argentea*, plants used to treat diabetes in South Africa (Oral).

Publications being prepared from the thesis:

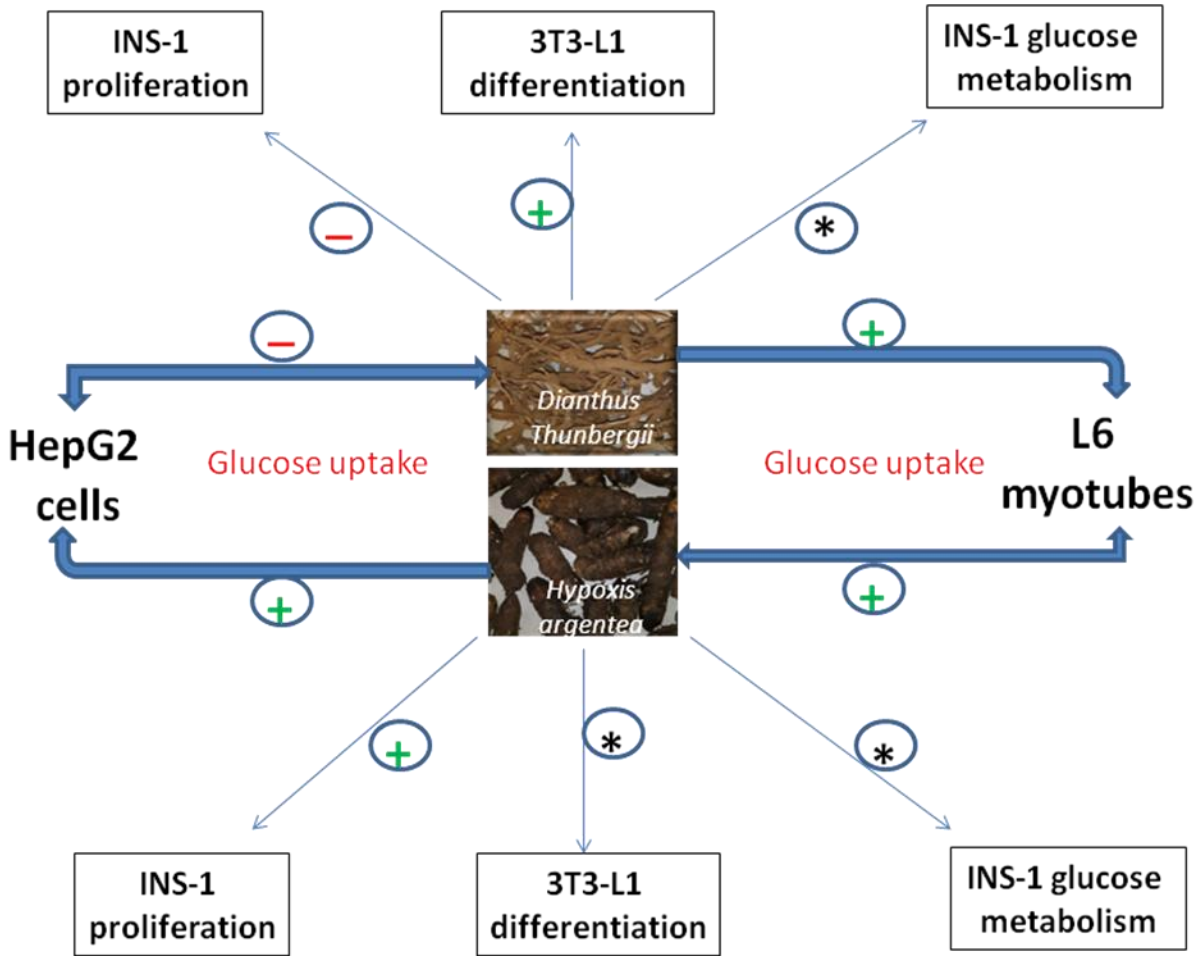
1. Phytochemical composition and antioxidant activities of *Dianthus thunbergii* (Hooper) and *Hypoxis argentea* (Harv. ex Baker) – plants used for the management of diabetes mellitus in Eastern Cape, South Africa.

2. Terpenoid profiles of the essential oils of the underground parts of *Dianthus thunbergii* and *Hypoxis argentea* are affected by pre-distillation drying.

