Antidiabetic activity and mechanism of action of extracts of *Brachylaena elliptica* (Thurb.) DC. and *Brachylaena ilicifolia* (Lam) Phill & Schweick

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DECLARATION

I Idowu Jonas Sagbo, declare that this thesis and the work therein submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture, Department of Biochemistry and Microbiology was carried out by me and it has never been submitted for any degree at this University or any other University.

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LIST OF ABBREVIATIONS

α	Alpha
ADA	American Diabetes Association
ADP	Adenosine Diphosphate
AGEs	Advanced Glycation End Products
AKT	Protein kinase B
AMPK	Adenosine monophosphate –activated protein kinase
ATCC	American Type Culture Collection
ATP	ATP Adenosine triphosphate
β	Beta
BHT	Butylated Hydroxy Toluene
BSA	Bovine Serum Albumin
CBL	Cbl Associated Protein
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase-IV
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's Minimal Essential Medium
NFK_B	Nuclear factor

	kappa B
nM	NanoMolar
NO	Nitric oxide
FBS	Fetal Bovine Serum
FCS	Fetal calf serum
FFA	Free Fatty Acid
GABA	Gamma aminobutyric acid
GDM	Gestational diabetes mellitus
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-Like Peptide-1
GLUT	Glucose Transporter
HCl	Hydrochloric acid
IC_{50}	Inhibitory concentration at 50%
IDDM	Insulin dependent diabetes
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
IRS1	Insulin Receptor Substrate 1
IRS2	Insulin Receptor Substrate 2
m	Milli
M	M -Molar
MAPK	Ras/Mitogen-Activated Protein Kinases

MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
NaCl	Sodium chloride
NaCO3	Sodium carbonate
NADH	Nicotinamide Adenine Dinucleotide
NIDDM	Non-insulin-dependent diabetes mellitus
P	Probability
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline (without Ca^{2+} and $Mg2^{+}$)
PI3K	Phosphoinositide 3-kinase
PDKI	Phosphatidylinositol-3,4,5-triphosphate-dependent kinase
PIP2	phosphatidylinositol -4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5- triphosphate
PH	Pleckstrin homolog
PKA	Protein kinase A
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PP	Pancreatic Polypeptide
RAS	Rat sarcoma protein
rcf	Relative centrifugal force
rpm	Revolutions per minute
RPMI	RPMI 1640 Roswell Park Memorial Institute culture medium
SD	Standard deviation

SHC	Adaptor protein with src-homology
SUR	Sulfonylurea receptor
Tris	Tris (hydroxymethyl) aminomethane
μ	Micro
WHO	World Health Organisation

ABSTRACT

International Diabetes Federation (IDF) estimated that there are 415 million people globally that are suffering from diabetes and this figure is likely to double by 2040. In South Africa, the number of people suffering from diabetes is believed to be rising steadily and the current antidiabetic therapies include the use of expensive pharmaceutical drugs which after display numerous adverse side effects. Herbal medicine is an alternative treatment strategy used by many rural populations for the management of diabetes, which is cost-effective and with minimal reported side effects. The aim of this study was to validate the antidiabetic activity and to elucidate the possible mechanisms of action of the aqueous leaf extracts prepared from two plants, *Brachylaena elliptica* and *Brachylaena ilicifolia*, used by traditional healers in the Eastern Cape, South Africa for the treatment of diabetes.

The current pharmaceutical drugs target site, including carbohydrate digestion, regulation of blood glucose levels through insulin levels or activating glucose uptake in liver and muscle. The plants extracts were therefore evaluated for their potential to mimic or replace these drugs. The inhibibitory effects on alpha amylase, alpha glucosidase, DPP-IV activity and pancreatic lipase were evaluated at various concentrations of the plant extracts. To evaluate the suitability of these extracts for cell based *in vitro* assay as well as to determine the safety of the plant extracts for human consumption, cytotoxicity towards HepG2 cells was assessed using the MTT assay. The effect of both plant extracts on glucose uptake in HepG2 and L6 cells, lipid accumulation in 3T3-L1 preadipocytes and NO production in RAW macrophages were investigated. Both plant extracts were also tested for INS-1 proliferation using the imageXpress®Micro XLS analysis. The MTT reduction assay was also used to investigate the effect of extracts on INS-1 glucose metabolism and as a reflection of insulin secretion. The inhibitory activity of the crude extracts

of both plants on protein glycation, collagenase activity and CYP3A4 interaction were also evaluated

The crude extracts of both plants displayed no significant inhibition on alpha amylase, alpha glucosidase, dipeptidyl peptidase-IV, lipase, protein glycation and collagenase compared to relevant standard inhibitors, acarbose, EGCG, Diprotin A, Orlistart and aminoguanidine. The mild inhibition of *B. elliptica* on alpha glucosidase activity at the highest concentration of the extract tested, was considered as not physiologically relevant. The aqueous extracts of both plants showed < 40% cell death in HepG2 cells even at high concentration and hence support the use of the plants by traditional healers. The extracts of *B. elliptica* at 25 µg/ml, displayed a proliferative effect on INS-1 cells when compared to the negative control and *B. ilicifolia* at 25 µg/ml. The treatment of HepG2 cells with 100 µg/ml *B. elliptica* and *B. ilicifolia* extracts revealed a higher glucose uptake of 121 % and 123 % respectively when compared to the pharmaceutical drug berberine. However, both plants extracts displayed weak significant glucose uptake in L6 muscle cell line at all the tested concentrations when compared to insulin at 6 µg/ml. The crude extracts of both plants demonstrated no significant effect on 3T3-L1 lipid accumulation and INS-1 glucose metabolism. The extracts of both plants strongly reduced NO production in RAW macrophages at the highest concentration (100 µg/ml) tested in this study, which at least in part, may be explained by the flavanoids and phenols content of these plants. In addition, the crude extracts of both plants displayed weak significant inhibition on the CYP3A4 activity indicating that both plant extracts do not interfere with the cytochrome P450 activation or metabolism of anti-diabetic drugs, that may be taken together with the plant extracts.

In conclusion, the results obtained from this study revealed that both plants could possibly exert their hypoglycemic effect via interactions with insulin receptor, thereby enhancing glucose metabolism as well as maintaining and repairing the health state and function of the pancreatic beta cells. The findings in this study, therefore provide evidence that *B. elliptica* and *B. ilicifolia* possess antidiabetic properties, and support their folkloric use for the management of diabetes. However, due to the potential toxicity of both plant extracts, they must be prescribed with caution.

Keywords: *B. elliptica*; *B ilicifolia*; diabetes melltus; toxicity; glucose metabolism; protein glycation;

INTELLECTUAL PROPERTY AGREEMENT STATEMENT COMPLIANCE STATEMENT

This thesis intended to be used for information dissemination; therefore no part of this study in any form has been commercialized. The study, investigates the medicinal ability of *Brachylaena elliptica* and *Brachylaena ilicifolia* to the immediate community and the entire Eastern Cape Province of South Africa.

Supervisor signature Student signature

CHAPTER 1

General Introduction and Literature Review

CHAPTER 1

1.1 Diabetes mellitus

Diabetes mellitus is known as a serious, complex condition, a group of disorders that have hyperglycemia and glucose intolerance as their hallmark, due to insulin deficiency or to the impaired effectiveness of insulin's action, or both (Deutschländer, 2010). It is also described as a chronic disease associated with elevated levels of glucose in the bloodstream which causes glycation of the body protein leading to severe complications (Rang *et al*., 1991; Vaidehi & Yogalakshmi, 2015). Complications include neuropathy (gradual damaging of the nerves), retinopathy (gradual damaging of the eyes), nephropathy (gradual damaging of the kidneys), polyuria, visual blurriness, disproportionate thirst, fatigue, irritability, weight loss and hypertension (Forbes & Cooper, 2013). Presently, there is no cure for diabetes however by controlling blood glucose levels through the use of oral hypoglycemic drugs, insulin injection and life style modifications such as healthy diet, medication and exercise, the complications associated with diabetes can be reduced. In recent times, attention has been centered on various antidiabetic plants used in the treatment of diabetes (Rahimi, 2015) by virtue of their bioactive compounds with antidiabetic properties.

1.2 Types of diabetes mellitus

There are basically three different types of diabetes mellitus namely: insulin dependent diabetes mellitus (IDDM or type I diabetes), non-insulin dependent diabetes mellitus (NIDDM or type II diabetes) and gestational diabetes mellitus (GDM or type III diabetes) (ADA, 2006).

1.2.1 Insulin dependent diabetes mellitus (IDDM or type I diabetes)

Type 1 diabetes is the most well-known kind of diabetes among children and adults. It is caused by the lack of insulin production and is usually characterized by insulin antibodies or islet cells or to an autoimmune process that leads to the destruction of beta- cell of pancreas (Notkins, 2002). Insulin dependent diabetes mellitus (IDDM) can be rapidly fatal and is associated with frequent urine production (polyuria), thirst (polydipsia), weight loss, vision changes and fatigue (ADA, 2006). Patients affected by this type of diabetes are completely reliant on exogenous insulin to maintain a normal life style (ADA, 2006).

1.2.2 Non-insulin-dependent diabetes mellitus (NIDDM or type II diabetes)

Type II diabetes results from the inability of the body to produce enough insulin for proper functioning or insulin resistance in the liver, muscle or fat cell (insulin resistance). It is also characterized by hyperglycemia during fasting and after eating and relative insulin inefficiency. If type II diabetes is left untreated it might result in hyperglycemia which could lead to long-term microvascular and macrovascular complications such as neuropathy, nephropathy, retinopathy and atherosclerosis (Jain & Saraf, 2008). In adults, type II diabetes represents close to 90% to 95% of all diagnosed cases of diabetes (Olokoba *et al*., 2012)**.**

1.2.3 Gestational diabetes mellitus (GDM or type III diabetes)

Gestational diabetes is a common disorder that occurs mostly during pregnancy due to hormonal changes or insulin deficiency (Oyedemi, 2010). It affects about 7% of pregnant women every year and if not properly managed can increase the risk of developing type II diabetes for both the

mother and baby (ADA, 2000). In most cases gestational diabetes does not have any noticeable signs or symptoms (ADA, 2000).

1.3 Prevalence of diabetes mellitus

Diabetes mellitus is currently one of the most well-known metabolic diseases worldwide. It is the third or fourth leading cause of death in most developed nations after strokes, heart attacks and dementias (Carissa, 2016). In the last two decades the number of individuals diagnosed with diabetes has erupted to record high with further increase expected. The International diabetes federation (IDF) estimated that there are 415 million (one in 11 adults has diabetes) people globally that are suffering from this deadly disease and this figure is expected to rise to 642 million (one adult in ten will have diabetes) by 2040 (IDF, 2015). The World Health Organization also pointed out that if the current trend continues, diabetes will be the second highest killer by 2040 unless strong and intensive measures are made by governments, communities and individuals (WHO, 2016). These are part of a campaign to promote awareness of the burden of diabetes and the urgent need to step up action for its prevention and control. Globally, India leads in terms of the highest number of people with diabetes, followed by China and United States of America (Mascarenhas, 2016). Asia and Africa have been identified as regions with high diabetic populations which could increase above expected levels if not given urgent attention (www.idf.org/diabetesatlas). In 2015, there were 2.28 million cases of diabetes reported in South Africa (IDF, 2015) equivalent to 7.0% of adults between the age of 20 and 79 year (www.idf.org/diabetesatlas). The prevalence of diabetes is very high and common among the Indian community in South Africa estimated to be 11-13% as this group has a strong hereditary predisposition for diabetes (Centre for Diabetes and Endocrinology, 2014). This is

followed by 8-10% in the colored community, 5-8% among Africa community and 4% among the European community (Centre for Diabetes and Endocrinology, 2014). However, it has been reported by the IDF that the high rate of diabetes cases in South Africa is closely linked to the rapid cultural and social changes, increasing urbanization, ageing populations, unhealthy eating, diet changes and less physical activity (www.idf.org/diabetesatlas).

Nevertheless, despite the development of modern medicine, this resulted in the introduction of modern oral antidiabetic drugs. These drugs are very expensive beyond the reach of diabetic patients, especially those living in the rural areas and also cause adverse side effects (Inzucchi *et al*., 2012). Therefore, there is still need to search for natural product from plants that are less costly and as well, complementing or negating the adverse side effects of the currently used antidiabetic drugs.

1.4. Glucose metabolism

The body requires fuel to provide energy for its to function. The fuel the body needs originates from the food we eat, which are made up of carbohydrates (glucose and starch), proteins and fats. Glycolysis is a biological process which involves the enzymatic breakdown of carbohydrate (as glucose) by way of phosphate derivatives resulting in the production of pyruvate ($CH_3COCOO^- + H^+$) to provide cells with energy. When glucose enters the cell, the it undergoes a series of insulin-induced enzymatic reactions, which include glucose phosphorylation, glycogen synthesis and glucose oxidation (DeFronzo, 2004). The release of free energy from this process is used to fuel cells by forming the high energy adenosine-5'- triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH), which are essential for the proper

functioning of metabolic pathway in a cell. Once in the cell, glucose is phosphorylated by the hexokinase glycolysis enzymes to form glucose-6-phosphate. This glucose-6-phosphate is either stored (polymerized into glycogen or converted to fat) or catabolised in the glycolytic pathway to generate energy in the form of ATP (Mathews *et al*., 2000).

1.4.1 Glucose metabolism in skeletal, liver and adipose tissues

The homeostasis of glucose is maintained by three key organs/tissues; i.e. the skeletal muscle, liver and fat/adipose. The human body sustains the supply of glucose to the cells by maintaining a stable concentration of glucose in the blood (Brunner *et al*., 2009). Generally, glucose is mostly used by the brain and muscles. However, the brain cells use about 50% of the total glucose uptake in an insulin independent manner (DeFronzo, 2004). The liver and gastrointestinal tissues also account for about 25% of the total glucose uptake in an insulin independent manner, whereas the glucose uptake in the skeletal muscle and adipose tissues is insulin stimulated with roughly 80-85% of glucose uptake (DeFronzo, 2004).

The skeletal muscle has been considered to play a vital role in the maintenance of systemic glucose homeostasis and in regulating whole-body carbohydrate metabolism. In this tissue glucose is taken up from circulation in response to insulin and converted to lactate by glycolysis process or stored as glycogen. Glucose uptake in the skeletal muscle is facilitated by GLUT 4 (glucose transporter 4), and is then stored in the intracellular vesicles in the absence of insulin (Mueckler, 1994). During starvation states and when glucose concentration is low the skeletal muscle circulates fatty acids by β-oxidation and/or ketogenesis; glycogen stores are also re-

converted to glucose in the process called glycogenolysis. During starvation states, proteins are also broken down into amino acid by protease enzymes (DeFronzo, 2004).

The liver also plays a key role in normal glucose homeostasis. It takes up glucose through GLUT 2 (glucose transporter 2) localized at the plasma membrane (Karim *et al*., 2012). In addition, it also balances the uptake and storage of glucose through glycogenesis and the release of glucose via glycogenolysis and gluconeogenesis (Nordlie *et al*., 1999; Karim *et al*., 2012). During periods of starvation, glucagon is secreted which then activates the liver glycogen and thus leads to glucose synthesis via gluconeogenesis pathway resulting in the depletion of glycogen (Voet *et al*., 2002).

The Adipose tissue is one of the major sites for the uptake of glucose and fatty acids. It accounts for only 4-5% of glucose disposal in peripheral tissues. In the presence of insulin, the adipose tissue takes up lipids and glucose from the blood and stores them as triacylglycerols (Fielding & Frayn, 1998; Chellan, 2011). Adipose tissues secrete adipocytokines and control the release of free fatty acids from stored triglycerides (DeFronzo, 2004). The adipocytokines and free fatty acids help stimulate insulin sensitivity in the liver and muscle (DeFronzo, 2004; Chellan, 2011).

1.5 Insulin metabolism

1.5.1 The endocrine pancreas

The pancreas has two principal functions: an exocrine function that helps to produce and releases digestives enzymes (trypsin, chymotrypsin elastase, amylase and others) during digestion and an endocrine function which is responsible for the control of glucose metabolism (Deutschländer,

2010). The endocrine part of the pancreas is located in the islet cells that produce and release important hormones directly into the blood stream. These islets are comprised of four main cellular components namely: alpha (α-cells), beta (β-cells), deta (δ-cells) and pancreatic polypeptide (PP cells) (Nunemaker & Satin, 2005). Each cell type secretes an important hormone: the alpha cells secrete glucagon, inducing a catabolic effect by activating glycogenolytic activity in the liver; the beta cells produce insulin, which helps in the control of blood glucose level; the delta cells produce somatostatin, which suppresses both the insulin and glucagon release; and the PP cells contain a unique pancreatic polypeptide, of which its exact biological role remains unknown (Williams, 2014). The only physiological effects that are recognized in humans are the inhibition of gall bladder contraction and pancreatic enzyme secretion (Williams, 2014).

1.5.2 Insulin

The name insulin is derived from the Latin word "insula" for "island" from the cells that secrete the hormone in the pancreas (Patlat, 2002). Insulin is a polypeptide hormone produced by the β-cells in response to increase blood glucose levels. The main function of this hormone is to maintain normal glucose homeostasis by reducing hepatic glucose production via gluconeogenesis and glycogenolysis. It also helps in stimulating glucose uptake primarily by the skeletal muscle and to a smaller extent the adipose tissue. Insulin has a molecular weight of 5802 Daltons with two subunits (A and B-chain) linked together by disulphide bonds (Wilcox, 2005). The hormone is secreted in substantial amounts as preproinsulin in the ribosome of endoplasmic reticulum. The enzymatic cleavage of this preproinsulin molecule then leads to the formation of

proinsulin which later matures into equal amounts of insulin and C-peptide through the action of an enzyme called the endo-peptidases (Wilcox, 2005).

1.5.3 Mechanisms of insulin secretion

Insulin secretion in β-cells is triggered by the elevated levels of glucose in the blood. Physiologically, glucose enters the β- cell via GLUT 2 (Glucose transporter 2). Glucose is then phosphorylated by hexokinase IV (glucokinase) and undergoes glycolysis and further metabolized to yield ATP via the mitochondrial TCA cycle and oxidative phosphorylation causing an increase in the ATP to adenosine diphosphate (ADP) ratio (Figure 1). This increase inactivates the ATP-sensitive potassium channels (K^{+}_{ATP} channels), resulting in depolarization of the cell membrane. The voltage-gated calcium channels open, allowing calcium ions to flow into the cell. The ensuring influx of calcium ion leads to the exocytosis release of insulin containing granules to release insulin (Yang & Gillis, 2004; Chellan, 2011). Even though the secretion of insulin is initiated by some amino acids, fatty acid and ketone bodies the primary initiation is caused by the high concentration of glucose (De Marchi *et al*., 2014).

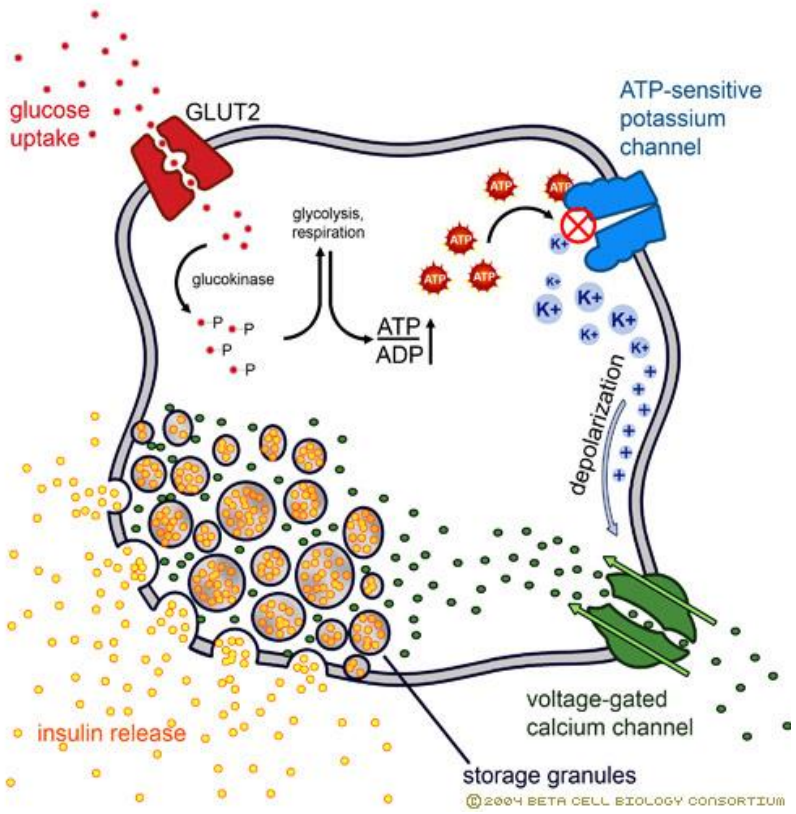

Figure 1: Mechanism of insulin secretion in normal pancreatic beta cell. (Taken from Chellan, 2011) .

1.5.4 Insulin degradation

Insulin degradation plays a vital role in regulating insulin activity by removing and inactivating the hormone, through hydrolysis of the disulphide connection between the A and B chain by the action of insulinase (Hulse *et al*., 2009). After this reductive cleavage further degradation by proteolysis occurs. The major site of insulin degradation and clearance is the liver and kidney (Duckworth *et al*., 2014). The liver is also accountable for eliminating insulin that is in the bloodstream while the kidneys are mainly responsible for eliminating insulin that is in normal circulation (Duckworth *et al*., 2014). The liver removes about 60% of the insulin released from the β cell by virtue of its location at the terminal site of the portal vein blood flow, while kidneys

removing approximately 50% of the pheripheral insulin. Additionally, the kidney removes about 50% of the circulating proinsulin and 70% of the c-peptide by glomerular filtration. The degradation of insulin also takes place within the insulin granule, and insulin is also degraded in other tissues after binding to the insulin receptor. According to Nolte and Karam (2001) the half-life of circulating insulin is estimated to be about 3-5 minutes.

1.5.5 Insulin receptor (IR)

Cell membranes are embedded with distinct cognitive molecules which function as receptors precisely to particular hormones, neurotransmitters and intercellular messengers. The actions of insulin in the muscle, liver and fat cells are facilitated by the insulin receptor (IR) (Pramfalk *et al*., 2004). The IR is situated in the plasma membrane and is described as a transmembrane, heterotetrameric protein that contains an insulin-stimulated tyrosine kinase activity (Kido *et al*., 2001). This receptor consists of two extracellular α-subunits that are each linked to a ß-subunit and to each other by disulfide bonds. The α-subunits contain the insulin binding sites while the β-subunit is comprised of an extracellular domain, a transmembrane domain and an intracellular domain which possesses the intrinsic tyrosine kinase activity (Figure 2).

