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ANTIDIABETIC PROPERTIES OF *CENTELLA ASIATICA* IN TYPE II DIABETIC RATS

BY

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Submitted in fulfillment of the academic requirements for the degree of Doctor of Philosophy in Biochemistry in the School of Life Sciences, College of Agriculture, Engineering and Sciences, University of KwaZulu-Natal (Westville campus), Durban, South Africa

Supervisor: **Dr Bubuya Masola**

Co-supervisor: **Prof Samson Mukaratirwa**

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCES

DECLARATION 1 - PLAGIARISM

I, Ayodeji Babatunde Oyenihi declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2 – PUBLICATIONS AND CONFERENCE/WORKSHOP ATTENDED

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

In all the publications included in this thesis, I was involved in the design of the studies, conducted the scientific experiments, performed data analysis and wrote the drafts of the manuscripts. The supervisors and co-authors made materials and analytical facilities available and made rigorous editing of the manuscripts in preparation for submission to journals.

PUBLICATIONS

Already published

Publication 1
Ayodeji B. Oyenihi, Nicole L. Brooks, Oluwafemi O. Oguntibeju, and Yapo Aboua (2014). Antioxidant-Rich Natural Products and Diabetes Mellitus: In Antioxidant-Rich Natural Products and Human Health. *In Tech* ISBN: 978-953-51-1215-0.

Publication 2
Ayodeji B. Oyenihi, Ademola O. Ayeleso, Emmanuel Mukwevho, and Bubuya Masola (2015). Antioxidant Strategies in the Management of Diabetic Neuropathy. *BioMed Research International* Volume 2015, Article ID 515042, 15 pages.

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Publication 3
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Publication 4
Ayodeji B. Oyenihi, Oluwafemi O. Oguntibeju, Bubuya Masola. Nephro- and neuro-protective actions of *Centella asiatica (L.) Urb.* in Type 2 diabetic rats: Possible roles of antioxidants and inflammatory cytokines *(In preparation).*

Publication 5
Ayodeji B. Oyenihi, Samson Mukaratirwa, Bubuya Masola. Effects of *Centella asiatica (L.) Urb.* on insulin signaling and glycogen metabolism (glycogen content, glycogen synthase and phosphorylase enzymes) in muscle of Type 2 diabetic rats *(In preparation).*

CONFERENCE/WORKSHOP ATTENDED

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Integrative and Organs Systems Pharmacology (IOSP) Workshop, A satellite meeting of World Congress of basic and clinical Pharmacology (WCP). 8th – 11th July, 2014, University of KwaZulu-Natal, Durban, South Africa.

Signature/Date:

PREFACE

I, **Ayodeji Babatunde Oyenihi** hereby declare that the thesis entitled **"Antidiabetic properties of *Centella asiatica* in Type II diabetic rats"** represents my original research conducted under the supervision of the under-listed supervisors and have not been submitted in any form for any degree to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

Candidate: Ayodeji Babatunde Oyenihi Signature/Date:

Supervisor: Dr Bubuya Masola Signature/Date:

Co-supervisor: Prof Samson Mukaratirwa Signature/Date:

ABSTRACT

Type II *diabetes mellitus* (TIIDM) continues to pose serious health challenges to medical practitioners and the general human population as the number of newly diagnosed patients increase relentlessly. Controlling glycaemia; neutralizing the over-activation of oxidative stress and inflammation; in addition to regulating the insulin signaling pathway and downstream effects are important mechanisms employed by many antidiabetic agents in the management of the disease. *Centella asiatica (L.) Urban* (CA) is an aromatic perennial herb that has been used in traditional folklore in Africa and Asia to treat various ailments including diabetes since time immemorial. Although CA has been reported to possess many pharmacological properties, there is paucity of pertinent information on its role on antioxidant capacity, inflammation, insulin signaling and downstream effects related specifically to TIIDM. Therefore, this research explored the *in vivo* antidiabetic potentials of methanol extract of CA leaves in a type II diabetic rat model that exhibits both insulin resistance and insufficiency.