3. The Antidiabetic properties of *Hypoxis argentea* Harv Ex Baker are mediated by the stimulation of glucose uptake in HepG2 and L6 cells without inducing adipogenesis in 3T3-L1 pre-adipocytes

4. *In vitro* cytotoxic and anti-proliferative activities of *Dianthus thunbergii* aqueous root extracts against HepG2 (human carcinoma) and INS-1 (murine insulinoma) cells

Appendix 5: Summary of the anti-diabetic properties shown by extracts of *D. thunbergii* and *H. argentea* in cell-based in vitro bioassays

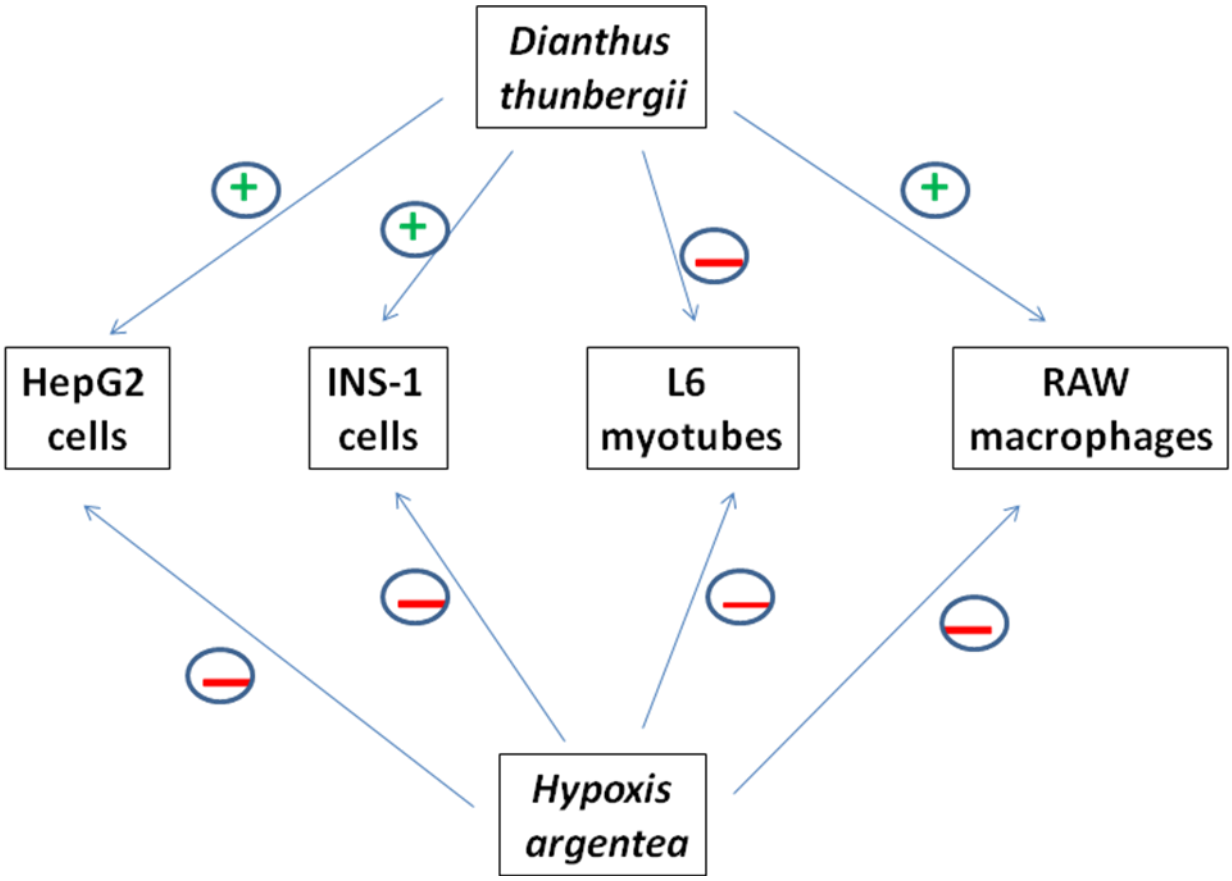


+ = stimulation

_ = inhibition

*** = no change**

Appendix 6: Toxicological properties of *D. thunbergii* and *H. argentea* in HepG2, INS-1 cells, L6 myotubes and RAW macrophages



+ = toxic

_ = non-toxic