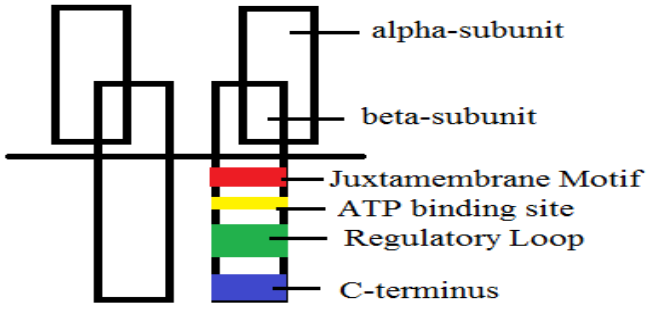

Figure 2: Model of the structure of the insulin receptor. The insulin receptor is a transmembrane receptor that is activated by insulin (taken from Suran, 2015).

1.5.6 Insulin signalling

Insulin is one of the most effective pleiotropic anabolic agents known (Porte, 2006). It helps in promoting the storage and synthesis of carbohydrates, proteins and lipids as well as inhibiting their breakdown and release into the circulation (Saltier & Kahn 2001). Insulin's increases of energy storage or utilization involves the regulated transport of glucose into the various cells types such as the muscle and adipose cells, mediated by GLUT 4 (glucose transporter 4) (Nedachi & Kanzaki, 2006). As illustrated in Figure 3 below, insulin binds to the α-subunit of the receptor leading to activation of the insulin receptor intrinsic tyrosine kinase activity in the β-subunits. This protein tyrosine kinase phosphorylates itself as well as targets different substrate adapters such as the insulin receptor substrates (IRS-1 and IRS-2), Cbl and $p52^{Sho}$ (Galic *et al*., 2005). The IRS-1 plays an important role by stimulating glucose uptake in the skeletal muscle and adipose tissue, whereas the IRS-2 functions mostly in the liver. The phosphorylated IRS serves as a multiside docking protein for enzymes such as the phosphatidylinositol-3-kinase (PI3-K) and other proteins that lack enzymatic activity but which link the IRS-1 and other intracellular signaling systems, for example the adaptor protein Grb2, which connects with the RAS/MAPK (mitogen activated protein kinase) pathway (Sesti, 2006).

Phosphatidylinositol-3-kinase (PI3-K) is a heterodimeric lipid kinase possessing a significant role in the metabolic and mitogenic actions of insulin (Elliot, 2009). It is also reported to promote the translocation of the glucose transporter protein, protein synthesis and the control of hepatic gluconeogenesis (Burks & White, 2001;Wilcox, 2005). Activation of PI3K, phosphorylates membrane phospholipids (Phosphatidylinositol biphosphate) into phosphatidylinositol triphosphates (PIP_3) (Anderson *et al*., 2001; Hickman *et al*., 2002 ;

Bugianesi *et al*., 2005; Shaodong, 2014) (Figure 3). The phosphatidylinositol triphosphates (PIP_3) travels along the membrane to activate a PIP_3- dependent protein kinase (PDK-1), an isoform of protein kinase C (PKC), thereby leading to the activation of an Akt, which is also known as protein kinase B (PKB). The Akt stimulates movement of the glucose membrane transporter (GLUT 4 or 2) resulting in increase in glucose uptake, glycogen synthase and glycogen formation. Table 1 shows the distribution and function of GLUT 1-5.

The MAPK cascade is mainly involved in gene regulatory responses in insulin-sensitive tissues and does not play any role in the control of glucose transport (Elliot, 2009). The Ras/MAPK pathway is largely involved in facilitating cell growth, cellular differentiation and cell survival (Taniguchi *et al*., 2006; Shaodong, 2014). The MAPKs belong to a family of intracellular protein kinases, whose activity is regulated by phosphorylation of their activation loop on conserved threonine and tyrosine residues (Pimienta & Pascual, 2007). This pathway is activated by a plentiful array of stimuli; thereby phosphorylating numerous proteins such as the transcription factors, the cytoskeletal proteins, kinases and other enzymes that influence gene expression, cell division, cell survival metabolism and cell morphology (Figure 3) (Qi & Elion, 2005).

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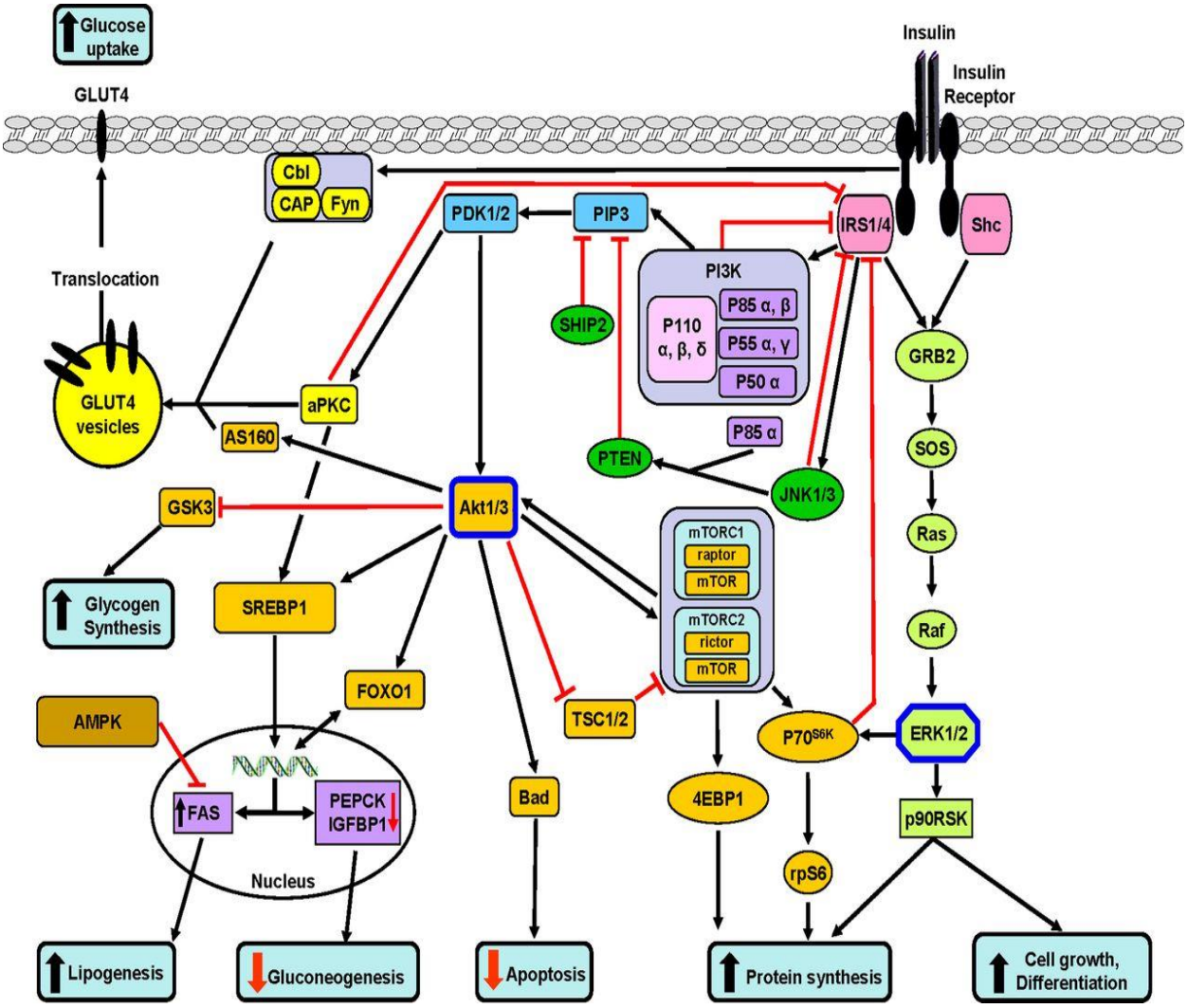

Figure 3: Signal transduction pathway activated by insulin binding to the insulin receptor (taken from www. Biochemj.org).

Table 1: The glucose transporters (GLUTs) and their tissue distribution and functions

GLUCOSE TRANSPORTER	TISSUES	FUNCTION
GLUT -1	All tissues, most especially red blood cells and the brain	Basal glucose uptake
GLUT-2	Liver, gut, kidney and pancreatic beta cells	Regulation of insulin release and other aspects of glucose homeostasis.
GLUT-3	Kidney, placenta, brain	Uptake into neurons and other tissues.
GLUT-4	Skeletal muscle and adipose tissues	Insulin- facilitated uptake of glucose
GLUT-5	Kidney, intestine, testes and red blood cells	Absorption of fructose

1.6 Oxidative stress and diabetes mellitus

Oxidative stress occurs as a results of excessive reactive oxygen species (ROS) in the absence or presence of reduced antioxidant substances, resulting in lipid peroxidation, structural deterioration and instability of the macromolecules, thus affecting proper cellular signaling pathways (Pop-Busui *et al.*, 2006) (Figure 4). Researchers reported that oxidative stress plays a major role in the pathogenesis of several degenerative diseases such as heart disease, cataracts, cognitive dysfunction, cancer and diabetes (Pietta, 2000). In diabetes, persistent hyperglycemia causes increased production of free radicals most especially the reactive oxygen species (ROS).

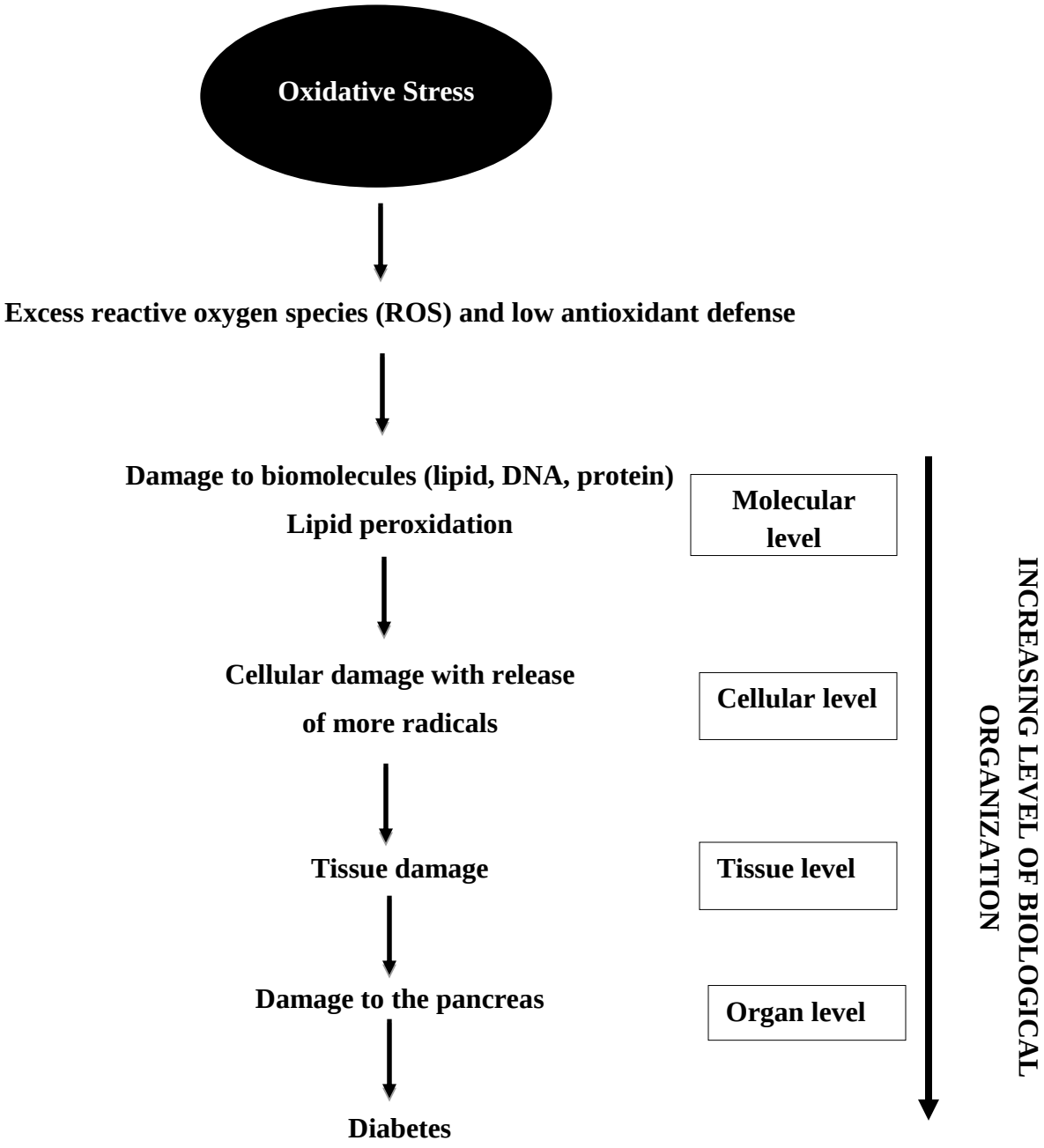

Figure 4: Pathways indicating the negative effects of oxidative stress, leading to the development of diabetes mellitus (adapted from Mousinho, 2013).

Oxidative stress is also implicated in the development of diabetic complications like nephropathy, retinopathy and microangiopathy (Fridlyand & Philipson, 2006; Kaneto *et al*., 2007 ; Ge *et al*., 2008). However, an increase in oxidative stress also causes a reduction of the GLUT-4 expression which in turn impaires the glucose uptake in skeletal muscles and adipose tissues and as well damaging the pancreatic beta cells, which results in an impaired secretion of insulin (Rudich *et al*., 1998 ; Maddux *et al*., 2001) .

Humans employ the use of antioxidants systems to protect themselves against the onslaught of free radicals and oxidative stress. Antioxidants reduce the formation of free radicals thereby neutralizing them and in order to limit their harmful effects. It has been reported that antioxidant may retard the onset and decrease the incidence of diabetes mellitus (Sakai *et al*., 2003). Scientific evidence have also shown that antioxidants also prevent the development of insulin resistance (Evans *et al*., 2002 ; Robertson, 2006; Kawano *at al*., 2009). The antioxidant defence system includes the endogenous enzymatic and non-enzymatic antioxidant defence systems. The endogenous enzymatic antioxidant system includes the catalase, glutathione perioxidase, superoxide dismutase and glutathione reductase enzymes while the non-enzymatic antioxidant defense system includes coenzymes Q, carotenoids, cofactors, vitamin A, C, and E, glutathione and biflavonoids. These antioxidant systems work together using different mechanisms of action and against the various free radicals and different stages of oxidative stress (Maritim *et al*., 2003). However, plants have substantial amounts of antioxidants, a property that can be used for the management of diabetes due to the presence of polyphenolic compounds such as phenols, flavanols, flavonoids, alkaloids and proanthocyanidins (Pietta, 2000). Therefore, targeted antioxidant therapies based on the mechanisms of diabetes induced oxidative stress, might be

worth taking into consideration as part of the important therapeutic strategies to control diabetic complications.

1.7 Treatment option for diabetes mellitus

1.7.1 Western medicine and limitations in the treatment of diabetes mellitus

Oral hypoglycemic drugs have been employed for the treatment of diabetes. However, these oral anti-diabetic drugs have prominent side effects and do not alter the cause of diabetic complications (Rang *et al*., 1991; Inzucchi *et al*., 2012). The most important pathological process during the development of diabetes involves three key organs/tissues which include the pancreatic islets, liver and skeletal muscles. It has been reported that almost all oral anti-diabetic drugs are targeted to three organs (Dey *et al*., 2002). Presently, there are six available categories of oral hypoglycemic agents namely; the sulfonylureas, the meglitinides, the thiazolidinediones, the biguanides, and the alpha amylase and alpha glucosidase inhibitors (Nolte & Karam, 2001). The major mode of action for each group is discussed below, with more information on metformin which was used in this study as a control.

1.7.2. Sulfonylureas

These anti-diabetic drugs are known as endogeneous insulin secretagogues since they act by stimulating insulin secretion from the pancreatic β-cells (Mousinho, 2013). Sulfonylureas bind to the sulfonylurea receptor-1 (SUR-1), expressed on the pancreatic beta cell membranes, thereby inhibiting the efflux of potassium ions through the channels which are causing depolarization (Mousinho, 2013). Depolarization, in turn, opens a voltage-gated calcium then channel that leads to the influx of calcium, and this rise in intracellular calcium then stimulates the release of insulin (Kokil *et al*., 2010). These drugs effectively reduce the elevated levels of blood glucose

in patients suffering from type II diabetes in the short term. They can be used as combined therapy with other classes of antidiabetic drugs. Sometimes, they are used in combination with the longer-acting insulin as part of a daytime-sulphonylurea-night-time-insulin regimen (Bösenberg & Van Zyl, 2008). Diabetic patients using this drug start with a low dose, and the dosage can then be up-titrated at intervals of 2- 4 weeks to achieve an optical glycemic control (Bösenberg & Van Zyl, 2008). However, it has been proven that these antidiabetic drugs do not reduce the long term complication of diabetes and may also stimulate appetite hence resulting in weight gain (Pleuvry, 2005). Examples of drugs in this class are tolbutamide, tolazamide, acetohexamidechlorpropamide, glyburide, glipizide, glibenclamide and glimepiride.

1.7.3 Meglitinides

Meglitinides are another class of insulin secretagogues. These drugs lower the elevated levels of glucose in the blood by increasing the release of insulin from the β-cell pancreatic (Dornhost, 2001). This occurs by modulating the beta cells to secrete insulin by regulating an efflux of potassium through potassium channels. Meglitinides have no direct effect on insulin exocytosis as it is in sulfonylureas (Nolte & Karam, 2001). These anti-diabetic drugs may be used alone or in combination with meformin. This class of antidiabetic drugs are taken by diabetic patients shortly before meal to boost the insulin response to each meal (Iheanyi, 2010). If a meal is skipped the medication is also skipped. Typical examples of the anti-diabetic drugs in this class are prardinic and starlic.

1.7.4 Thiazolidinediones

Thiazolidinediones are another class of oral antidiabetic drugs that help reduce insulin resistance in the muscle and liver. Thiazolidinediones activate the peroxisome proliferator-activated receptor (PPAR) gamma, in the cell nucleus by binding to it, where it acts as an agonist (Suzuki *et al*., 2010). Examples of drugs in this class include troglitazone, rosiglitazone and pioglitazone. However, troglitazone was the first thiazolidinedione to be endorsed, yet was later withdrawn from the market because of the reports of liver failure in people who were using it (Hinterthuer, 2008; Iheanyi, 2010). But liver failure has not yet been reported with rosiglitazone and pioglitazone. Both rosiglitazone and pioglitazone are rapidly absorbed after a meal.

1.7.5 Biguanides

In 1950, three typical examples of biguanides became available for the treatment of diabetes namely, phenformin, buformin and metformin. It was only in 1970 that phenformin and buformin were discontinued due to their association with lactic acidosis and increased cardiac mortality (Nolte & Karam, 2001).

Metformin is derived from the French lilac, *Galega officinalis* L., a perennial herb known for centuries to lessen diabetes symptom (Deutschländer, 2010). It was only in 1995 that the clinical trials of metformin were completed (Deutschländer, 2010). These oral antidiabetic drugs improve the peripheral insulin effect at the musculature and inhibit hepatic gluconeogenesis by enhancing glucose uptake in peripheral cells. It does not increase weight and also has the advantage over other oral anti-diabetic drugs such as insulin and sulfonylureas in treating hyperglycemia. Metformin has been reported to possess some undesirable side effect such as

heart failure, hepatic impairment, gastrointestinal disturbances and renal impairment (Hanefeld, 1998; Mousinho, 2013).

1.7.6 Alpha amylase and alpha glucosidase inhibitors

This class of anti-diabetic drugs sometimes called "starch blockers", block the action of enzymes that break down carbohydrates in the upper part of the small intestine. These enzymes are located in the brush border cell that lines the intestine. Miglitol and acarbose are examples of drugs in this category. Acarbose and miglitol are regarded as competitive inhibitors of the alpha glucosidase enzymes responsible for the breakdown of complex carbohydrates into smaller sugars like glucose, and in order for carbohydrate to be absorbed. These two compounds help slow down carbohydrate digestion and delay glucose absorption which results in a smaller and slower rise in postprandial blood glucose levels after a meal (Mousinho, 2013). However, the binding capacity of acarbose and miglitol differ in that they both target alpha-glucosidases such as sucrase, maltase, glycoamylase and dextranase while miglitol only targets isomaltase and beta-glucosidase, and alpha amylases being targeted by acarbose (Kokil *et al*., 2010). In addition, this class of oral-antidiabetic drugs can also be used in combination therapy with other classes of diabetes drugs due to their novel mechanism of action. However, it has been reported that one of the biggest setbacks to these oral antidiabetic drugs is their side effects. The prominent side effects include nausea, bloating, diarrhoea, flatulence and abdominal pains (Nolte & Karam, 2001).

In general, adverse side effects associated with western medicine are not always satisfactory in maintaining normal glucose level. In view of this, therefore there is need to search for anti-

diabetic medicinal plants that can complement or nagate adverse side effects of the currently used oral antidiabetic drugs (Marles & Farnsworth, 1994).

1.8 Herbal medicine in the treatment of diabetes mellitus

Herbal medicine has been considered to be very acceptable as part of the medical intervention for treatment of diabetes mellitus. In the last few years, there has been a growing interest in the field of herbal medicine for diabetes both in developing and developed countries, due to their antidiabetic activity, lesser cost and probable fewer side effects (Hasani-Ranjbar *et al*., 2009). In South Africa, there are few herbal products available in the market for the maintenance of blood glucose level. The most commonly used herbal products include Probetix, Diabecinn, Cinnachrome and Minna. However, these herbal products are developed from many indigenous antidiabetic medicinal plants, for example, Probetix, a commonly used herbal supplement was developed from the leaf extracts of the indigenous shrub, *Sutherlandia frustescene*. Scientific research done on this plant extract has found out that it reverses insulin resistance and decreases the intestinal glucose uptake (Chadwick *et al*., 2007).