Adult male Sprague-Dawley rats were randomly divided into 5 groups: normal control rats (NC); diabetic control rats (DC); CA-treated (500 mg/kg body weight) diabetic rats (D500); CA-treated (1000 mg/kg body weight) diabetic rats (D1000) and metformin-treated (300 mg/kg body weight) diabetic rats (DME). TIIDM was induced in rats by administering 10% fructose in drinking water *ad libitum* for 14 days followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight). Treatments were by oral gavage for the duration of 30, 60 and 90 minutes for acute study; and daily for 14 days for the sub-chronic study. Biochemical techniques such as spectrophotometry, Luminex xMap, Western blot and radiochemistry were used to determine level or activity of mediators of oxidative stress, inflammation, insulin signaling and glycogen metabolism in blood, liver, kidney, brain and muscle in rats. Histopathological examination of pancreatic sections of rats from experimental groups was performed. Gas chromatography-mass spectroscopy (GC-MS) analysis was used to characterize the crude methanol extract of CA.

The induction of TIIDM in rats caused significant elevation in malondialdehyde (MDA) levels; reduced the antioxidant capacity and altered the levels of pro-inflammatory and anti-inflammatory cytokines in liver, kidney and brain in comparison to normal rats. In addition, diabetes induced alterations in the expression of proteins, phospho-Akt, glycogen synthase (GS) and glycogen phosphorylase (GP) in muscle in rats. The activities of GS and GP were altered

leading to reduction in glycogen concentration in liver and muscle of diabetic rats when compared to normal rats. However, treatments of diabetic rats with CA for 14 days lowered high blood glucose level; reduced MDA production; decreased the levels of pro-inflammatory cytokines and improved antioxidant capacity in tissues of diabetic rats compared to diabetic control rats. In addition, insulin-independent effects of crude extract of CA on expression of phospho-Akt, GS, GP proteins and activities of GS and GP may result in increased glycogen levels in muscle of diabetic rats. The increase in glycogen level in liver in CA-treated rats can also be associated with changes in the activities of GS and GP. Furthermore, the daily treatment of diabetic rats with CA for 14 days ameliorated diabetes-induced abnormalities in pancreatic β islets that can lead to increase in insulin secretion/actions. The GC-MS chromatogram of the crude methanol extract of leaves of CA revealed the presence of peaks representing over 50 compounds with matching similarity index (SI) greater than 70% in the National Institute of Standards and Technology (NIST) library. Some of the medicinal compounds identified by GCMS analysis in the crude extract of CA include ascorbic acid, asiatic acid, oleanolic acid, stevioside, stigmasterol and α-humulene.

In summary, the present data revealed promising potential of *Centella asiatica (L.) Urban* leaves in the management of type II *diabetes mellitus.* This could be due to the anti-hyperglycaemic, anti-hyperlipidaemic, antioxidant and anti-inflammatory properties of the medicinal phyto-compounds present therein.

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LIST OFABBREVIATIONS

AAPH	2, 2'-Azobis (2-methylpropionamidine) dihydrochloride
ABTS	2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AGEs	Advanced glycated end-products
Akt/PKB	Protein kinase B
BRU	Biomedical Resource Unit
BSA	Bovine serum albumin
bwt	Body weight
CA	*Centella asiatica (L.) Urb.*
CAM	Complementary/alternative medicines
CDNB	1-chloro-2, 4-dinitrobenzene
$CuSO_4.H_2O$	Copper sulphate
D1000	Diabetic rats treated with *Centella asiatica (L.) Urb.* at 1000 mg/kg body weight dose
D500	Diabetic rats treated with *Centella asiatica (L.) Urb.* at 500 mg/kg body weight dose
DC	Diabetic control rats
DME	Diabetic rats treated with metformin at 300 mg/kg body weight dose
DTNB	5′, 5′-Dithiobis- (2-nitrobenzoate)
DTT	Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBG	Fasting blood glucose
FFA	Free fatty acids
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectroscopy

GDM	Gestational *diabetes mellitus*
GP	Glycogen phosphorylase
GPx	Glutathione peroxidase
GS	Glycogen synthase
GSH	Reduced glutathione
GSK-3	Glycogen synthase kinase 3
GST	Glutathione S-transferases
HDL-c	High-density lipoprotein cholesterol
i.p	Intraperitoneal
IDF	International Diabetes Federation
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LDL-c	Low-density lipoprotein cholesterol
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MDA	Malondialdehyde
MES	2-(N-morpholino) ethanesulfonic acid
MOPS	3-(N-morpholino) propanesulfonic acid
NC	Normal control rats
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST	National Institute of Standards and Technology
OGTT	Oral glucose tolerance test
p-Akt	Phospho-Akt

p-GS	Phospho-GS
PI-3K	Phosphoinositide-3 kinase
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluoride
PTP	Protein tyrosine phosphatase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
STZ	Streptozotocin
TEMED	N, N, N, N –Tetramethylethylenediamine
TG	Triglycerides
TIDM	Type I *diabetes mellitus*
TIIDM	Type II *diabetes mellitus*
TM	Traditional medicines
TNF	Tumor necrosis factor
TPTZ	2, 4, 6-tri [2-pyridyl]-s-triazine
Trolox	6-hydroxy-2, 5, 7, 8-tetra-methylchroman-2 carboxylic acid
UDPG	Uridine diphosphate glucose
VLDL	Very low-density lipoprotein
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Although diabetic conditions have been described in ancient Egyptian and Indian literature since 1500 BC, extensive knowledge and understanding of the complex nature of the disease only began in the 21st century (Nwaneri, 2015). In the Ebers Papyrus, a medical condition described as excessive thirst and urination was reported. This was confirmed by Indian physicians around the same period noting that the excess urine excreted from the patients was in fact very sweet. It was in 230BC that the word '*diabetes*' was first coined by the Apollonius of Memphis to describe the medical condition which literarily means "to pass through" in the Greek language. At first, diabetes was thought to be only a disease of the kidneys with no clear-cut distinctions between symptoms of diabetes and those caused by other diseases (Grmek, 1995). Later on, Matthew Dobson (1713–1784) accurately reported that diabetes is much more a disease of the whole body and not just the kidneys, further describing the concept of diabetes-associated high blood sugar in patients (Zajac et al., 2010). Thomas Cowley in 1788 observed a relationship between diabetes and pancreatic diseases before the islet cells of the pancreas were discovered by Paul Langerhans (1849-1888) in 1869.

John Rollo (1749-1809) in 1798 added the Latin word '*mellitus*' translated 'honey' to emphasize the sweetness of the urine from diabetic patients. Johann Frank (1745-1821) was credited with distinguishing *diabetes mellitus* from *diabetes insipidus* (non-sweet urine) (Nwaneri, 2015). A major breakthrough however, came when insulin (from crude pancreatic extracts) was used to successfully lower high blood glucose in diabetic dogs and a young boy between 1921 and 1923 by Frederick G. Banting, John J.R. MacLeod, Charles H. Best and James Collip (Zajac et al., 2010). Subsequently, experiments to isolate and purify insulin in mass quantities increased with tremendous success achieved in its use in the management of *diabetes mellitus*. Interestingly, human insulin was the first protein to have its complete amino acid sequence determined (Sanger, 1959). However, it was not until the 1930s that crucial biochemical differences between the largely accepted lack of insulin secretion in type I *diabetes mellitus* and asymptomatic insulin resistance in type II *diabetes mellitus* was described (Tattersall, 2010). Presently, type II *diabetes mellitus* occurs in approximately 90% of all diabetic patients worldwide resulting in life-reducing or life-threatening microvascular and macrovascular complications (International Diabetes Federation, 2015).