In this regards, there has been a lot of success with the use of plant species to cure and control the different kinds of diabetes and its complications. Much success has only been observed in animal model (streptozotocin (STZ) and alloxan induced diabetic rat) at different dosages to evaluate their antidiabetic potentials. Nevertheless, only a few numbers of these plant species have been reported in different parts of the world for their antidiabetic activity (Edwin *et al*., 2008). In South Africa, many medicinal plants have been scientifically investigated for the treatment of diabetes. Some of the plants for which scientific data could be obtained to support

their antidiabetic properties are listed in Table 2. However, a large number of plants have not yet been studied scientifically for their efficacy in the treatment of diabetes mellitus and as is recommended by the World Health Organization. A lack of such scientific evidence also exists for the two plants, *B. elliptica* and *B. ilicifolia*, investigated in this study (Deutschländer, 2010).

Table 2: Some of the plants scientifically investigated in South Africa for the treatment of diabetes

Plant	Family	Plant part used	Mechanism of action	References
Artemsia afra jacq. ex wild	Asteraceae	Leaves	Displayed inhibitory activity against alpha amylase and alpha glucosidase	Nkobole, 2009
Albuca setosa	Hyacinthaceae	Bulb	Increased glucose uptake in C2C12 and 3T3-L1	Odeyemi, 2016
Brachylaena discolour (DC)	Asteraceae	Leaves, root and stem	Increased glucose uptake in chang liver, C2C12 and 3T3-L1	van de Venter *et al*., 2008
Burbine frustecens (L.) wild.	Asphosdelaceae	Whole plant	Increased glucose uptake in chang liver and C2C12	Mkhomo, 2010
Clausena anisata Wild	Rutaceae	Leaves and Root	Displayed inhibitory activity against alpha amylase and alpha glucosidase	van Huyssteen *et al*., 2011
Cinnamonium cassia (Nees & T .Nees) j. Presi	Lauraceae	Leaves and bark	Increase insulin secretion from INS-1 cells	Boaduo, 2010
Cissampelos capensis (L.)	Menispermaceae	Leaves	Increased glucose uptake in 3T3-L1	van de Venter *et al*., 2008
Euclea natalensis A. DC	Ebenaceae	Root and bark	Displayed inhibitory activity against alpha amylase and alpha glucosidase	Nkobole, 2009
Euclea undulata Thurb.var mytina (Burch)	Ebenaceae	Stem-bark and root-bark	Increased glucose uptake in chang liver, C2C12 and 3T3-L1	Deutschländer *et al*., 2009
Hypoxis hemerocallidea Fisch, C.A. Mey.α Ave-call.	Hypoxidaceae	Leaves and bark	Displayed inhibitory activity against alpha amylase and alpha glucosidase	Boaduo , 2010
Pteronia divanicata (P.J. Bergius) Less	Compositae	Whole plant	Exhibited inhibitory activity against alpha amylase and alpha glucosidase	Deutschländer *et al*., 2009
Sutherlandia frustescens (L) RBr.	Fabaceae	Leaves and Shoot	Prevented insulin resistance in chang liver	Williams *et al*., 2013
Sclerocarya birrea (A.Rich)	Anacardiaceae	Bark	Increased insulin secretion from INS-1 cells	Mousinho, 2013
Strychnos henningsii (Gilg)	Longaniaceae	Bark	Increased glucose uptake in C2C12 and 3T3-L1	Oyedemi *et al.*, 2012

i. *Brachylaena elliptica*

Brachylaena elliptica belongs to the family Asteraceae. Locally, it is commonly known as isiduti in Xhosa, iphahle and uhlunguhlungu in Zulu. It is a shrub or small tree (4 m tall), with a light grey to brown bark that becomes rough with age. The plant is dispersed from Port Elizabeth, Eastern Cape Province to Durban, KwaZulu-Natal Province. The leaves are lanceolate, elliptic to ovate, dim green above and white felted beneath (Figure 5). The species occurs in bushveld on rough outcrops and alongside the edge of the evergreen forest. Poles from this species are utilized as fence posts; the sticks have been purportedly used to start a fire by friction. The leaves, which are intensely bitter tasting, are reportedly used traditionally (Van Wyk & Van Wyk , 1997) and valued by the Zulu and Xhosa for the management of diabetes. The infusion of the leaves is used as a gargle and mouthwash (Coates Palgrave, 1984).

ii. *Brachylaena ilicifolia*

Brachylaena ilicifolia belongs to the family Asteraceae. Locally, it is commonly known as umphahla in Xhosa and iGqeba in Zulu. It is a shrub or small tree (3 to 4 m in height) with grey to brown bark. The leaves are often on short lateral branches, small, narrowly oblong, lanceolate to ovate, green above and covered with whitish-green hairs beneath. The plant is distributed from Port Elizabeth Eastern Cape Province to Durban, KwaZulu-Natal Province, in bush and scrub forest (Coates Palgrave, 1984). The leaves of *B. ilicifolia*, are extremely bitter, and are used traditionally to treat the various diseases including diabetes (Coates Palgrave, 1984). The infusion and decoction of the leaves are used to treat diarrhoea (Olajuyigbe & Afolayan, 2012).

In spite of the acclaimed folkloric use of *B. elliptica* and *B. ilicifolia* as an antidiabetic agent, on this note, there is still lack of scientific evidence to substantiate the claim. Both plants are normally soaked in water and the concoction taken orally. Thus, there is a need to authenticate these acclaimed uses/properties by traditional healers for the treatment of diabetes. Previous work on *B. elliptica* and *B. ilicifolia* have focused on antioxidant, phytochemical and antibacterial activities (Sagbo, 2015) but there is no information about antidiabetic, probable mechanisms of action and the toxicity effect of these plants.

Brachylaena elliptica *Brachylaena ilicifolia*

Figure 5: Medicinal plants used in this study.

1.9 Rational and justification for this study

Diabetes mellitus (DM) is becoming an increasing concern all over the world. It is a major health problem with its recurrence expanding each day in many nations. The prevalence of this disease worldwide constitutes a global health burden (Boyle *et al*., 2001). In South Africa, the disease is

commonly attained in the most prolific period of life. However, there is a lack of awareness and increased susceptibility to DM among the South African populace due to dietary intake and inactive lifestyles. In particular, the current medication is not readily available to many rural populations and many of these medications are frequently reported to have adverse side effects (ADA, 2006). Diabetic patients have therefore resorted to the use of antidiabetic plants with fewer reported side effects (Nyarko *et al*., 1999). However, the majority of these traditional plants have not been scientifically validated for their efficacy in the treatment of diabetes mellitus. Based on ethnobotanical surveys, *B. elliptica* and *B. ilicifolia* are commonly used plants for the management of diabetes among local healers (Deutschländer, 2010) yet no scientific research has confirmed the efficacy of these plants. Therefore, determination of their efficacy is very important as these plants may play a significant role in the management of diabetes mellitus.

1.10 Hypothesis

It is hypothesized that the *Brachylaena elliptica* and *Brachylaena illicifolia* used have hypoglycemic activity by activating/enhancing glucose uptake in liver and/or muscle cells.

1.11 Aim of this study

To validate the antidiabetic activity and to elucidate the possible mechanism of action of the aqueous leaf extracts prepared from *Branchylaena elliptica* and *Brachylaena ilicifolia*

1.12 Specific objectives

- To evaluate the cytotoxic and proliferative effect of *Brachylaena elliptica* and *Brachylaena ilicifolia.*

- To explore the antidiabetic mechanism of *Brachylaena elliptica* (Thurb.) DC. and *Brachylaena ilicifolia* (Lam) Phill & Schweick using the target directed *in vitro* screening.

- To evaluate the potential of the extracts of *B. elliptica* and *B. ilicifolia* for alleviating secondary diabetic complications and synergistic effect on the metabolism of diabetic drugs.

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CHAPTER 2

In vitro evaluation of cytotoxic and proliferative effects of aqueous extracts of *Brachylaena elliptica* and *Brachylaena ilicifolia*

Part of this Chapter has been presented orally at the First International Conference of the Society for Medicinal Plants and Economic Development (SOMPED)

Conference presentation title: *In vitro* antidiabetic activity and mechanism of action of extract of *Brachylaena elliptica* (Thurb.) DC.

CHAPTER 2

2.1 INTRODUCTION

Medicinal plant preparations administered by traditional healers are assumed to be safe and promote healthy treatment of various diseases such as inflammation, GIT syndrome, depression, diabetes (Chen *et al.,* 2011*).* In recent times, there has been several reports regarding the adverse effects allegedly arising from the use of medicinal plants due to contamination, content of heavy metals, poor quality control, poisonous phytochemicals, cytotoxic, genotoxic and organ toxicity (Jordan *et al*., 2010; Ina & Brvar 2014). Some of the adverse effects purportedly reported include vomiting, diarrhoea, ulcers, loss of appetite and abdominal pains (Voncina *et al*., 2014). These adverse effects could be attributed to the presence of phytochemical compounds that are cytotoxic or genotoxic or may have carcinogenic effects as well as using incorrect dosages for treatment of diseases. Phytochemicals from some medicinal plants are associated with the toxicities of liver, heart, kidney, central nervous system (Jordan *et al*., 2010). Therefore, it is important to evaluate the toxicity of all medicinal plants and to determine the safe dosages to use in treating various diseases. This study was therefore designed to investigate the safety or potential risks of *B. elliptica* and *B. ilicifolia* and to determine their effect in pancreatic beta cell mass.

2.2 MATERIAL AND METHODS

2.2.1 Collection of plant materials

The leaves of *B. elliptica* were collected from a thick forest in Amathole District in Eastern Cape, while *B. ilicifolia* leaves were collected from around Grahamstown also in the Eastern Cape. The plants were identified by their vernacular names and later confirmed at the Department of Botany, University of Fort Hare by Prof. D.S. Grierson and voucher specimens with their corresponding numbers (BRA (47) 8936 for *B. elliptica* and BRA (47) 1512 for *B. ilicifolia*) were deposited at the Griffin Herbarium of the University of Fort Hare.

2.2.2 Preparation of extracts

The leaves of each plant were oven dried at 40^{o}C to constant weight and then ground to a homogeneous powder using an electric blender (Waring Products Division, Torrngton, USA). Sixty (60) grams of each powdered plants were extracted separately in 1000 ml of distilled water. They were then filtered using a Buchner funnel and Whatman No. 1 filter paper. The extracts were frozen at -40^{o}C and dried for 48 h using a freeze dryer to give a yield of 9 g and 8.1 g for *B. elliptica* and *B. ilicifolia* respectively. The dried extracts were stored at -4^{0} C and later reconstituted in DMSO just before various bioactivity determinations.

2.2.3 Cell lines, media, reagents and assay kits

HepG2 liver cells and INS-1 cells were obtained from Highveld Biological and ATCC respectively. The Eagle's minimum essential medium (EMEM), MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide were obtained from Sigma Aldrich, South Africa

whereas, fetal calf serum (FCS) and PBS were obtained from Lonzas Biologics. All other reagents used in this study were of analytical grade and purchased from Sigma or Merck Chemicals.

2.2.4 Maintenance of cell cultures.

All cell cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. HepG2 cells were replenished with growth medium every 2-3 days, consisting of RPMI 1640 medium supplemented with 10% fetal calf serum. The INS-1 cells were cultured in RPMI containing 5% fetal calf serum. Both cell lines were sub-cultured after 90% confluence was reached.

2.2.5 Basal cytoxicity assay (MTT assay)

The MTT assay was determined using a modification method described by Mosmann (1983). The HepG2 liver cells were seeded into 96-well plates at a density of 8000 cells per well within a volume of 100 µl. The cells were left to attach overnight and then treated with 100 µl of standards or plant extracts at various concentrations (50 µg/ml, 100 µg/ml and 200 µg/ml) to specific well. After 48 hrs of incubation at 37^{o}C, the spent medium was removed from the cells and 100 µl EMEM medium containing 10% FCS and 0.5 mg/ml MTT was added and further incubated for 3 h at 37^{o}C. The medium was later aspirated and MTT crystal (purple formazan) was then dissolved in DMSO (200 µl/well). The absorbance was read at 540 nm using a microplate reader (Multiscan MS, Labsystem). The percentage cell death was calculated using the following equation:

$$\% \text{ Cell death} = 1 - \left(\frac{Absorbance\ of\ test\ well}{Average\ of\ the\ untreated}\right) X\ 100$$

2.2.6 INS-1 proliferation assay using ImageXpress®Micro XLS analysis

The cell proliferation assay was carried out using a modification method described by Sirenko *et al*. (2015). The INS-1 cells were cultured in RPMI containing 5% FCS, seeded into 96-well plates at a density of 8000 cells per well, with a volume of 100 µl, The cells were left for 16 h to attach overnight and then treated by adding 100 µl of the plant extracts at various concentrations (12.5 µg/ml, 25µg/ml and 50 µg/ml). A well was also treated with gamma-aminobutyric acid (GABA) (10 µg/ml) instead of the plant extracts to serve as a positive control. The cells were then stained with 10 µl of the Hoechst staining solution (10 µg/ml in PBS) for 10 minutes. After 10 minutes, the staining solution was removed and 10 µl of propidium iodide (PI) solution (10 µg/ml in PBS) was added and incubated for 10 minutes. The cells were then observed under ImageXpress®Micro (Molecular Devices) automated epifluorescent microscope. The images were later acquired and analyzed using a Multi-wavelength Cell Scoring application module to calculate the percentage of cells positive for each dye (Hoechst 33342 for total cell counts and propidium iodide for dead cell counts).

2.2.7 ImageXpress®Micro (Molecular devices) configurations

The cell proliferation assay was performed on image Xpress micro widefield high content screening system (Molecular Devices) (Figure 6). The system configuration includes: the camera 4.66 megapixel scientific Scmos; the objectives: Nikon 10x Plan Flour, 10x Plan Fluor DL (Phase constrast), 20x Super Plan Fluor ELWD ADM cc0-2mm (phase constast), 40x Super Plan Fluor ELWD ADM cc 0-0.2mm (phase constrast); the light source (solid state light source, 380 nm (DAPI) -680 (cy5) 3- log dynamic range); the filter cubes (colourimetric: Red, Green, Blue); the sample compatibility (slides, Multiwall plates (6-through to 1536 wells), glass or plastic); the

analysis software (metaXpress software); the environmental control (temperature (Ambient to 40^{o}C), CO_2 (5-10%), humidity); the phase contrast transmitted light: Nikon 100W pillar diascopic illuminator with TEC ELWD condenser; Phl, Ph1 and Ph2 selectable phase rings.

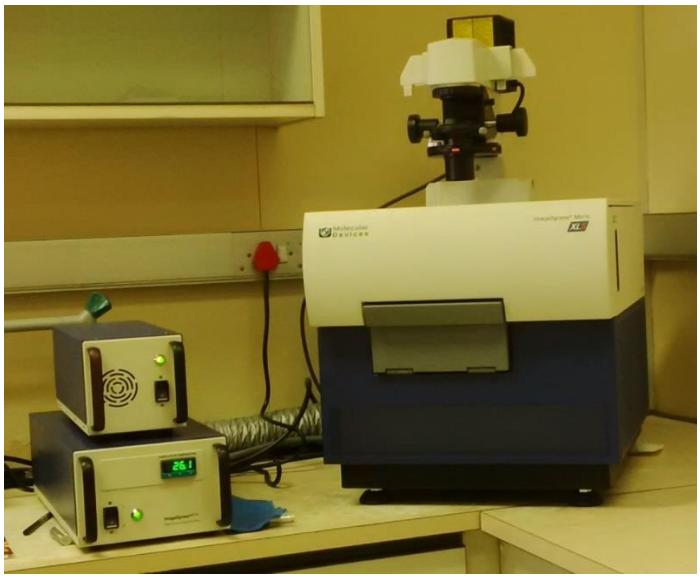


Figure 6: The ImageXpress® Micro widerfield High-Content Imaging System

2.2.8 Statistical analysis

The statistical analysis was carried out with a one way analysis of variance (ANOVA) and the difference between samples were determined by a Duncan's Multiple Range test using the minitab program (12.11.1). The data were expressed as the mean ± standard deviation and values were considered significant at $P < 0.05$.

2.3 RESULTS AND DISCUSSION

2.3.1 Cytotoxicity (MTT assay)

The MTT assay is one of the most commonly used assays for *in vitro* cytotoxicity testing. It is a sensitive, quantitative and reliable colorimetric assay that measures cell viability, cell proliferation and activation in cell population using a 96-well plate format. This assay is based on the ability of the cellular mitochondrial dehydrogenase enzyme in living cell to reduce the yellow water-soluble substrate (3-(4, 5-dimethylythiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium hydrobromide (MTT) into a dark/ purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a ranges of cell lines (Azurah *et al*., 2011). Ideally, this assay also enables one to determine the concentrations of each treatment that are not toxic to the specific cell line thereby using the concentration that are not toxic for further experiment.

In this study, the effect of *B. elliptica* and *B. ilicifolia* were observed in HepG2 cells using an MTT assay (Figure 7). The crude extracts of both plants displayed low levels of toxicity of up to doses of 200 µg/ml in the HepG2 cell lines in a dose dependent manner. However, the toxicity level remains below 50% cell death, even for the highest dosage used (Figure 7). It was observed that *B. elliptica* showed a significantly higher toxicity at all dosages when compared to *B. elliptica* at the same concentration. The IC_{50} value (concentration that can cause 50 % cell death) for *B. elliptica* and *B. ilcifolia* was calculated to be 250 µg/ml and 340 µg/ml, respectively. It has been reported that toxicity from plants may be attributed to the presence of alkaloids, glycosides, saponins, polyacetylenes (Majak, 2001; Orech *et al*., 2005). Previous phytochemical reports for

the *B. elliptica* and *B. ilicifolia* revealed the presence of low levels of alkaloids and saponins in both plants (Sagbo, 2015). Therefore, the low levels of toxicity observed in this study could be attributed to the presence of alkaloids and saponins observed in the previous study (Sagbo, 2015).

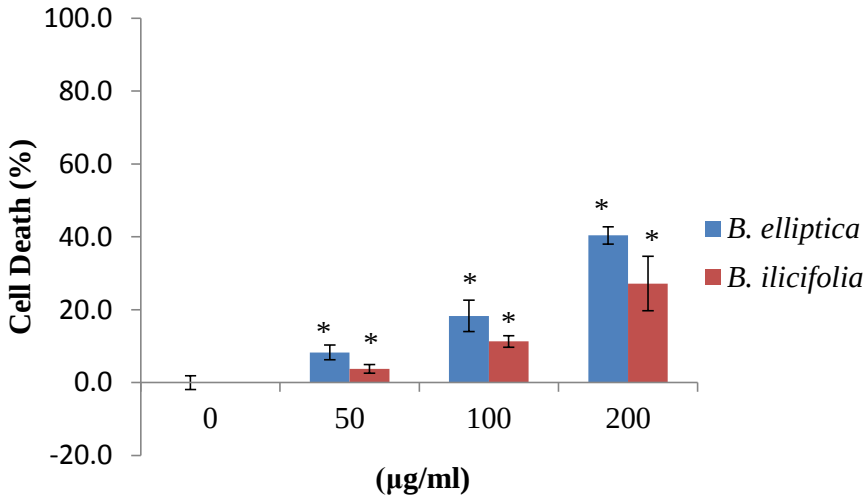


Figure 7: Determination of the cytotoxicity effect of the aqueous extract of *B. elliptica* and *B. iIicifolia* on HepG2 liver cells, using MTT assay. Data are expressed as % control ± SD (n = 4). (*) indicates a significant increase relative to the untreated control ($p < 0.05$).

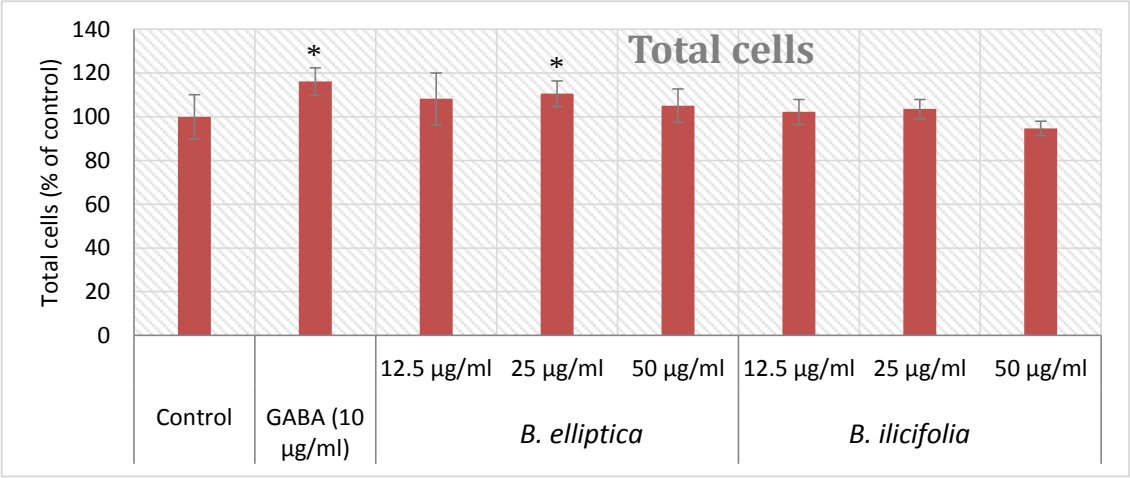
2.3.2 INS-1 proliferation assay

The ImageXpress Micro XLS system is a wide field automated microscope capable of fluorescent, transmitted light and phase-constrast imaging. It is used for the high content analysis of fixed- or live-cell assays such as tissues and small organisms. The system combined with specific softwares, provides a fast and robust platform to translate new discoveries into scientific breakthroughs. The proliferation of INS-1 cells is a well-established model to assess the beta-cell proliferation. Therefore, an increase in INS-1 cells indicates cell proliferation in a non-toxic

environment while a decrease in cell proliferation indicates cell death due to toxicity in the experimental cultures.