Diabetes mellitus (DM) is now understood to be a complex, heterogenous, chronic metabolic disorder with multiple causes and its frequency is increasing yearly in most countries. The International Diabetes Federation (IDF) reported that DM caused 5 million deaths in 2015 with the number of diabetic adults set to increase from the current 415 million to 642 million in 2040 which may results in serious public health and economic challenges (International Diabetes Federation, 2015). Yet, an estimated 193 million diabetic people remain undiagnosed with almost 80% of this population from low to middle income countries. According to the Society of Endocrinology, Metabolism and Diabetes in South Africa, the prevalence of diabetes is estimated to be 14%, 13%, 6%, and 6% in the Coloured, Indian, African and European communities respectively (Levitt, 2009). Also, the occurrence of type II DM is estimated to be about 17%, 6%, and 6% in the Indian, African, and White/Coloured populations (Bradshaw et al., 2007, Amod, 2012). A major feature of DM is chronic or intermittent high level of glucose in the blood (hyperglycaemia) which is accompanied by impaired carbohydrate, protein and lipid metabolism due to absolute or relative deficiency in insulin secretion or insulin action (Bastaki, 2005).

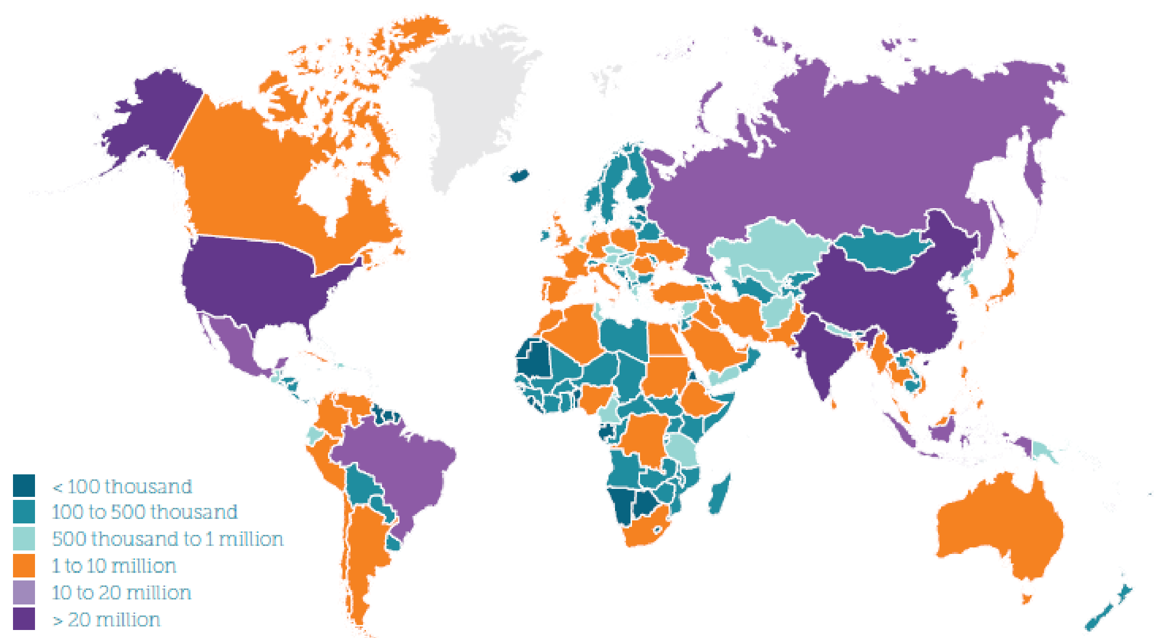


Figure 1.1: Approximate distribution of people (age 20-79) living with diabetes as at 2015 (International Diabetes Federation, 2015)

Table 1.1: Approximate prevalence and mortality rates in sub-Saharan African countries with the most number of diabetic patients (Age 20-79) in 2015

Countries	National prevalence of diabetes	Population diagnosed with diabetes	Estimated population of undiagnosed diabetes	Number of diabetes-related deaths
South Africa	7.0	2,286,000	1,396,800	57,319
DR Congo	5.3	1,762,900	1,257,900	32,417
Nigeria	1.9	1,564,700	949,900	40,815
Ethiopia	2.9	1,333,200	951,300	23,145
Tanzania	3.5	822,800	591,500	17,698
Cameroon	5.3	567,300	344,400	14,998
Kenya	2.2	478,000	287,700	8,722
Uganda	2.5	400,600	285,800	11,341
South Sudan	6.6	376,600	268,700	6,405
Madagascar	3.3	372,000	265,400	5,580