The effect of aqueous *B. elliptica* and *B. ilicifolia* on INS-1 cell proliferation in the presence or absence of GABA was examined using the ImageXpress Micro XLS system. The results (Figs, 8A and B) revealed that *B. elliptica* showed significant increases in INS-1 cells compared to the negative control (untreated control) at 25 µg/ml. However, at the highest concentration, no significant increases were seen. *B. ilicifolia* showed no significant increases at all the concentration used in this study. *B. elliptica* not only showed proliferation of INS -1 cells, but also showed low levels of dead cell (< 1%). However, *B. ilicifolia* had no effect on proliferation (Figure 8A) even though higher levels of dead cell were observed (Figure 8B). None of the extracts evaluated showed a higher level of INS-1 proliferation than the positive control (GABA) at all the tested concentrations. However, it could be deduced from this study that the aqueous leaf extracts of *B. elliptica* may possess a mitogenic effect or able to induce the expression of growth stimulating factors such as insulin-like growth factors (IGF-1), growth hormone (GH) and prolactin which have been reported to stimulate or enhance beta cell proliferation (Gahr *et al*., 1999). This is the first study to examine the effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on INS-1 cell death and proliferation. However, aqueous and methanol extracts of *Brachylaena discolor* have been reported to possess a proliferative effect on Hela cell lines (Mellem, 2013) which supports the result obtained for *B. elliptica*. This result gives an indication that the aqueous leaf extract of *B. elliptica* has a proliferative or anti-apoptotic effect or both and could benefit patients suffering from diabetes.

A



B

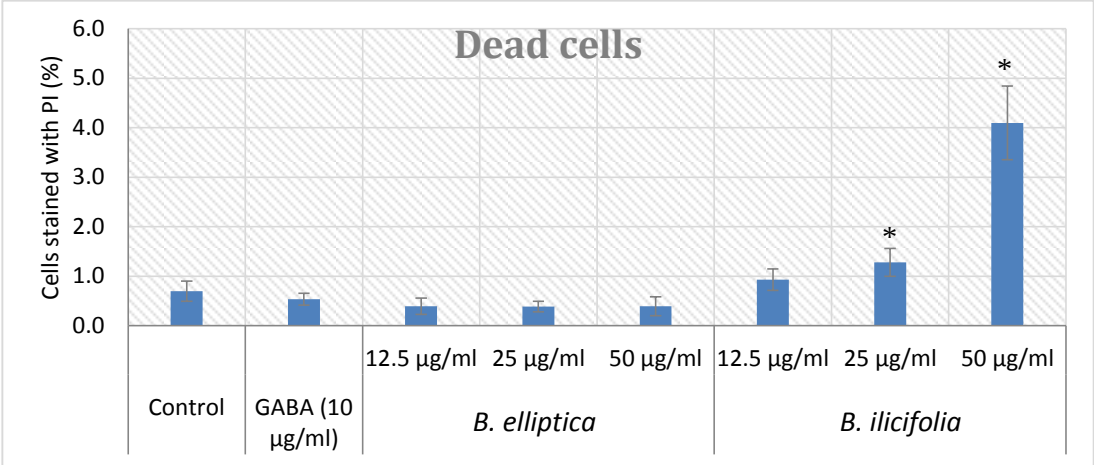
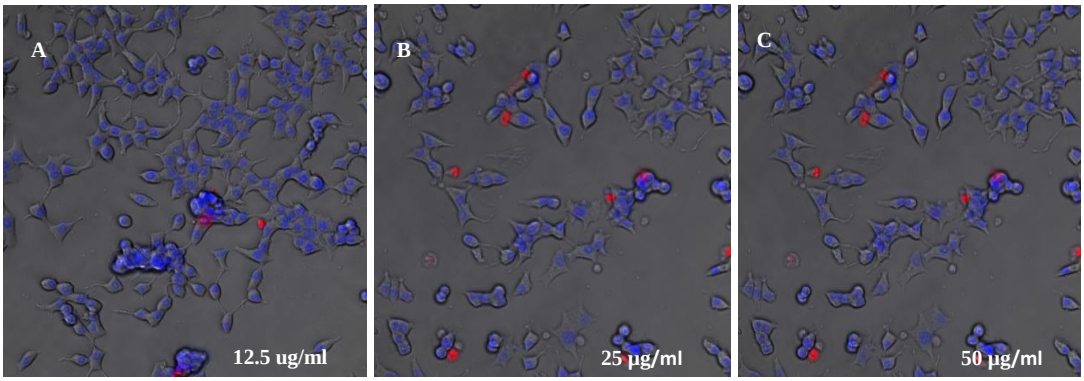
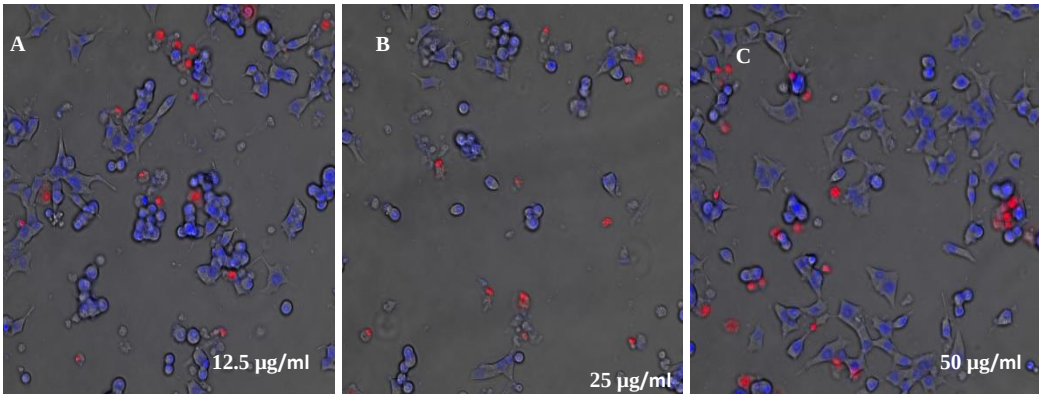


Figure 8A and B: The effect of *B. elliptica* and *B. ilicifolia* on the proliferation of INS-1 beta cells. INS-1 cells were cultured with or without the plant extract for 48 hours, and the total cells and dead cells (cells stained with PI) were then determined using the ImageXpress Micro XLS analysis with a Hoechst 33342 dye for total cell counts and propidium iodide for dead cell counts. Data are expressed as mean ± S.D. (n= 8). (*) indicates a significant increase relative to the untreated control ($P < 0.05$).

B. elliptica



B. ilicifolia



GABA

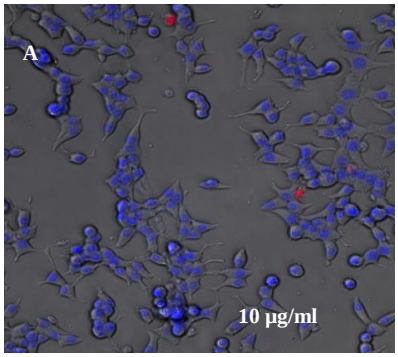


Figure 9: Fluorescence images of the Hoechst 33258 and propidium iodide stained INS-1 cells when treated with plant extracts at various concentrations compared to the positive control. The light blue staining (Hoechst) indicates live cells while the red staining (Propidium iodide) indicates dead cells. GABA was used as a positive control for cell proliferation.

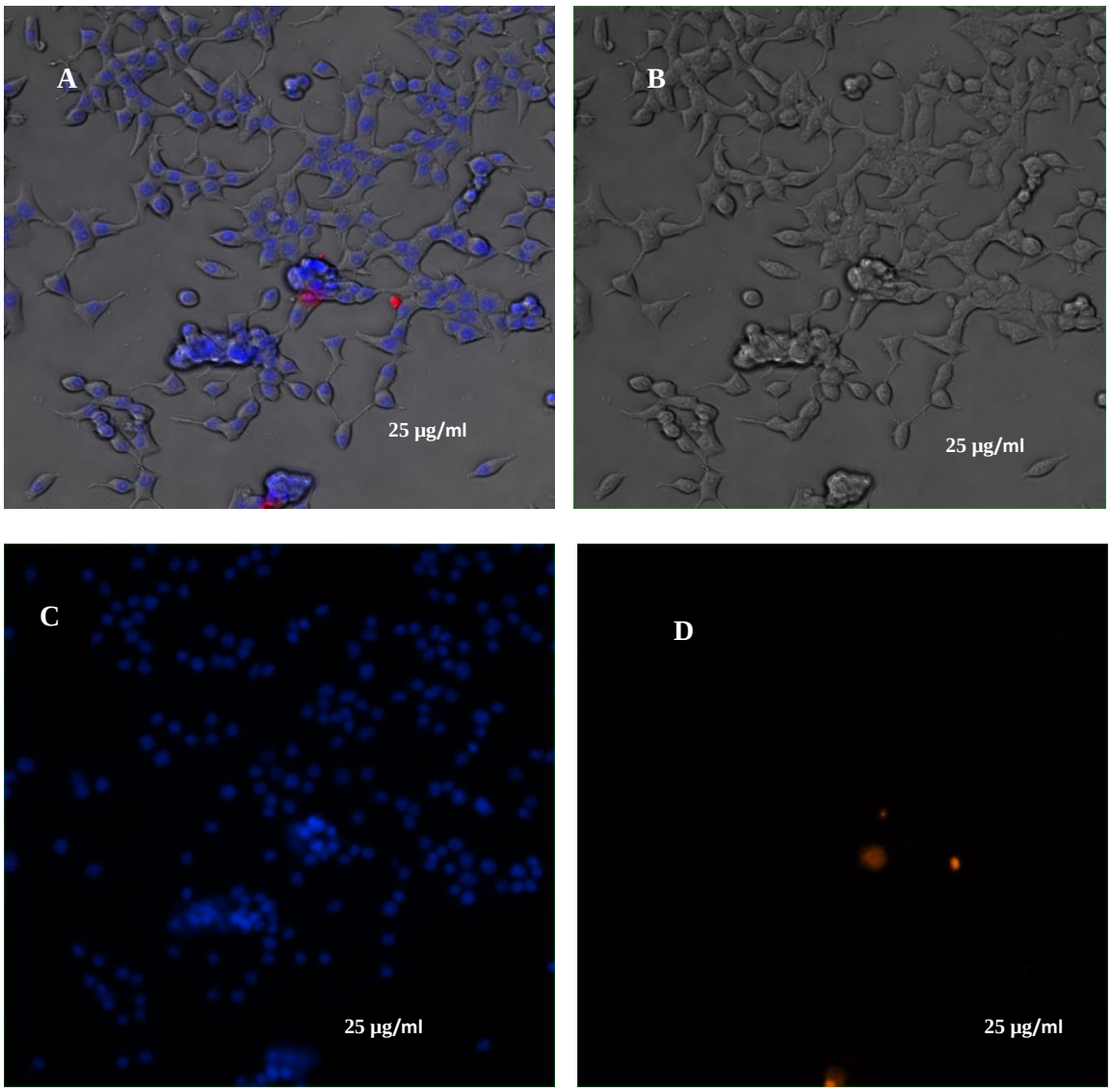


Figure 10: *B. elliptica* induced proliferation in INS-1 cells at 25 µg/ml, after staining with Hoechst 33258 and propidium iodide. (A: fluorensence image of live and dead cells; B: phase-constrast image of live and dead cells; C: fluorensence image of live cells; D: fluorensence image of dead cells). All images of INS-1 cells are presented at a final magnification of 10x obtained by the ImageXpress and computer-generated enlargement.

2.4 CONCLUSION

The Aqueous extracts of both plants showed low level of toxicity in HepG2 cells at the doses investigated thus supporting the traditional healers' claim that these plants are safe for human consumption. However, further studies using different toxicity models are recommended to further confirm their claims. The crude extract of *B. elliptica* showed highly promising proliferative effects in INS-1 cells which indicate that the dietary intake of this plant may be a promising strategy for diabetes prevention. The extract of *B. ilicifolia* showed some levels of toxicity in INS-1 cells at the concentration investigated but did not inhibit proliferation, indicating a possible different mechanism through which it could function as an anti-diabetic treatment.

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Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1–2), 55–63. http://doi.org/http://doi.org/10.1016/0022-1759(83)90303-4

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CHAPTER 3

Exploring the antidiabetic mechanism of *Brachylaena elliptica* (Thurb.) DC. and *Brachylaena ilicifolia* (Lam) Phill & Schweick using target directed *in vitro* screening

This Chapter has been presented orally at the 25th Congress of the South African Society of Biochemistry and Molecular Biology (SASBMB).

Conference presentation title: Exploring the antidiabetic mechanism of *Brachylaena elliptica* (Thurb.) DC. and *Brachylaena ilicifolia* (Lam) Phill & Schweick using target directed *in vitro* screening.

CHAPTER 3

3.1 INTRODUCTION

The use of medicinal plants as antidiabetic remedies has recently gained popularity and many people believe that natural products are mostly associated with fewer side effects (Khan *et al*., 2012). Evidence has suggested that the antidiabetic properties of many medicinal plants is mostly attributed to their ability to stimulate glucose uptake and restoring the function of pancreatic beta cells thereby causing an increase in insulin production (Mathews *et al*., 2000; Ugochukwu & Babady 2003; Abesundara *et al*., 2004 ; Malviya *et al*., 2010; Odeyemi 2016). Some plants with antidiabetic properties are also reported to inhibit intestinal carbohydrates digestive enzymes and regulation of different biochemical pathway, such as glycolysis, Krebs cycle, gluconeogenesis, glycogen synthesis, AMPK pathway (adenosine mono-phosphate kinase) (Babu *et al*., 2004; Hussain *et al.*, 2004; Gholap & Kar, 2004; Chung *et al*., 2010; Jayaprasad *et al*., 2016). These plants are very rich in flavonoids, phenolics, coumarins, terpenoids, tannins, steroids, glycosides, anthoroquinones which allow them to perform different mechanisms of action in the treatment of diseases (Prabhakar & Mukesh 2008; Jayaprasad *et al*., 2016). In literature, there are more than 400 plant species having antidiabetic properties, but the complete mechanisms of action and efficacy of these plants have not been reported (Sujatha & Jamimo 2012).

In the previous chapter, the cytotoxic and proliferative effect of aqueous extracts of *B. elliptica and B. ilicifolia* were evaluated. In this chapter, the antidiabetic mechanisms of both plant extracts were studied using a target directed *in vitro* screening. The inhibition effects of both plant on alpha amylase, alpha glucosidase, pancreatic lipase, dipeptidylpeptidase –IV and nitric

oxide (NO) production in lipopolysaccharide (LPS) stimulated RAW macrophage cells were investigated as possible therapeutic targets. In addition, this study also investigated the effects of both plant extracts on glucose uptake in hepatic (HepG2) and myoblasts (L6) cell lines. These two cell lines were chosen based on the role hepatic and skeletal muscle play in the regulation of blood glucose homeostasis and because they also possess the different glucose transporters that respond to insulin stimulation.

3.2 MATERIALS AND METHODS

3.2.1 Collection of plant materials

The leaves of *B. elliptica* and *B. ilicifolia* were collected as described in section 2.2.1.

3.2.2 Preparation of extracts

Preparation of plants extraction was done as described in section 2.2.2.

3.2.3 The alpha-amylase inhibition assay

The alpha amylase assay was performed according to the method described by Zhizhuang *et al*. (2005). Fifteen microliter (15 µl) of the plant extracts at different concentrations (50 µg/ml – 200 µg/ml) (diluted in a phosphate buffer) was added to 5 µl of enzyme porcine pancreatic solution into 96-well plate. After 10 mins of incubation at 37°C, the reaction was initiated by adding 20 µl of starch solution and further incubated for 30 min at 37°C. The reaction was then stopped by adding 10 µl 1M of HCl to each well followed by 75 µl of iodine reagent. A blank containing phosphate buffer (pH 6.9) instead of the extract, and a positive control (acarbose 64 µg/ml) were

prepared. No enzyme control and no starch control were included for each test sample. The absorbance was measured at 580 nm and the percentage inhibitory activity was calculated by using the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ the\ untreated (Control)}{Absorbance\ of\ the\ test\ well}\right) X\ 100$$

3.2.4 The alpha-glucosidase inhibition assay

The alpha glucosidase inhibition assay was determined according to a modification of the method described by Sancheti *et al.*, (2010). Five microlitre (5 µl) of the plant extract (50 µg/ml – 200 µg/ml) was added to 20 µl of 50 µg/ml α-glucosidase enzyme into a well of a 96-well plate. Sixty microlitre (60 µl) of a 67 mM potassium phosphate buffer (pH 6.8) was then added. After 5 min of incubation, 10 µl of the 10 mM ρ-Nitrophenyl-α-D-Glucoside solution (PNP-GLUC) was added and further incubated for 20 min at 37°C. After incubation, 25 µl of a 100 mM sodium carbonate (Na_2CO_3) solution was added and the absorbance was measured at 405 nm. A blank and sample blank were also prepared by adding 5 µl of the deionised water instead of plant extracts and 20 µl of deionised water instead of enzyme respectively. Epigallocatechin gallate (10 µg/ml) was used as a positive control. The percentage inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ the\ test\ well}{Absorbance\ of\ the\ untreated\ (control)}\right) X\ 100$$

3.2.5 The Lipase inhibition assay

The lipase inhibition assay was performed according to the method described by Lewis & Liu, (2012). Ten microlitre (10 µl) of the plant extract or positive (Orlistat, 50 µg/ml) or negative

control (distilled water) was added into the well of a 96-well plates. Porcine pancreatic (10 mg/ml) was freshly prepared in 50 mM Tris HCl buffer (pH 8.0) and centrifuged to remove any insoluble materials, was then added at 4 times the volume to each of the sample (40 µl). After 15 min of incubation, 170 µl of a substrate solution (20 mg pNPP in 2ml isopropanol added to 18 ml 50 Mm TrisHCl buffer (pH 8.0) containing 20 mg gum Arabic, 40 mg sodium deoxycholate and 100 µl Triton X-100) was then added and incubated for 25 min at 37°C. The absorbance was then measured at 405 nm using a Biotek® PowerWave XS spectrophotometer and the percentage inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ the\ test\ well}{Absorbance\ of\ the\ untreated\ (control)} \right) X\ 100$$

3.2.6 The DPP -IV inhibition assay

The DPP-IV inhibition assay was carried out according to a modification of the method described by Al-masri *et al*. (2009). Briefly, thirty-five microlitre (35 µl) of the plant extract (50 µg/ml – 100 µg/ml) or positive control (Diprotin A, 50 µg/ml) was added to 15 µl of a human recombinant DPP-IV enzyme solution (50 µU/µl in Tris buffer) into the well of 96-well plate. After 5 min of incubation at 37^{o}C, 50 µl of 20 mM ρNA substrate (Gly-Pro- ρNA) dissolved in Tris buffer was added to initiate the reaction and further incubated for 30 min at 37°C. After incubation, 25 µl of a 25% acetic acid solution was added to stop the reaction and the absorbance was then measured at 410 nm. A blank and sample blank were also prepared by adding 35 µl of buffer instead of plant extracts and 15 µl of buffer instead of enzyme respectively. The percentage inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ the\ test\ well}{Absorbance\ of\ the\ untreated\ (control)} \right) X\ 100$$

3.2.7 Maintenance of cell cultures

All cell cultures were incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. The RAW 264.7 macrophages cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing l-glutamine supplemented with 10% FCS and 1% PSF solution. HepG2 cells were replenished with growth medium after every 2-3 days, consisting of RPMI 1640 medium supplemented with 10% fetal calf serum. The L6 myoblasts cells were cultured in antibiotic-free growth medium consisting of RPMI 1640 supplemented with 10% fetal calf serum. All the cells were sub-culture after 90 % confluence were attained.

3.2.8 Inhibition of nitric oxide production in RAW macrophage cells

Inhibition of nitric oxide production in RAW macrophages was determined using a modification of the method described by Yang *et al*. (2009). The RAW macrophage cells were seeded into a 96-well culture plate at a density of 5 000 cells per well and allowed to attach overnight. The spent culture medium were removed and replaced with fresh medium containing a different concentration (12.5 µg/ml – 100 µg/ml) of the extracts to give a total volume of 50 µl per well. Aminoguanidine (4 µg/ml) was used in place of the extracts, as a positive control. Fifty microlitre (50 µl) of lipopolysaccharides (LPS) containing medium (100 µg/ml) was added to all the wells except for those of the blank. After 18 h of incubation, 50 µl of the medium was removed and transferred into a new 96 well plate into which 50 µl of the Griess reagents was added and incubated for 11 min. Then the absorbance was measured at 540 nm using a Multiscan MS microtitre plate reader (Lab systems). The toxicity test (Mosmann, 1983) was performed on the remaining cells by adding 100 µl of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide) solution to obtain a final concentration of 0.5 mg/ml. After 15 min of incubation, the spent culture medium was removed and 100 µl of DMSO was added to solubilise the MTT crystal. The absorbance was then measured at 540 nm using a Multiscan MS microtitre plate reader (Labsystems).

3.2.9 Glucose utilization experimental procedure on HepG2

The glucose utilisation in HepG2 cells was determined by the method described by van de Venter *et al.,* (2008). HepG2 cells were seeded at a density of 6 000 cells per well into a 96-well culture plate (Nunc, Denmark) and allowed to adhere and grow in a humidified incubator with 5% CO_2 at 37°C for three days. Two cell free rows were also included to serve as blanks. On day three after seeding, without changing the medium, 10 µl of plant extract at a concentration dose of 25 µg/ml and 100 µg/ml were added to each well. After 48 hrs incubation, the spent culture medium was removed and replaced with a 25 µl incubation buffer (RPMI medium diluted with PBS, 0.1% BSA and 8 mm glucose) and further incubated for an additional 3 h at 37°C. Metformin (0.1 µg/ml) and berberine (18 µg/ml) were used as positive controls while the negative control (untreated) contained only the incubation buffer without treatment. After incubation, 10 µl of the incubation medium was removed from each well and transferred into a new 96-well plate into which 200 µl of glucose oxidase reagent (SERA-PAK Plus, Bayer) was added to determine the concentration of glucose in the medium. After 15 min of incubation at 37^{o}C, the absorbance was measured at 492 nm using a Multiscan MS microtitre plate reader (Lab systems). The amount of glucose utilized was calculated as the difference between the cell free and cell containing wells. Cell viability in the representative wells was then determined using the MTT assay (Mosmann, 1983).

3.2.10 Glucose utilization experimental procedure on L6 myoblasts

Glucose utilisation in L6 myoblasts cells was determined according to the methods described by van de Venter *et al*., (2008). The L6 cells were seeded into 96-well culture plates at a density of 3 000 cells/well and allowed to adhere until a 90% confluence was reached. Two cell free rows were also included to serve as blanks for glucose utilisation assay. After 90% confluence, the culture medium was removed and replaced with DMEM containing 2% FBS and cultured for an additional five days. Fourty-eight hours (48 hrs) prior to the glucose utilisation assay, the culture medium was replaced and 10 µl of plant extract at various concentrations of 12.5 µg/ml, 25 µg/ml and 50 µg/ml was added to separate wells. A column was also treated with insulin (4 µg/ml) instead of the plant extracts to serve as a positive control. The cells were incubated in the presence of the extract for an additional 48 h. After the incubation period, the spent medium was then removed and replaced with a 25 µl incubation buffer containing an RPMI medium diluted with PBS, 0.1% BSA and 8 mM glucose and incubated for a further 3 h at 37°C. Five microliter (5 µl) of the incubation medium was removed from each well and then placed into a new 96-well plate into which 200 µl of glucose oxidase reagent (SERA-PAK Plus, Bayer) was added per well to determine the concentration of glucose in the medium. After 15 min of incubation at 37^{o}C, the absorbance was measured at 520 nm using a Multiscan MS microtitre plate reader (Lab systems). The amount of glucose utilized was calculated as the difference between the cell free and cell containing wells. Cell viability in the representative wells was also determined using the MTT assay (Mosmann, 1983) and as is described in section 3.2.8

3.2.11 Lipid accumulation in 3T3-L1 preadipocytes

Lipid accumulation in 3T3-L1 preadipocytes cells was determined by the method described by Oyedemi *et al*. (2012). The 3T3-L1 preadipocytes were seeded at a density of 6 000 cells per well into a 48-well culture plate (Nunc, Denmark) and allowed to grow until 100% confluence was reached. Two days post-confluence, the preadipocytes were treated for an additional two days with 0.1 µM insulin or various concentrations (12.5 µg/ml, 25 µg/ml and 50 µg/ml) of plant extracts or rosiglitazone (0.4 µg/ml). The cells were then cultured for an additional ten days in a normal culture medium (DMEM with 10% FBS) and the medium replaced every two to three days. After ten days, the spent culture medium was removed and gently washed with PBS. The cells were then allowed to fix at room temperature for approximately 1 hour by adding 500 µl per well of a 10% formaldehyde in PBS. The fixed solution was aspirated and later stained by adding 200 µl of a pre-warmed oil red working solution (6 ml of stock solution (0.5g oil red dye in 100 ml isopropanol) in 4 ml of distilled water) for 15 min at 37^{o}C. After 15 min of incubation, excess dye was extensively washed with water and the plate dried in an oven at 37^{o}C. The dye was further extracted by adding isopropanol (250 µl per well) after which 200 µl was transferred to a 96-well plate and the absorbance measured at 520 nm using a Multiscan MS microtitre plate reader (Lab systems).

3.2.12 Glucose metabolism as a reflection of insulin secretion

The glucose metabolism assay as a reflection of insulin secretion in INS-1 cells using an MTT (tetrazolium) colorimetric assay was determined according to the method decribed by Janjic & Wollheim (1992). The INS-1 cells were cultured in RPMI containing 5% FCS. Cells were seeded into 96-well plates at a density of 8000 cells per well, with a volume of 100 µl. The cells were

left to attach overnight and treated with plant extracts (100 µg/ml) or PBS (which serve as control) in the presence or absence of glucose (20 mM). After 48 hrs of incubation at 37^{o}C, the spent medium was removed from the cells and 100 µl of EMEM medium containing 10 % FCS and 0.5 mg/ml MTT was added and further incubated for an additional 30 min at 37^{o}C. The medium was later aspirated and MTT crystal (purple formazan) dissolved in DMSO (200 µl/well). The absorbance was read at 540 nm using a microplate reader (Multiscan MS, Lab system).

3.3 RESULTS AND DISCUSSION

3.3.1 Alpha amylase inhibition assay

Anti-diabetic drugs that retard glucose absorption after a meal through the inhibition of carbohydrate hydrolyzing enzymes, namely alpha amylase and alpha glucosidases, are recognized to be efficient in decreasing postprandial hyperglycemia. Alpha amylase breaks down complex dietary carbohydrates (starch) to oligosaccharides and disaccharides, which are eventually converted into monosaccharide by alpha glucosidase. An Inhibition of these two enzymes limits carbohydrate digestion by delaying the process of carbohydrate hydrolysis and absorption, making the inhibitor useful in the management of diabetes. The ability of aqueous extracts of *B. elliptica* and *B. ilicifolia* to reduce the postprandial level of glucose by inhibiting these two digestive enzymes was examined in this study.

The results of the crude extracts of both plants showed no significant inhibition of the alpha amylase enzyme at all the tested concentrations when compared to acarbose which was used as

the positive control (Figure 11). *B. ilicifolia* however, showed a weak inhibition (< 5%) at 200 µg/ml but stimulated the enzyme at 50 µg/ml and 100 µg/ml. The extract of *B. elliptica* stimulated the enzyme at all the tested concentrations used in this study (50 µg/ml, 100 µg/ml and 200 µg/ml).

To date, this is the first study to investigate the effect of crude extracts of *B. elliptica* and *B. ilicifolia* on the activity of alpha amylase. The acetone extract of *Schkuhria pinnata* was also reported to have no significant inhibition on alpha amylase enzyme (Deutschlander *et al*., 2009). It is also reported that compounds that possess strong inhibition of pancreatic alpha amylase activity have similar side effect to that of acarbose (Bischoff, 1994). The results obtained in the present study for alpha amylase however, could be desirable, as previous studies reported that total or excessive inhibition of the pancreatic alpha amylase caused abdominal discomfort (Kwon *et al*., 2006; Cho *et al*., 2011).

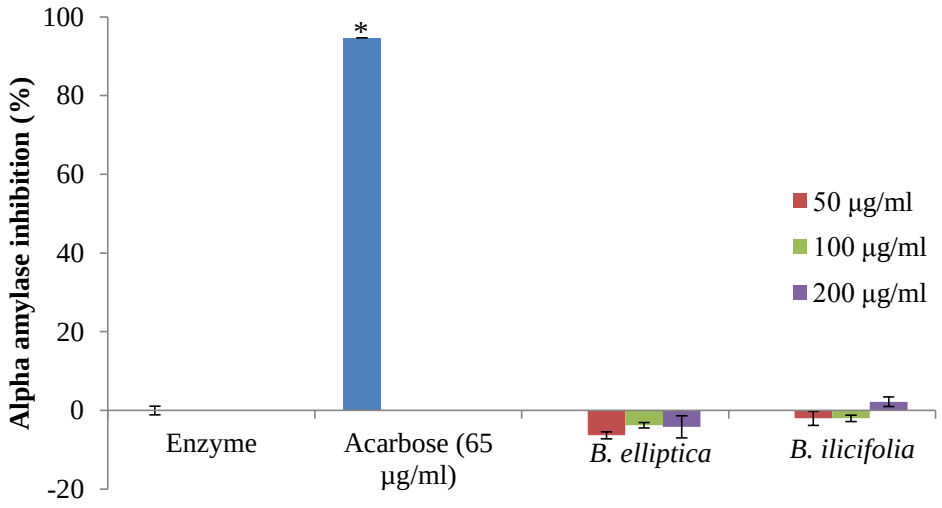

Figure 11: Potential inhibitory effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on alpha amylase activity (%). Data expressed as mean ± SD (n = 4). (*) indicates a significant increase relative to the untreated control (Enzyme) ($P < 0.05$). No significant increase relative to the positive control (acarbose) was noted.

3.3.2 Alpha glucosidase inhibition assay

Alpha-glucosidase is a membrane bound enzyme present in the epithelium of the small intestine, responsible for facilitating the absorption of glucose by catalyzing the hydrolytic cleavage of oligosaccharides or disaccharides into absorbable monosaccharides. Inhibiting this enzyme slows down the elevation of blood sugar following a carbohydrate meal (Lebovitz, 1997). In this study, the inhibitory potential of aqueous leaf extracts of *B elliptica* and *B ilicifolia* against the alpha glucosidase enzyme was evaluated (Figure 12). The crude extracts of both plants displayed a significantly weaker inhibitory activity compared to EGCG (57.5%), at all the concentrations tested (50 µg/ml, 100 µg/ml and 200 µg/ml). At the highest concentration (200 µg/ml) investigated, *B. elliptica* inhibited alpha glucosidase by 32.2%, which was significantly higher ($P< 0.05$) than that of *B. ilicifolia* (22.9%), at the same concentration. It was also observed that, at the lower dosage (50 µg/ml), the extract of *B. elliptica* exhibited a significant higher (21.6%)

inhibition on alpha glucosidase when compared to *B. ilicifolia* (16.5%). These results showed that the crude extracts of both plants exhibited a < 40% inhibition of the intestinal alpha glucosidase and therefore the IC_{50} was not determined. This is similar to the previous report by Deutschländer *et al*. (2009) for the acetone extract of *Pteronia divaricata* which showed a low inhibitory effect of the α-glucosidase enzymes. Other studies have also shown that plant polyphenols inhibit alpha glucosidase activities (Haysteen, 1983; Kim *et al*., 2000). Polyphenols such as tannins, anthocyanins and flavonoids are known to possess potent alpha glucosidase inhibitory activities (Toda *et al*., 2000). The weak inhibitory effect of both plants extracts on α-glucosidase could therefore be attributed to the presence of polyphenols such as flavonoids and tannins.

It can therefore, be deduced from this study that the antidiabetic properties of both plants extract probably function through other mechanisms and not through the inhibition of carbohydrate digestive enzymes.

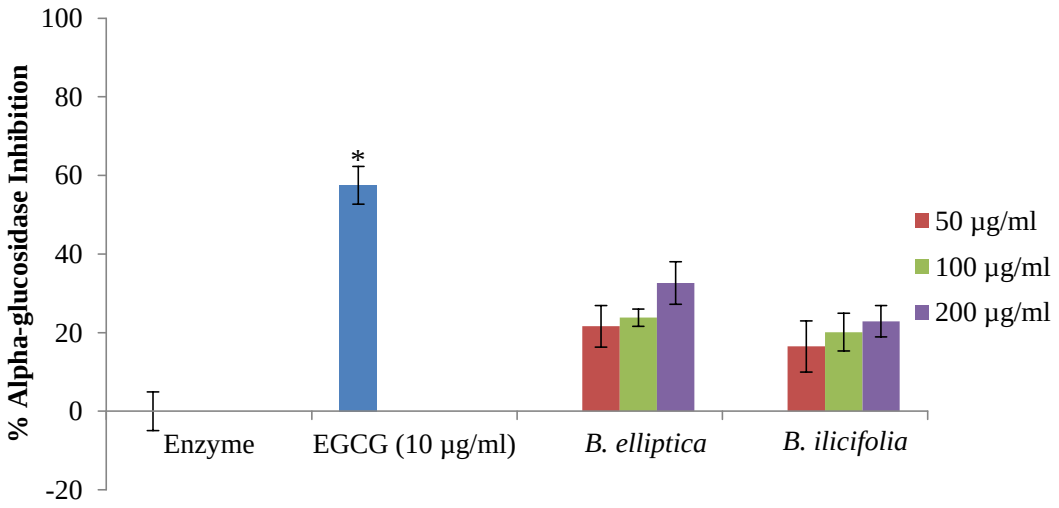

Figure 12: The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on alpha glucosidase activity. EGCG: epigallocatechin gallate. Data expressed as mean ± SD, (n = 4). (*) indicates a significant increase relative to the untreated control (Enzyme) ($P < 0.05$). No significant increase relative to the positive control (epigallocatechin gallate) was noted.

3.3.3 Lipase inhibition assay

Pancreatic lipase is known as the principal lipolytic enzyme synthesized and secreted by the salivary glands and the pancreas. It plays an important role in the efficient digestion of triglycerides. The pancreatic lipase is accountable for the hydrolysis of 50–70% of the total dietary fats (Mukherjee, 2003). Pancreatic lipase inhibition is one of the most broadly studied mechanisms for the determination of the potential efficacy of natural products as anti-obesity agents (Seyedan *et al.*, 2015). Obesity is a major risk factor for the type II diabetes, cardiovascular disease, dyslipidemia (increased levels of total cholesterol, low-density lipoprotein and triglycerides with decreased levels of high-density lipoprotein) and gallbladder disease (Mukherjee, 2003). Inhibition of pancreatic lipase was chosen as the criteria for therapeutic efficacy since such inhibition would serve two functions, i) it would provide an adjunctive therapy to the pharmacological agents and ii) would lessen systemic adverse reactions

by acting topically in the GI tract in order to decrease fat absorption. In this present study, the results of the crude extracts of *B. elliptica* and *B. ilicifolia* were investigated using orlistat (18 µg/ml) as the positive control (Figure 13). The results obtained displayed no significant inhibition from both plants extracts on the pancreatic lipase enzyme at all the concentrations tested compared to orlistat, a known lipase inhibitor. In comparison, *B. elliptica* showed weak significant inhibition at all the concentrations tested (50 µg/ml, 100 µg/ml and 200 µg/ml) in a dose dependent manner when compared to *B. ilicifolia* that stimulated the enzyme at all concentrations investigated in this study. Ong *et al*. (2014) reported similar results when they investigated the effect of methanolic extract of *Youngia japonica* and *Vernonia amyglalina* on inhibition of the pancreatic lipase activities *in vitro*. The extracts of both plants did not exhibit inhibitory effect on pancreatic lipase, with extracts of *Vernonia amyglalina* actually showing increased enzyme activity at 100 µg/ml. This finding corroborated with the results of this present study in that *B. elliptica* and *B. ilicifolia* exhibited no significant inhibition on the pancreatic lipase however, *B. ilicifolia* showed an increased enzyme activity at 100 µg/ml. Ethanolic extracts of *Baccharis trimera* (Asteraceae) which also belong to the Asteraceae family showed a low significant inhibition (<17%) on pancreatic lipase (De Souza *et al*., 2011). These findings therefore, indicate that the *B. elliptica* and *B. ilicifolia* plants may act as antidiabetic agent though not as inhibitors of the lipases.

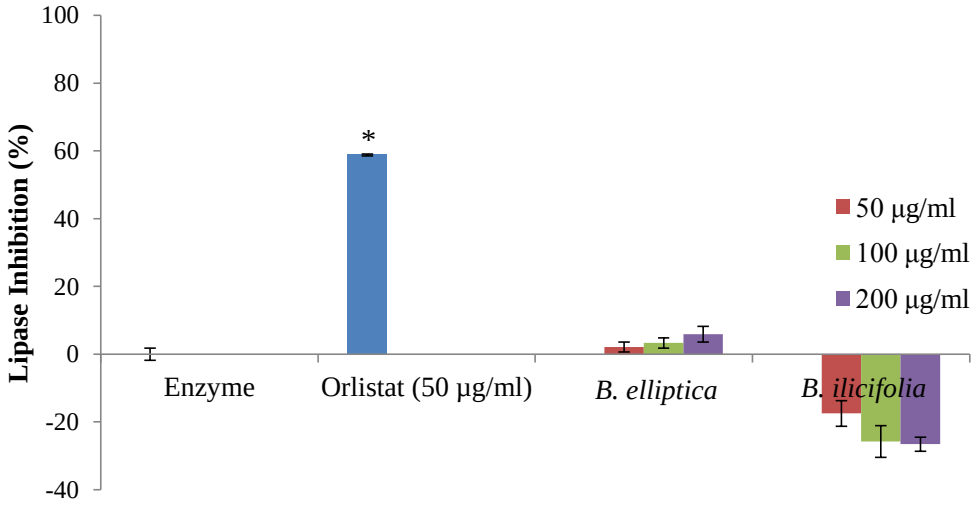

Figure 13: The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on pancreatic lipase activity (%). Data expressed as mean ± SD (n = 4). (*) indicates significant increase relative to the untreated control (enzyme) ($P < 0.05$). No significant increase relative to the positive control (orlistat) was noted.

3.3.4 DPP – IV inhibition assay

The dipeptidyl peptidase IV (DPP-IV) enzyme plays a crucial role in glucose metabolism. It destroy or degrades incretin hormones such as the gastric inhibitory polypeptide (GIP) or glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 (GLP-1) (Mclntosh *et al*., 2005). These hormones are peptide hormones released by the digestive tract in response to nutrient ingestion and play an important role in glucose homeostasis by stimulating insulin secretion, β-cell growth and differentiation, and the inhibition of glucagon secretion (Mclntosh *et al*., 2005). Reduction of incretin (GIP and GLP-1) degradation by DPP-IV activity represents an important theurapeutic strategy to control type 2 diabetes. The aqueous leaf extracts of *B. elliptica* and *B. ilicifolia* showed no significant inhibition against the DPP-IV enzyme at all tested concentrations when compared to the positive control diprotin A (75.8%) (Figure 14). The observed results also showed that, *B. elliptica* displayed a very low significant inhibition (0.3 ±

1.3) at the lowest (50 µg/ml) dosage when compared to *B. ilicifolia* that stimulated DPP-IV enzyme at all the tested concentrations. Nevertheless, none of the plant extracts evaluated shows inhibition that was as potent as that of the diprotin A (75.8%), a known DPP-IV inhibitor. This high inhibitory activity observed in DPP-IV enzyme by diprotin A might be due to the tripeptide specificity and purity. Maes *et al*. (2007) reported that diprotin A affects the catalytic activity of DPP-IV in the human central nervous systems, CD-26 of the immune system. The non-inhibitory effect of the *B. elliptica* and *B. ilicifolia* extracts are contradictory to the results reported for *Berberis aristata, Szygium cumini* and *Mangifera indica* which were claimed to be novel DPP-IV inhibitors (Davy *et al*., 2000; Sedo *et al*., 2002; Yogisha & Rveesha, 2010; Singh & Jatwar, 2012;). The antidiabetic mechanism of *B. elliptica* and *B. ilicifolia* are therefore not through the inhibition of DPP-IV.

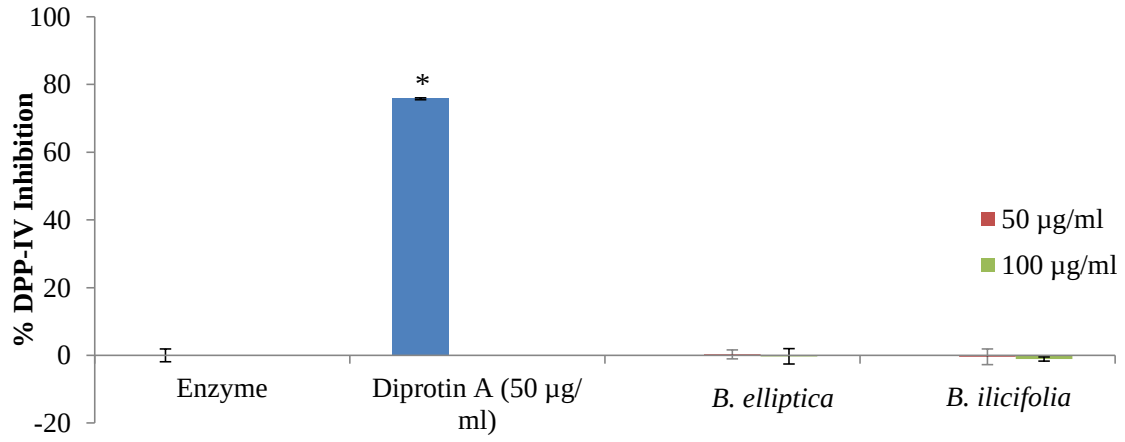

Figure 14 : The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on DPP-IV activity (%). Data expressed as mean ± SD (n= 4) (*) indicates a significant increase relative to the untreated control (Enzyme) ($P < 0.05$). No significant increase relative to the positive control (diptotin A) was noted.

3.3.5 Inhibition of nitric oxide production in RAW macrophage cells

Nitric oxide (NO) is a small, short-lived free radical that easily spreads from its production site within a cell to a different site of action (Aktan, 2004). It acts as a cytoprotective molecule for host defence responses against different pathogens, for example, bacteria, fungi, viruses and parasites (Bogdan *et al*., 2000). Under normal conditions, NO plays a very vital role in the up regulation of several pathophysiological processes such as vasodilatation (widening of blood vessels), neurotoxicity and neurotransmission (Moncada *et al*., 1991; Nakagawa and Yokozawa, 2002). However, it has been reported that an over production of NO causes tissue damage linked with acute and chronic inflammations (Taira *et al*., 2009). Adipose tissue inflammation has been recognised as a contributory factor in the development of diabetes and obesity (Boutens & Stienstra, 2016). Therefore, an attenuation of adipose tissue inflammation is consequently identified as a potential therapeutic target in the treatment of diabetes. In the present study, aqueous extracts of *B. elliptica* and *B. ilicifolia* were evaluated for their possible inhibitory potential on NO production in the lipopolysaccharides (LPS)-stimulated RAW macrophage cells and NO production was measured as nitrate concentration in the culture medium (Figure 15).

The crude extracts of both plants significantly inhibited the NO accumulation in LPS activated RAW macrophage cells in a concentration dependent manner, but their effect was significantly lower than that of aminoguanidine (0.96 ± 0.2), a known inhibitor of NO production (Figure 15). When compared to the untreated control, the pre-treated cells induced with LPS, released a higher level of NO in the medium at both concentrations of 12.5 µg/ml and 25 µg/ml respectively. Treatment of RAW macrophage cells with *B. elliptica* significantly reduced NO formation at a concentration of 50 µg/ml (4.9 ± 0.3) when compared to *B. ilicifolia* (5.1 ± 0.9) at

the same concentration. It was also observed that at the highest concentration (100 µg/ml) investigated; there was no significant difference between both plants by showing reduction in NO formation. The number of viable activated macrophages was not significantly affected by the crude extracts of both plants as determined by MTT assays, thus indicating that an inhibition of the NO production was not due to cell death (data not shown). The results of this present study are in agreement with other studies of extracts from the *Brachylaena* family which reported an inhibition of the nitric oxide production (Chougouo *et al*., 2016). Ethanol extracts of *Artemisia* were also reported to reduce NO production in RAW macrophages at 50 µg/ml, which is in accordance with the results of this present study (Chougouo *et al*., 2016). Studies have also suggested that plant secondary metabolites such as flavonoids, phenols, polyacetylenes and lignans inhibit various inflammatory facilitators including nitric oxide (Chen *et al*., 2008; Choi *et al*., 2009; Paoletti *et al*., 2009). Therefore, the NO inhibitory effects observed in the present study could be attributed to the presence of polyphenolics, predominantly flavonoids and phenols in the extracts of both plants.

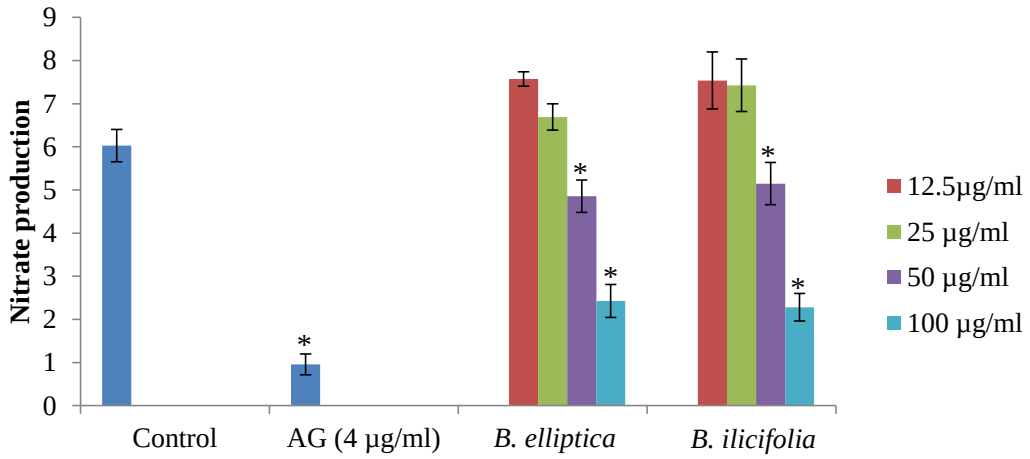

Figure 15: Inhibitory effects of aqueous extracts of *B. elliptica* and *B. ilicifolia* on NO production by LPS-stimulated RAW macrophage cells. Concurrent MTT assay indicates no significant toxicity under the experimental conditions (Data not shown) Data expressed as mean ± SD (n = 4). (*) indicates significant decrease relative to the untreated control ($P < 0.05$). No significant decrease relative to the positive control (aminoguanidine) was noted.

3.3.6 Glucose utilisation in HepG2 liver cells.

The results of the effect of *B. elliptica* and *B. ilicifolia* in HepG2 glucose utilization is shown in Figure 16. Metformin (0.1 µg/ml) and berberine (18 µg/ml) were chosen as positive controls due to their known effect on glucose uptake and metabolism in hepatic cells. In this study, a significant reduction in the level of glucose in the medium was observed at the concentrations used (25 µg/ml and 100 µg/ml), for both the *B. elliptica* and *B. ilicifolia* extracts. This is an indication that both plant extracts caused a significant increase in the glucose uptake in HepG2 when compared to berberine (108%) and the untreated control. It was also observed that the effects of both plants and berberine were less marked in HepG2 compared to metformin (164%). This effect of metformin stimulation was expected as it is known to enhance glucose utilisation in hepatic cells. This suggests that the activation of glucose utilisation is the primary effect of metformin and the suppression of gluconeogenesis through AMP-activated protein kinase may

be a secondary effect. MTT assays showed no significant toxicity for metformin berberine and *B. ilicifolia* (Figure 17). However, *B. elliptica* showed low levels of toxicity (< 10%). Metformin, berberine and *B. ilicifolia* activate a proliferation of the cells, which could be as a result of the stimulation of growth factors.

The effect of aqueous leaf extracts of both plants on glucose utilisation in the HepG2 cells has not been previously reported in literature. The results of this study support the findings of van de Venter *et al*. (2008) who reported that the aqueous and organic extracts of *Brachylaena discolour* showed a good glucose utilisation activity in Chang liver with no or very low toxicity recorded. Previous reports have also shown that medicinal plants exhibit hypoglycemic and antidiabetic activity due to the presences of phytochemicals such as phenols, terpernoids flavonoids, flavanols, proanthocyanidin and glycosides (Ahmad *et al*., 2000; Anila *et al*., 2002 ; Mankil *et al*., 2006 ; Sanni, 2007). Therefore, the glucose uptake observed in HepG2 for both plants could be attributed to the presence of some polyphenolics compounds reported in both plants (Sagbo, 2015) or due to the activation of the insulin signalling cascade, resulting in stimulation of GLUT 2 that facilitates the translocation of glucose into the cell. It can be deduced from this study that an enhancement of glucose uptake in hepatic cells could possibly be the mechanism through which both plants function as antidiabetic agents.

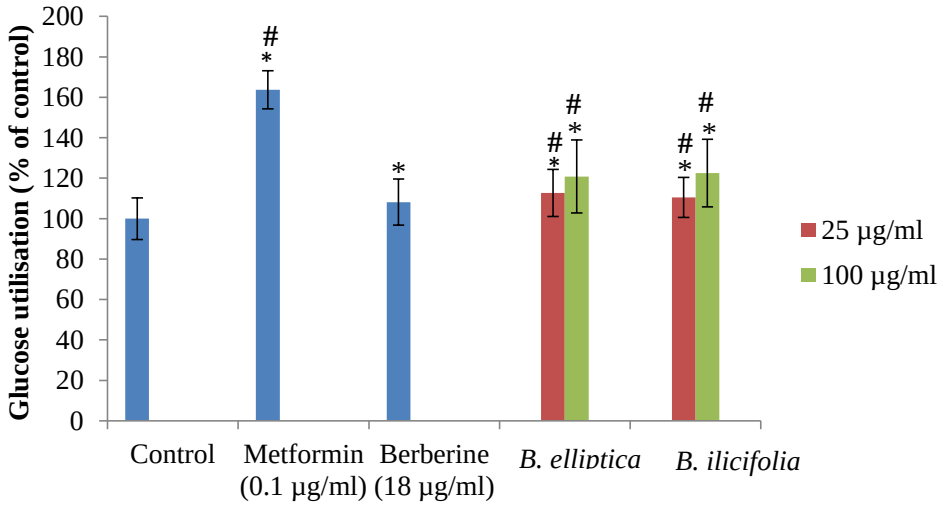

Figure 16: Effect of *B. elliptica and B. ilicifolia* on glucose utilization in HepG2 hepatocytes**.** Cells were treated for 48 hrs in the presence or absence of varying concentration of both plant extracts. Data expressed as mean ± SD (n = 4). (*) indicates a significant increase relative to the untreated control ($P < 0.05$). (#) indicates a significant increase relative to the positive control (berberine) ($P < 0.05$). No significant increase relative to the metformin (positive control) was noted.

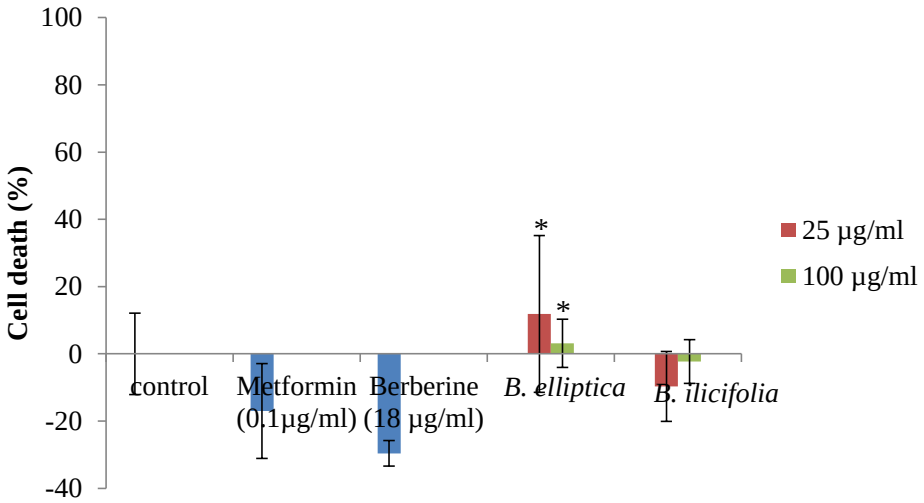

Figure 17: MTT assay of *B. elliptica* and *B. ilicifolia* extracts in the HepG2-liver cells. Data represents the mean ± SD (*n* = 4). (*) indicates a significant increase relative to the untreated control ($p < 0.05$).

3.3.7 Glucose utilisation in L6 myoblast

Muscles are the main sites for disposal of ingested glucose in humans (Abdul-ghani & Defronzo, 2010). In the skeletal muscle, insulin enhances glucose uptake by stimulating the translocation of glucose transporter (GLUT 4) from an intracellular compartment to the plasma membrane through the PI3-kinase pathway. However, evidence have suggested that muscle accounted for more than 80% of the total insulin mediated glucose uptake in the postprandial state (Kumar and Dey, 2003). Defects in insulin stimulated skeletal muscle glucose uptake are common pathological states in type II diabetes (Dachani *et al*., 2012). Many researchers have reported the use of an L6 cell model to elucidate the mechanisms of glucose uptake in muscles (Gupta *et al*., 2010; Das & Devi, 2015; Odeyemi, 2016). The L6 cell model represents a good model for glucose uptake in skeletal muscle as it possesses an intact insulin signalling pathway and expression of the insulin sensitive GLUT 4 (Gupta *et al*., 2010). Therefore, this study investigated the effect of *B. elliptica* and *B. ilicifolia* on glucose uptake in L6 muscle cells (Figure 17).

Insulin was used as positive control due to its known effects on glucose uptake in muscle cells. The results revealed a low reduction in the level of glucose in the medium at all the tested concentrations for *B. elliptica* and *B. ilicifolia* extracts, indicating that both extracts showed a weak significant increase in the glucose uptake in L6 muscle cells. At the highest (50 µg/ml) concentration tested in this study, *B. ilicifolia* showed a slight significant ($P < 0.05$) increase (111%) in glucose uptake in the L6 cells when compared to *B. elliptica* (107%) and the untreated control (Figure 18). Nevertheless, none of the plant extracts and untreated control evaluated caused a significant increase in glucose uptake when compared to the positive control, insulin

(129 %). Concurrent MTT assays indicated no significant toxicity under this experimental condition but rather proliferative effects (Figure 19) by both plant extracts and insulin which could be because both extracts and insulin induced the expression of growth factors (Figure 19). Research undertaken by Erasto *et al*. (2009) reported a significant increase in glucose uptake in C_2C_{12} muscle cells for an aqueous extract of *Vernonia amygdalina* at 50 µg/ml. Aqueous leaf extracts of *Brachylaena discolor* were also found to show a significant increase in glucose utilization in C_2C_{12} muscle cells at 12.5 µg/ml (van de Venter *et al*., 2008). These finding contradict the results obtained in this present study in that crude extracts of *B. elliptica* and *B. ilicifolia* showed only a weak increase in glucose uptake in L6 cells. Therefore, it can be deduced from this study that the antidiabetic mode of action of these plant extracts is not through glucose uptake in skeletal muscle.

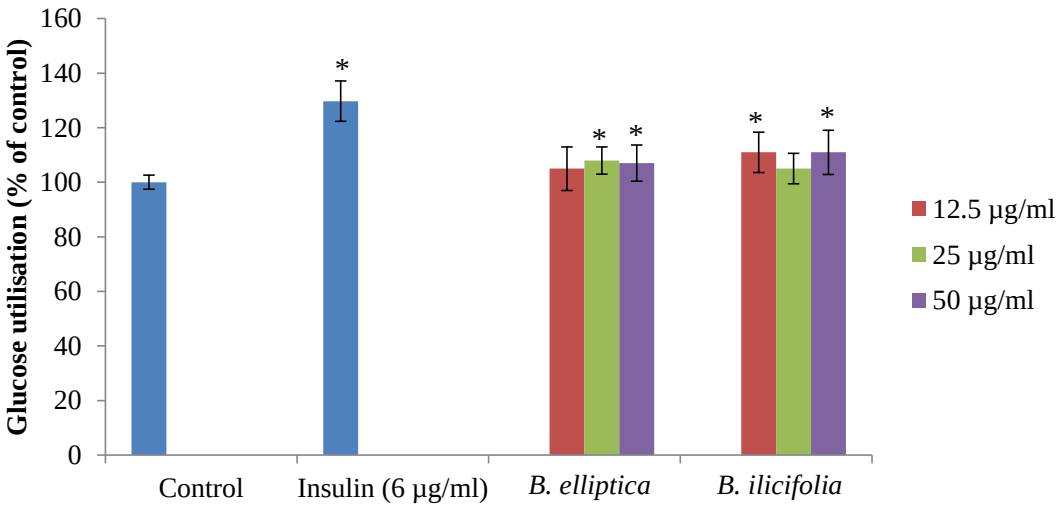

Figure 18: Effect of *B. elliptica* and *B. ilicifolia* on glucose utilization in L6 myoblast. Cells were treated for 48 hrs in the presence or absence of varying concentration of plant extracts. Data expressed as mean ± SD (n= 4). (*) indicates a significant increase relative to the untreated control ($p < 0.05$). No significant increase relative to the positive control (insulin) was noted.

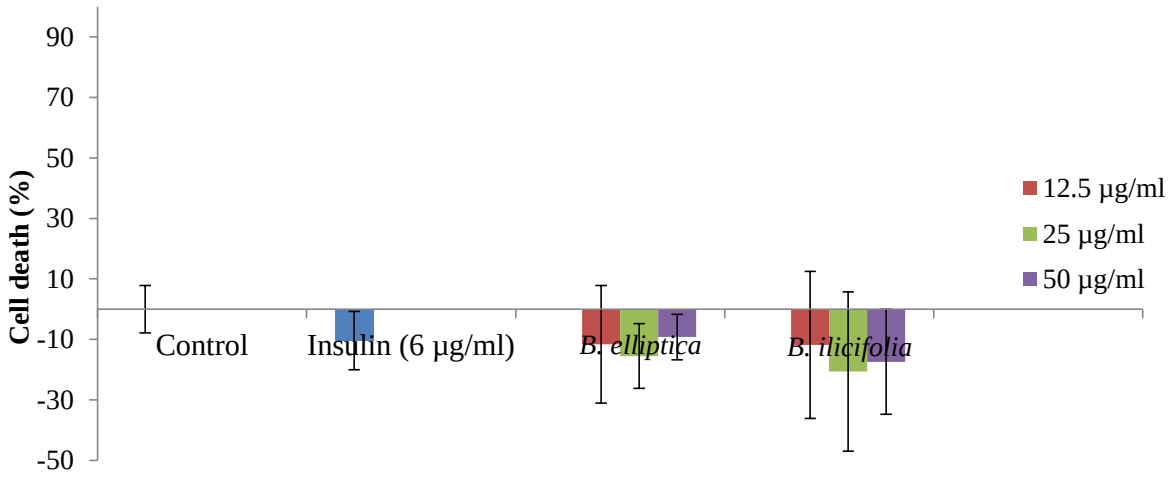

Figure 19: MTT assay of *B. elliptica* and *B. ilicifolia* extracts in the L6 muscle cells. Data represents the mean ± SD (*n* = 4). No significant toxicity relative to the untreated control was noted.

3.3.8 Lipid accumulation in 3T3-L1 preadipocytes

Adipose tissue plays a significant role in the upkeep of whole body insulin sensitivity and subsequently glucose homeostasis (Jung & Choi, 2014). However, excess blood glucose and free fatty acids are channelled into triglyceride thereby attenuating the ectopic deposition of lipid in organs such as the liver, muscle and pancreas (Jung & Choi, 2014). Adipocytes represent the primary cell type of adipose tissue thereby playing a major role in maintaining energy homeostasis. Persistent exposure of adipocyte to over-nutrition promotes adipocyte dysfunction, which is characterized by decreased insulin responsiveness of adipocyte and increased lipolysis which contribute to the development of insulin resistance and overt diabetes (Goossens, 2008).

In this study, the effect of aqueous extracts of both plants on triglyceride accumulation in 3T3-L1 preadipocyte differentiation was examined using Oil-red-O staining (Figure 20). The crude

extracts of both plants did not exhibit a significant effect on lipid accumulation in the 3T3-L1 cells at the concentrations (12.5 µg/ml and 25 µg/ml) tested when compared to untreated cells. In general, *B. ilicifolia* extracts reduced lipid droplet accumulation in 3T3-L1 but weak at the highest concentration (50 µg/ml) tested by 7% compared to *B. elliptica*, untreated cell and rosiglitazone respectively. However, rosiglitazone significantly induced lipid accumulation in the 3T3-L1 cells. This is in agreement with finding from previous study which reported significant increase in lipid accumulation in 3T3-L1 when treated with rosiglitazone (Oyedemi *et al*., 2012). Rosiglitazone is a standard antidiabetic drug used as an insulin sensitizer, by binding to peroxisome proliferator-activated receptor-gamma (PPARγ) and making fat cells more responsive to insulin. As a PPARγ agonist, rosiglitazone enhances adipogenesis, which is accompanied by an increasing lipid content of the differentiating cells (Hutley *et al*., 2003; Wang *et al*., 2007). Studies have also shown that 3T3-L1 cells express PPARγ thereby exhibiting a hypoglycemic effect via PPARγ (Verma *et al*., 2004 ; Nazemzadeh, 2012).

It can be concluded from this study that the observed results give an indicated that the crude extracts of *B. elliptica* and *B. ilicifolia* might not be a good therapeutic agent in lowering excessive triglyceride accumulation associated with a variety of metabolic disorders.

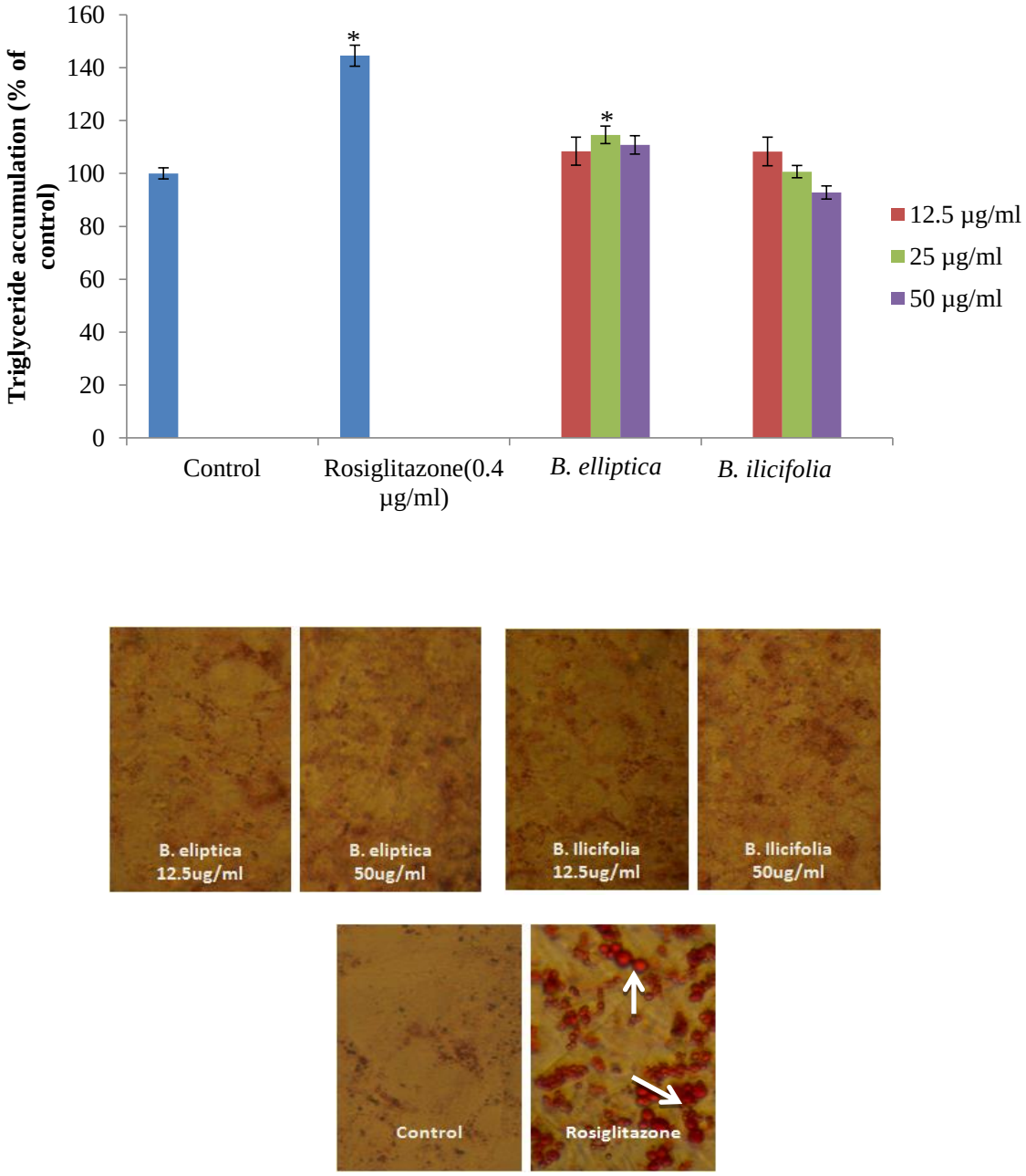

Figure 20: Effect of *B. elliptica* and *B.ilicifoli* extracts on triglyceride accumulation in 3T3-L1 preadipocytes. Two days post-confluence preadipocytes were treated for an additional two days with 0.1µM insulin and varying concentrations of plants extracts or rosiglitazone. Cells were cultured in normal culture medium for an additional ten days. Lipid accumulation was visualized by staining with Oil red O. Arrows indicate lipid accumulation. Data expressed as mean ± SD (n= 4). (*) indicates a significant increase relative to the untreated control (control) ($p < 0.05$). No significant decrease relative to untreated control was noted.

3.3.9 Glucose metabolism as a reflection of insulin secretion

In pancreatic beta cells, insulin secretion depends on glucose metabolism. When blood glucose concentrations are high, pancreatic beta cell metabolisms are activated through the glucose transporter 2 (GLUT 2) (Westerlund & Bergsten, 2001). Glucose is then phosphorylated by hexokinase IV (glucokinase) and undergoes glycolysis and further metabolized to yield ATP via the mitochondrial TCA cycle and oxidative phosphorylation causing an increase in the ATP to adenosine diphosphate (ADP) ratio. The resulting increase in the ATP/ADP ratio decreases the permeability of the ATP-sensitive K^+ (KATP) channels leading to depolarization of the cell (Yeom *et al*., 2006). The subsequent influx of Ca^{2+} through voltage-dependent channels triggers some insulin storing granules to secrete insulin through the process of exocytosis (Yang & Gillis, 2004). However, an impaired glucose metabolism in pancreatic β-cells has been reported to be implicated in the development of insulin secretion (Westerlund & Bergsten, 2001). Therefore, the effect of plant extracts on INS-1 glucose metabolism as a reflection of insulin secretion was investigated using an MTT colorimetric assay (reduction of tetrazolium salts to formazan) (Figure 21).

In the light of considering the unique metabolic feature of MTT, it is particularly suitable for assessing the beta cell function, thereby capable of modulating its glycolytic and oxidative rates as a function of extracellular glucose concentration. Several lines of studies have also suggested that MTT reduction reflects an indirectly mitochondrial oxidative processes of living cells (Takahashi *et al*., 2002). The exact cellular mechanism by which MTT (tetrazolium salts) is transformed to formazan production in the cells is partly understood, but likely involves a reaction with NADH or similar reducing molecules that transfer electrons to MTT. Electron

transfer from mitochondrial succinate dehydrogenase complex has been suggested to be coupled with the production of formazan (Janjic & Wollheim, 1992).

In this study, the results of the crude extracts of both plants on INS-1 glucose metabolism as a reflection of insulin secretion were investigated (Figure 21). The results obtained displayed no significant effect on INS-1 cell glucose metabolism for both plant extracts when compared to the untreated control indicating that both plant extracts at 100 µg/ml did not increase the formazan production and therefore did not increase glucose metabolism. However, it is possible that by increasing the concentration, the crude extracts of both plants would increase the formazan production. These results vastly contrast, those found by Jung *et al*., (2006) who that reported stevioside, which is found in plant *Stevia rebandiana* (Asteraceae) acts by stimulating insulin secretion via direct action on the beta cells of pancreatic islet cells. It can be concluded that both plants extracts do not function as antidiabetic agents by inducing insulin secretion in beta cells in response to high blood glucose levels.

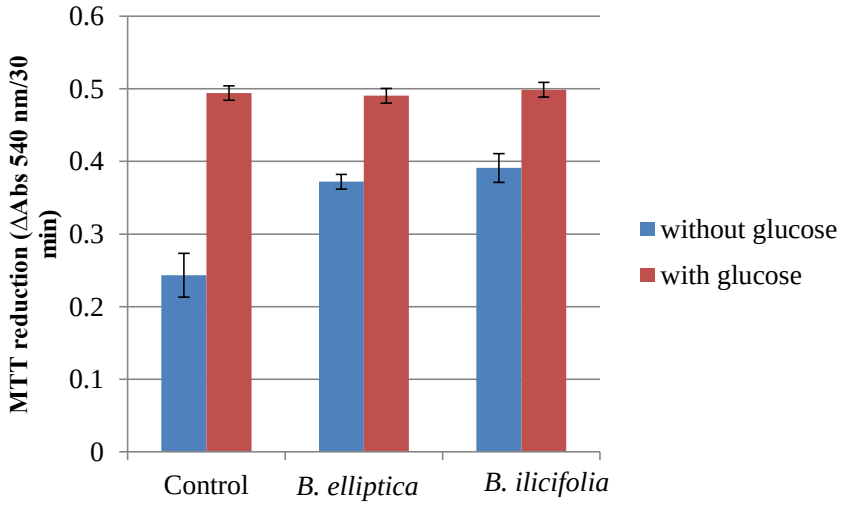

Figure 21: The effect of *B. elliptica* and *B. ilicifolia* on MTT reduction in INS-1 cells. Data are expressed as mean ± SD (n = 6). No significant increase relative to the untreated control (with glucose) was noted.

3.4 CONCLUSION

The extracts of *B. elliptica* and *B. ilicifolia* displayed no significant inhibition on alpha amylase, alpha glucosidase, dipeptidy peptidase-IV and lipase compared to the inhibition show by the positive controls, acarbose, EGCG, diprotin A and orlistart respectively. Mild inhibition of *B. elliptica* on alpha glucosidase activity at high concentrations, is not physiologically relevant. The extracts of both plants strongly reduced NO production in RAW macrophages at the highest concentrations tested in this study, which may at least in part, be explained by the presence of flavanoids and phenols in these plants. However, both plants displayed no significant effect on triglyceride accumulation in 3T3-L1 or glucose metabolism in INS-1 cells. Treatment of L6 cells revealed no significant increase in glucose uptake. However, the enhanced glucose uptake in HepG2 cells may represent a mechanism through which the aqueous leaf extracts of *B. elliptica* and *B. ilicifolia* improve glycemic control. The crude extract of both plants could possibly exert

their hypoglyceamic effect in HepG2 cells via interaction with the insulin receptor, resulting in stimulation of the GLUT 2 cascade.

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CHAPTER 4

Evaluation of *B. elliptica* and *B. ilicifolia* aqueous potential to alleviate secondary diabetic complication and synergistic effect on the metabolism of diabetic drugs

Part of this Chapter has been presented orally at the First International Conference of the Society for Medicinal Plants and Economic Development (SOMPED)

Conference presentation title: *In vitro* antidiabetic activity and mechanism of action of extract of *Brachylaena elliptica* (Thurb.) DC.

CHAPTER 4

4.1 INTRODUCTION

Diabetes complications are initiated by the accumulation of advanced glycation end products (AGE's) in various tissues. Studies have shown that AGEs accumulation plays a major pathogenic link between hyperglycemia and diabetic complication (Jakuš & Rietbrock, 2004). During diabetes mellitus AGE's are formed at an accelerated rate because of the hyperglycemic condition in the body (Kumar *et al*., 2004a; Kumar *et al*., 2004b). Glycation of proteins and its further oxidation changes the protein conformation and stability. It also induces protein aggregation and immobilization through cross-linkage (Watala *et al*., 1996; Kumar *et al*., 2004a ; Kumar *et al*., 2004b;). Collagen is one of the key targets for AGE's formation (Dorsey, 2012), causing damage to the collagen and thereby contributing to the development of atherosclerosis, diabetes and coronary diseases (Soldatos & Cooper, 2006). Diabetic patients demonstrate reduced capability in acute wound healing. These patients are also susceptible to develop chronic diabetic foot ulcers, a serious diabetic complication. However, studies have shown that collagen plays a very critical role in all phases of wound healing (hemostasis, inflammation, proliferation and remodelling) and as well as provide the strength of the healing tissue (Gosain & DiPietro, 2004; Mathieu *et al*., 2006). Subsequently, any procedure or process that results in the degradation or loss of integrity of this protein is likely to have significant health related issues such as diabetes complication. Collagenase enzymes by any means results in the destruction of collagen. Thus, inhibitors of collagenase and protein glycation may be an effective strategy in reducing diabetic complication.

Plant extracts generally contain several bioactive components, many of which have unknown biological activity. These bioactive components can possibly mimic, enhance or lessen the effects of co-administered prescription drugs resulting in synergistic or antagonistic effects through concurrent interaction on the same therapeutic target or by affecting the metabolic stability of prescription drugs (SØrensen, 2002). Also, competitive or antagonistic interactions can reduce the therapeutic ability of prescription drugs, leading to treatment failure (Fang, 2011). Previous studies have shown the involvement of herbal products in the inhibition of liver and intestinal drug metabolising enzymes such as cytochrome P450 (CYP450) (Pal and Mitra, 2006; Zhou *et al*., 2004; Odeyemi, 2016). Cytochrome P450 is mostly abundant in the gut, responsible for metabolism of xenobiotics in the intestine (Gavhane, 2012). It has emerged as a major determinant factor in the manifestation of several drug interactions that can lead to drug toxicities, adverse drug reactions and reduced pharmacological effect. Identifying whether the plants under study act as enzyme substrates, inhibitors or inducers can prevent clinically significant drug interactions from occurring.

4.2 MATERIALS AND METHODS

4.2.1 Collection of plant materials

The leaves of *B. elliptica* and *B. ilicifolia* were collected as described in section 3.2.1

4.2.2 Preparation of plant extracts

Preparation of plants extraction was done as described in section 3.2.2.

4.2.3 Protein glycation assay

Briefly, 50 µl of protein solution (100 mg gelatin in 5 ml of distilled water) was added to 10 µl of glyceraldehyde solution (222 mg in 5 ml distilled water) in black microplates. The plate was then sealed and incubated at 37^{o}C for 24 hour. After incubation, 40 µl of the plants extracts at concentrations of 50 µg/ml and 100 µg/ml were added. A blank containing distilled water instead of the extracts served as a negative control (untreated) while aminoguanidine (20 mM) was used as a positive control. The fluorescence was then measured at 370 nm (excitation); 440 nm (emission). The experiments were performed in triplicate and the percentage inhibition was then calculated using the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{Fluoresence\ of\ test\ well}{Fluoresence\ of\ negative\ control}\right) X\ 100$$

4.2.4 Collagenase inhibition assay

Briefly, 10 µl of enzyme was added to 10 µl of the plant extract at various concentrations (12.5 µg/ml, 25 µg/ml and 50 µg/ml) while EDTA (6 µg/ml) was used as positive controls. Ten microlitre (10 µl) of gelatine (2 mg/ml) followed by 10 µl of 50 mM Tris-HCl buffer (pH 7.4) were added to the reaction mixture. The resulting mixture was incubated at 37^{o}C for 1 hour. After incubation, 20 µl of Coomassie brilliant blue was added and then centrifuged at 500 rcf for 5 min. After the supernatant was removed, 50 µl of 40% methanol/ 10% acetic acid was added to wash the pellet. The pellet was then dissolved in 50 µl of DMSO. The absorbance was measured at 540 nm using a Multiscan MS microlitre plate reader (Lab systems). The experiments were performed in triplicate and the percentage inhibition was then calculated using the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{Absorbance\ of\ the\ untreated (Control)}{Absorbance\ of\ the\ test\ well}\right) X\ 100$$

4.2.5 Cytochrome P450 (3A4) Inhibition assay

The effects of the plant extracts were screened against a recombinant human CYP3A4 enzyme activity using the vivid CYP assay kits (BOMR substrate CYP3A4 red) according to the manufacturer's instructions. The kit contained the vivid CYP450 reaction buffer, the CYP450 BACULOSOMES reagent, the vivid fluorescent substrate, the vivid fluorescent standard, the vivid regeneration system (Part no. P2878; 333 mM glucose-6-phosphate and 30 U/mL glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate, pH 8.0), and 0.5 ml vivid $NADP^+$ (Part no. P2879; 10 mM $NADP^+$ in 100 mM potassium phosphate, pH 8.0). The vivid regeneration system and vivid $NADP^+$ were stored at -80^oC.

4.2.6 Preparation of stock

All the reactant mixtures were allowed to thaw for 10–15 min. The vivid substrates were reconstituted using an anhydrous acetonitrile and fluorescent standards were reconstituted using DMSO and DMSO/water (1:1) respectively.

4.2.7 Preparation of standard curve

Briefly, 100 µl of different concentrations of the vivid fluorescent standards (0 – 500 nM) were prepared in a 96 well plate, in duplicate. Fluorescence was measured using a BioTek's Synergy™ 4 Multi-Mode Microplate Reader (Winooski, VT, USA) and used to plot the standard curve. A volume of 200 µl of 500 nM vivid fluorescent standard was added into the first two wells (A1 and A2) of a 96 well plate while 100 µl of the reaction buffer was added to each of the remaining

well in column 1 and 2. Hundred microlitre (100 µl) of the vivid fluorescent standard from the first two well (A1 and A2) were then transferred into the well below (B1 and B2) containing 100 µl of the reaction buffer making it a two – fold dilution. This dilution step was then repeated until the last wells (H1 and H2) which were used as a blanks contained only the reaction buffer without the vivid fluorescent standard. The resulting concentrations of the fluorescent standard concentrations were 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 0 nM.

4.2.8 Reaction procedure

The plant extracts were prepared at 2.5 times the extract concentration (100 µM) within 1X CYP450 reaction buffer while ketoconazole (230 µM and 92 µM) was used as a positive control. Forty microlitre (40 µl) of the plant extracts or positive control were added to the relevant wells of the black 96-well plates. Fifty microlitre (50 µl) of the master pre-mix (P450 Baculosomes Plus reagent and vivid regeneration system in 1X vivid CYP450 reaction buffer) was then added to each well and incubated for 10 minutes at room temperature to allow the compounds to interact with the P450. After incubation, the reaction was then initiated by adding 10X substrate (10 µl) consisting of BOMR substrate and $NADP^+$ diluted in reaction buffer. The reaction mixture was incubated for 30 min at room temperature. The reaction was stopped by the addition of 50 µl of 0.5 M Tris base. The fluorescence was measured at 550 nm (Excitation) and 590nm (Emission) using a Biotek® Synergy MX fluorimeter. The same procedure was applied for the standards. A solvent control was also prepared; this contained only the reaction buffer instead of the sample. The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{C - B}{A - B}\right) X\ 100$$

Where,

C: the fluorescence intensity observed in the presence of the sample

A: the fluorescence observed in the solvent control

B: the fluorescence intensity observed in the presence of the positive inhibition control

4.3 RESULTS AND DISCUSSION

4.3.1 Protein glycation assay

Protein glycation involves a complex series of nonenzymatic reaction between reducing sugars and amino groups in proteins and lipoproteins. Over a period of time, glycation intermediates undergo a series of irreversible reactions through highly reactive dicarbonyl intermediates to form a complex family of stable covalent adducts called advanced glycation end-products (AGEs). The structural and functional integrity of the affected molecules become perturbed by these modifications.

In this study, the inhibition of protein glycation by either *B. elliptica* or *B. ilicifolia* was determined and indicated. The results showed that both plants extracts displayed weak (< 10 %) but significant inhibition of protein glycation at all the tested concentrations in a dose dependent manner, when compared to the positive control, aminoguanidine (65.7 %) (Figure 22). *B. elliptica* showed a significantly higher (8.2 %) inhibition of protein glycation at 100 µg/ml when compared to *B. ilicifolia* (7.7 %) at the same concentration. Studies have shown that some

polyphenols may inhibit formation of protein glycation (Rice-Evans *et al*., 1995; Sang *et al*., 2007; Edeas *et al*., 2010). Verzelloni *et al*. (2011) also reported that phenolic compounds inhibit the formation of glycation by preventing the binding of dicarbonyls to protein amino group. Thus, the weak inhibition observed in this study may be attributed to the presence of phenolic compounds reportly in both extracts (Sagbo, 2015). Kim and Kim (2003) reported a correlation between the inhibition of glycation and DPPH scanvenging activity as compounds that scavenge free radicals to the same extent could have a widely different capacity to inhibit protein glycation. Their finding supported the results of this study in that both extracts weakly inhibited the formation of protein glycation although they both showed strong DPPH radical scavenging activity (Sagbo, 2015). Therefore, findings of this study indicate that crude extracts of both plants do not have the ability to reduce diabetic complications and probably suggest that a combination therapy of both extracts would be most appropriate.

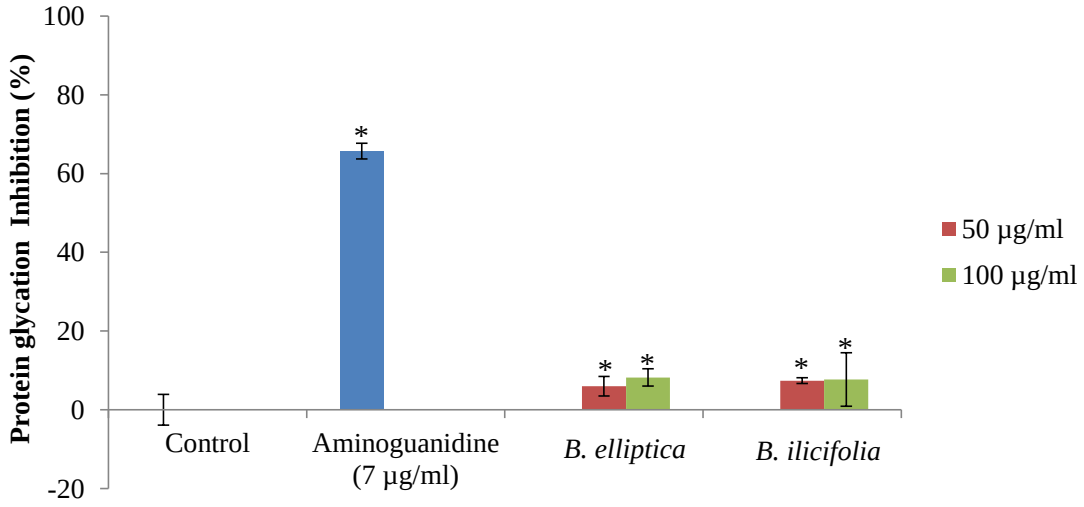

Figure 22: The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on protein glycation (%). Data expressed as mean ± SD (n = 4). (*) indicates significant increase relative to the untreated control (control) ($p < 0.05$). No significant increase relative to the positive control (aminoguanidne) was noted.

4.3.2 Collagenase inhibition assay

Collagen plays an important role in the wound healing process thereby controlling functions necessary to heal a wound, functions such as cell differentiation, cell shape, migration and synthesis of a number of proteins (Brett, 2008). Collagenase is a metalloproteinase enzyme which is responsible for the breakdown of collagen (Raffetto & Khalil, 2008). The effect of the aqueous extracts of *B. elliptica* and *B. ilcifolia* on collagenase was investigated (Figure 23). Both plant extracts displayed a weak inhibition on collagenase which was significantly less when compared to EDTA (75.7 ± 1.9), a standard known inhibitor of collagenase. *B. ilicifolia* significantly inhibited collagenase at all the tested concentrations in a dose-dependent manner when compared to *B. elliptica* that inhibited collagenase in a dose-decreasing manner. However, none of the extracts evaluated in this study, showed more potent inhibition than EDTA. This is in agreement with the finding of Thring *et al*., (2009) who reported a weak significant inhibition for *Silybum marianum* (L) Gaetrn on collagenase. Therefore, it could be deduced from this study that both plant extracts might not be considered as potential candidates in the search for lead compounds for the management of diabetic complications.

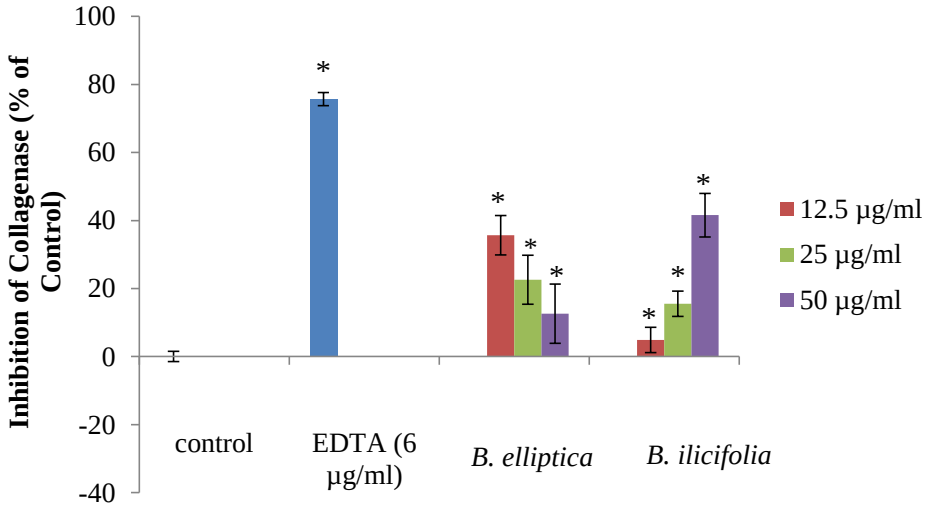

Figure 23: The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on collagenase activity (%). Data expressed as mean ± SD (n= 4). (*) indicates significant increase relative to the untreated control (control) ($p < 0.05$). No significant increase relative to the positive control (EDTA) was noted.

4.3.3 Cytochrome P450 (3A4) Inhibition assay

The inhibitory effect of *B. elliptica* and *B. ilicifolia* on CYP3A4 activity was investigated in this study. The results of the standard curve showed that the assay was in a linear and in the range of 0 – 500 nM, (Figure 24). In this study, a weak inhibitory effect was observed on CYP3A4 enzyme by the extract from *B. elliptica* (9.4 %) when compared to *B. ilicifolia* (29.4%) at the concentrations investigated (Figure 25). Nevertheless, both plant extracts evaluated, showed a significantly less inhibition of CYP3A4 than the standard positive inhibitor, ketoconazole at 230 µM (88.5 %) and 92 µM (56.7 %). The weak inhibition observed on CYP3A4 by both extracts could be attributed to the presence of phytochemicals such as alkaloids, flavonoids and saponins which have been specifically reported to be implicated in plant-drug interaction (Schmiedlin-Ren *et al*., 1997; Havsteen, 2002; Hanapi *et al*., 2010). The finding from this study is in agreement with previous reports on medicinal plants reported to have weak inhibition on CYP3A4 enzyme

(Donovan, 2003; Markowitz *et al*., 2003; Donovan *et al*., 2004). Therefore, the observed results give an indication that *B. elliptica* and *B. ilcifolia* might not be potent inhibitors of the CYP3A4 drug metabolising enzymes implying low potential risk of drug interactions with the plant extracts, especially in patients who are suffering from diabetes and consume large amounts of these plant extracts

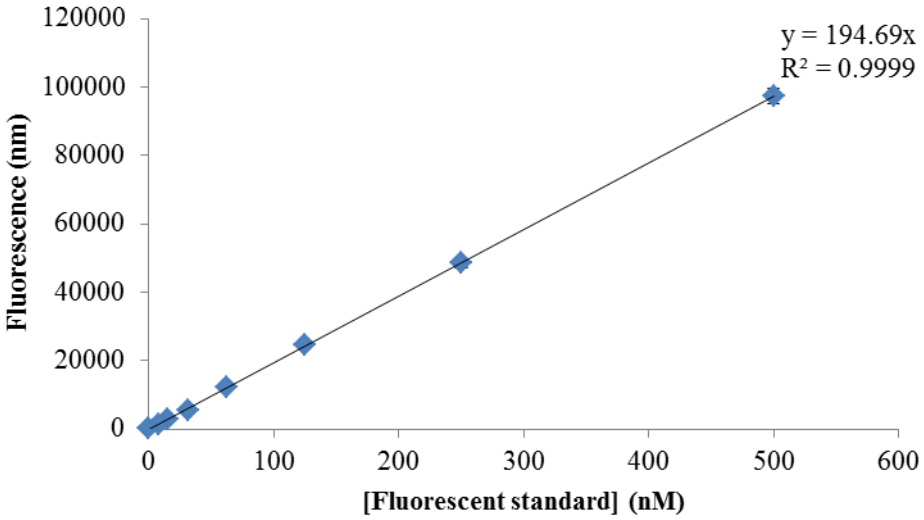

Figure 24: Vivid fluorescence CYP3A4 Starch standard curve. Data expressed as mean ± SD (n = 6).

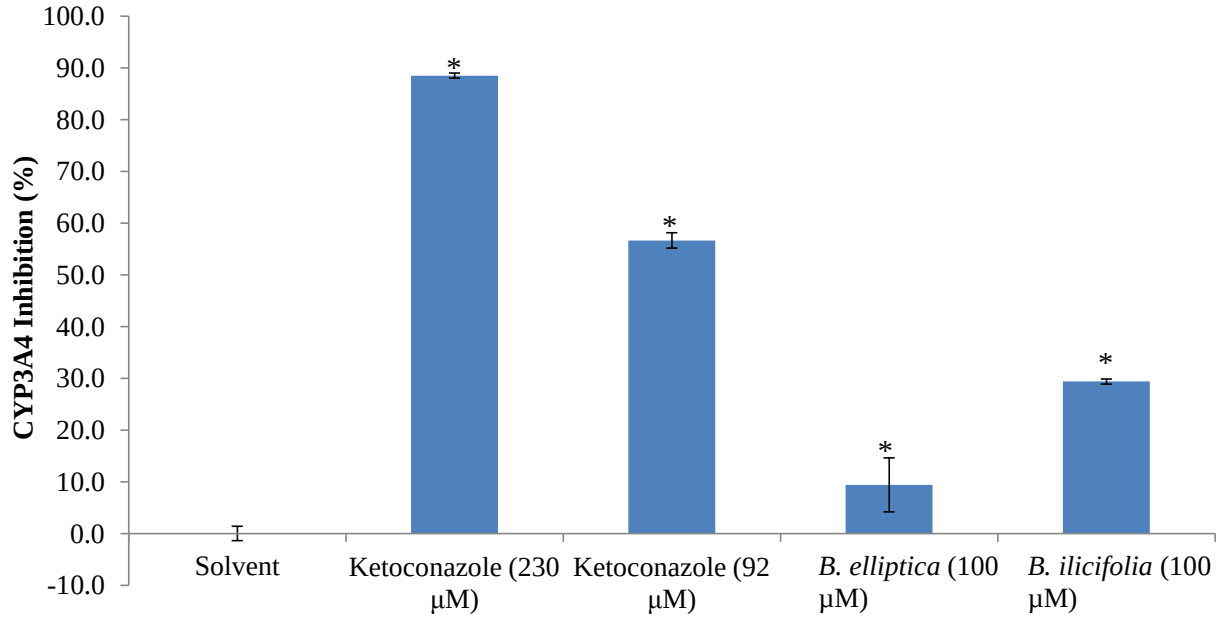

Figure 25: The effect of aqueous extracts of *B. elliptica* and *B. ilicifolia* on CYP3A4 activity (%). Data expressed as mean ± SD (n= 4). (*) indicates a significant increase relative to the untreated control (solvent) ($p < 0.05$). No significant increase relative to the positive controls (ketoconazole) was noted.

4.4 CONCLUSION

In summary, the aqueous extracts of these plants showed weak significant inhibitory effect on protein glycation, collagenase and CYP3A4 activity compared to the standard inhibitors, aminoguanidine, catechin, EDTA and ketoconazole respectively. The weak inhibition observed in protein glycation and collagenase activity suggests that these plants probably function through other antidiabetic mechanism as herbal remedy, but not through inhibition of glycated protein and collagenase which contribute to the development of diabetic complications. However, the weak inhibitory effect observed in CYP3A4 indicates that there might not be a potential risk of interaction if these plants are used with the conventional therapeutic product.

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CHAPTER 5

General discussion, conclusion and future studies

CHAPTER 5

5.1 GENERAL DISCUSSION

The present study investigated the *in vitro* antidiabetic properties and possible mechanisms of action of two indigenous plants, used by traditional healers to treat diabetic patients. The study was based on the fact that these plant extracts are being widely used as medicinal plants; however there is a lack of scientific evidence for their efficacy and potential mechanisms of action.

Cytotoxicity and proliferation

Less than 50% cell death was recorded for HepG2 cells treated with both plant extracts even at the highest dose of 200 µg/ml. Both plant extracts displayed low level of toxicity when compared to other plants used in previous studies that have shown significant toxicity (> 50% cell death) at different concentrations (17 µg/ml, 100 µg/ml, and 200 µg/ml) (Jansen *et al*., 2010; Muganga *et al*., 2010; More *et al*., 2012). The relative low level of toxicity exhibited by the extracts of these two plants raise prospects that aqueous extracts of both plants could be potentially safe for the users. Further *in vivo* investigations are required to elucidate or identify why the extracts are toxic and whether apopotosis is induced or not.

The crude extract of *B. elliptica* caused a significantly higher increase in INS-1 cell proliferation when compared to negative control and the *B. ilicifolia* extract, especially at the concentration of 25 µg/ml. Therefore, the proliferative effect observed in this study when compared to other plant from the same family (Asteraceae) that have shown proliferative effects at a concentrations of 100 µg/ml (Mellem, 2013) give an indication that the aqueous extract of *B. elliptica* may be used in preventing or alleviating hyperglycemia even at the low concentration levels, as a decline in

pancreatic beta cell mass is one of the major contributory factor in the development of diabetes (Baggio & Drucker, 2006; Oh, 2015).

Inhibition of digestive enzymes

Several pharmaceutical drugs are used to treat or manage diabetes and the mechanisms of action of these drugs are well known (Figure 26). These include the inhibition of alpha amylase, alpha glucosidase, and lipase and DPP-IV enzyme. The aqueous extracts of both plants of displayed no significant inhibition on alpha amylase, alpha glucosidase, lipase and DPP-4 when compared to the respective positive controls (Figure 26). The mild inhibition on alpha glucosidase activity observed in this study, by both plants extract, is probably not physiologically relevant.

Antioxidation potential

NO and its toxic metabolic peroxynitrate have been implicated in the pathogenesis of diabetes (Tangpong *et al*., 2008). Both plant extracts reduced NO production in RAW 264.7 cells probably through inhibition of the expression of iNOS. Further studies are required to confirm the inhibition of iNOS and the subsequent prevention of NO production during inflammation. Previous reports on antioxidant activity have suggested that both plant extracts possess strong antioxidant activity, but less marked when compared to the respective positive controls, vitamin C and butylated hydroxytoluene (BHT) (Sagbo, 2015). The presence of various phytochemical such as saponins, alkaloids, flavanols, proanthocyanidines, total phenol, flavonols, terpenoids have also been reported (Sagbo, 2015). The observed inhibition of NO production in RAW macrophage cells by both plant extracts, further confirm their potential antioxidant activity reported by Sagbo (2015).

Adipogenesis

A possible common link between diabetes and obesity is the adipocyte. Adipocyte plays a central role in whole-body energy metabolism, thereby storing excess lipid in the form of triglyceride, and releases free fatty acids in response to energy requirements in the fasting state (Jung & Choi, 2014). The capacity of adipose tissue to accommodate excess lipid can be exceeded in patients suffering from obesity resulting in the abnormal accumulation of lipid in other tissues, such as muscle, liver and pancreatic islet leading to physiological dysfunction (lipotoxicity) (Goossens, 2008). The results obtained for lipid accumulation in 3T3-L1 using oil red O staining indicated that the crude extracts of both plants displayed no significant reduction in lipid accumulation in the 3T3-L1 adipocytes. Therefore, it would be suggested the that both plant extracts might not be a good therapeutic agent in lowering lipid accumulation in patients suffering from obesity and diabetes.

Glucose metabolism as a reflection of insulin secretion

Impaired glucose metabolism in pancreatic β-cells has been reported to result in the reduction of insulin secretion (Matschinsky, 1996). In pancreatic beta cells, glucose metabolism is very important for glucose stimulated insulin release. Studies have shown that the rate of formazan production is correlated with glucose oxidation and glucose utilization in INS-1 cells and is therefore, correlated with glucose stimulated insulin release (Malaiisse, 1990; Janjic & Wollheim 1992). No significant increase in formazan production in INS-1 cells was observed for both plant extracts, and therefore they do not stimulate insulin secretion. The mechanism of action of these plants in treating diabetes cannot replace the use insulin injection to control diabetes.

Glucose uptake in liver and skeletal muscles

Glucose uptake is a crucial step in life-supporting processes since glucose is the main monosaccharide in nature that provides carbon and energy for almost all cells. The development of insulin resistance, which is linked to type II diabetes, leads to impaired glucose uptake in liver and skeletal muscle cells. In the present study, the crude extracts of both plant aqueous extracts significantly increased glucose uptake in HepG2 cells when compared to berberine. However, no significant glucose uptake was observed in L6 cells (skeletal muscles) when compared to the positive control, insulin. The observed results suggest that the aqueous extracts of both plants, therefore, mimic metformin by, increasing glucose uptake in the liver. Metformin belongs to the category of the biguanide class of oral hypoglycemic. Its exert its hypoglycaemic effect through activation of the AMP-activated protein kinase (AMPK) in the liver, which in turn may lead to various pharmacologic effects, including inhibition of glucose, lipid synthesis and also improved hepatic sensitivity to insulin (Foretz & Viollet 2011; Viollet *et al*., 2012). However, the mechanism by which metformin activates AMPK pathway is not yet understood (Nolte, 2009; Kokil, *et al*.,. 2010; Coughlan *et al.* 2014; Hart *et al.* 2016).

Alleviation of diabetic complications.

Protein glycation is one of the consequences of abnormal high blood glucose in patients suffering from diabetes (Ulrich & Cerami, 2001). Studies have shown that increased glycation and together with the build-up of tissue AGEs have been implicated in the pathogenesis of diabetic complications. This is mainly because they alter enzymatic activity, decrease ligand binding, modify protein half-life and facilitate crosslinking in collagen, by causing a loss of bulk elasticity and flexibility (Kostolanska *et al*., 2009). The aqueous extracts of both plants only

demonstrated weak inhibition on protein glycation and collagenase at all the concentrations investigated when compared to aminoguanidine and EDTA. Both plants are therefore might not be potential candidates in the prevention of diabetic complications.

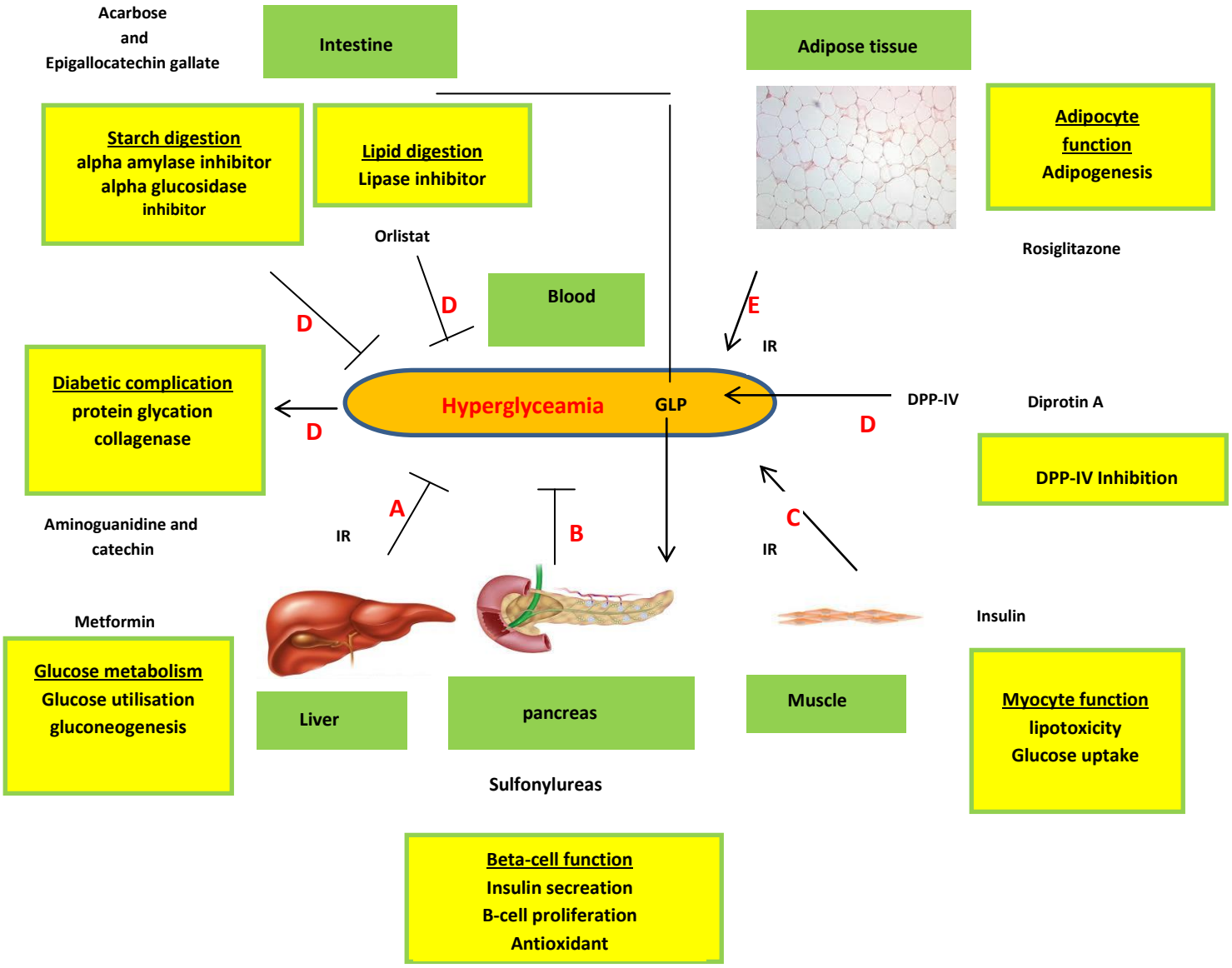

Figure 26: Schematic representation of the summary of the effect of *B. elliptica* and *B. ilicifolia* extracts on various model designs to stimulate specific antidiabetic target. (A: both plants enhance glucose uptake in HepG2 cells; B: *B. elliptica* stimulates proliferation while both plants reduced NO production in RAW macrophages and both plants showed no effect on INS-1 glucose metabolism; C: both plants displayed no significant increase in glucose uptake in L6 cells compared to insulin; D: both plants displayed no significant inhibition of specific enzymes compared to positive controls; E: both plants displayed no effect on lipid accumulation in 3T3-L1 cells). **(**IR = insulin resistance, DPP-IV= dipeptidyl peptidase IV and GLP = glucagon like peptide 1).

Interactions with drug metabolism pathways

Cytochrome P450s play a crucial role in drug metabolism and therefore interactions of co-administration of herbs with CYP enzymes (CYP1A2, CYP2A6, CYP2E1 and CYP3A4) may alter the activation and efficacy of drugs thereby causing potential adverse effects (Wang *et al*., 2015). The aqueous extracts of both plants showed weak inhibition of CYP3A4 at all the tested concentrations. Both plants may therefore be taken concurrently with pharmaceutical drugs which are activated or metabolized by cytochrome P450 with a low risk of pharmacokinetic interaction. Studies have shown that phytochemicals such as alkaloids and saponins inhibit various CYP450s subtypes (Hanapi *et al*., 2010; Wang *et al*., 2015). Therefore, the weak inhibition on CYP3A4 observed in this study, could be attributed to the low content of alkaloids and saponins reported in both plants aqueous extracts (Sagbo, 2015). Both plants extracts may therefore be beneficial to diabetic patients when they are consumed concurrently with the standard antidiabetic drugs which are metabolized by cytochrome P450.

Possible mechanism of action

Based on the findings observed in this present study, two possible mechanisms of action have been proposed (Figure 27):

1. The hypoglyceamic effect of *B. elliptica* and *B.ilicifolia* appears to be mediated via a mechanism that is similar to that of metformin, a biguanides antidiabetic drug.
2. The hypoglycemic effect of both plants may be associated with proliferative and for antioxidative effects via a mechanism similar to Sulphonylureas.

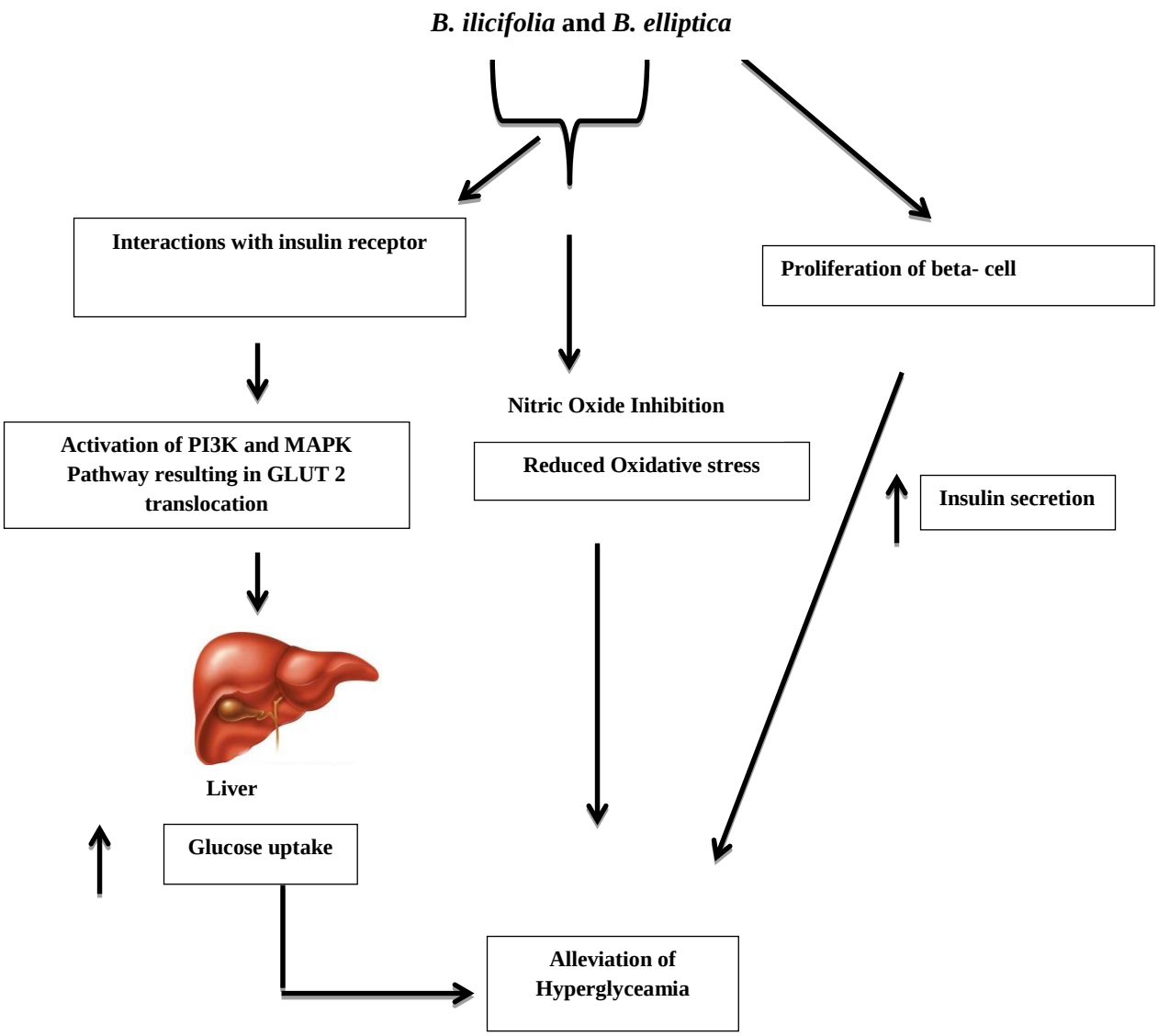

Figure 27: Proposed mechanism of antidiabetic and hypoglycemic effect of *B. elliptica* and *B. ilicifolia.*

5.2 CONCLUSIONS

The findings observed in this study suggest that *B. elliptica* and *B. ilicifolia* extracts exert their hypoglycemic activity independent of insulin and through restoring or maintaining the health and proper functioning of the beta cell and the pancreas. The possible mechanisms of their anti-diabetic action may be linked to the strong proliferative, anti-oxidative and interactions with insulin receptors, leading to the activation of the MAPK and P13K pathways, which results in the translocation of glucose transporters. This observed antidiabetic activity of these extracts might be due to their phytochemical constituents previously reported (Sagbo, 2015). The findings from this study therefore, support the folkloric usage of both plant extracts in the treatment of diabetes. However, due to the potential toxicity of both plant extracts they must be prescribed with caution. The results also support the use of these plant extract with other antidiabetic drugs that are metabolized by cytochrome P450.

5.3 FUTURE STUDIES

- The future work would be to investigate the effect of both plants extracts in a rat or mouse model for their hypoglycemic activity as the activity of some of these plant extracts may differ in *in vivo* studies.
- The exact mechanisms of action of these plants extracts will be evaluated to approve or disprove the speculation about their mechanism of action.
- Isolation and purification of the active compounds responsible for antidiabetic properties of *B. elliptica* and *B. ilicifolia* should be investigated.

- The effects of the different combinations of compound found within both plant extracts as well as observing the impact of these plants together with the other current antidiabetic medications will be investigated.

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APPENDICES

List of articles for publication from this thesis:

- **Idowu Jonas Sagbo and Graeme Bradley:** *In vitro* evaluation of cytotoxicity and proliferative effects of aqueous extracts of *Brachylaena elliptica* and *Brachylaena ilicifolia* using Imagexpress ImageXpress® Micro widerfield High-Content Imaging System (To be submitted to the African Journal of Traditional Complementary and Alternative Medicine (AJTCAM).

- **Idowu Jonas Sagbo and Graeme Bradley**: *In vitro* antidiabetic activity and mechanism of action of extract of *Bachylaena elliptica* (To be submitted to Journal of Ethnopharmacology).

- **Idowu Jonas Sagbo and Graeme Bradley**: Exploring the antidiabetic mechanism of *B. ilicifolia* (Thurb.) DC. Using target directed *in vitro* screening (To be submitted to International Journal of Molecular Sciences).