Adapted from International Diabetes Federation, (2015)

On the basis of etiology and clinical presentation, DM is classified mainly into three; type I *diabetes mellitus* also called insulin-dependent *diabetes mellitus* (IDDM), type II *diabetes mellitus* which is also known as non-insulin dependent *diabetes mellitus* (NIDDM) and gestational *diabetes mellitus*. Type I DM is generally characterized by the abrupt onset of severe symptoms, dependence on exogenous insulin to sustain life and proneness to ketosis even in the basal state. These have been shown to be caused by absolute insulin deficiency due to the inability of the pancreatic β-cells to produce insulin mostly because of auto-immune reactions (Atkinson et al., 2014, Chiang et al., 2014). In type II DM, there is inadequate insulin secretion in the pancreas or the body does not utilize the insulin produced efficiently. This may be due to insensitivity of insulin receptors or other intermediates in the insulin signaling pathways within cells of the body (Kohei, 2010). Hyperglycaemia and disturbances in energy metabolism as a result of impaired glucose uptake by cells are the major consequences of type II DM (Wilson and Islam, 2012). Important risk factors of developing type II DM are heredity,

unhealthy diet/eating habits and lack of regular exercise. People who are classified as obese or over-weight are equally at a high risk of developing type II DM. A situation where an individual has an elevated blood glucose levels but not high enough to be classified as diabetes is also known as pre-diabetes or impaired glucose tolerance (IGT). People diagnosed with IGT are usually susceptible to developing type II DM in future and exhibit very similar symptoms with the disease (Nathan et al., 2007). Gestational *diabetes mellitus* occurs when pregnant women are diagnosed with high blood glucose and it is currently affecting almost 1 in 7 live births (International Diabetes Federation, 2015).

DM results in various complications that increases the morbidity and mortality rates in patients. Microvascular complications may result from long term damage, dysfunction and failure of organs like liver (hepatopathy), kidneys (nephropathy), nerves (neuropathy), eyes (retinopathy), and testes (reproductive toxicity). Macrovascular complications are caused by chronic damage to the blood vessels becoming a major risk factor for coronary and cardiovascular diseases (Fowler, 2008, Forbes and Cooper, 2013).

Oxidative stress is a recurring central theme in hyperglycaemia, and pathways involved in the development of diabetic complications. The increase in oxidative stress in diabetes could be because of elevated blood glucose level, which upon auto-oxidation in the presence of transition metals, generates free radicals and damages the cell membrane through peroxidation of membrane lipids and protein glycation (Baynes, 2003). Chronic hyperglycaemia also results in decreased activities of antioxidant enzymes; increased oxidative phosphorylation, glycosylation of proteins; and activation of the hexosamine pathway (Kaneto et al., 2001). Alterations in the activities of key enzymes in the insulin signaling pathway have been shown to be a major hallmark in type II DM. An increase in oxidative stress may inactivate insulin receptor substrates (IRS) which in turn deactivates protein kinase B (PKB) through the downregulation of phosphoinositide-3 kinase (PI-3K). This leads to an increase in blood glucose as glycogen synthase enzyme that converts glucose to glycogen is markedly inhibited (Rains and Jain, 2011). Other mechanisms by which hyperglycaemia increases oxidative insult is through the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK)- stress-associated kinases (Mohamed et al., 1999, Ho et al., 2000).

The oxidative stress-inflammation pathway remains central in the pathogenesis of diabetes. It has been postulated that excess ROS activate inflammatory mediators or vice versa in a continuous molecular cascade within diabetic tissues promoting the development of complications. Diabetes-induced dysregulation of inflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-10, interferon (IFN)-γ, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)-α have also been documented (Esposito et al., 2002, Spitaler and Graier, 2002, Griendling and FitzGerald, 2003, Foss et al., 2007).

Glycaemic management with insulin has long been the mainstay for preventing the progression of diabetes and its complications (Inzucchi et al., 2015). Also, oral hypoglycaemic agents have been used in addition to insulin. These include insulin sensitizing drugs like biguanides (e.g. metformin), sulphonylureas (e.g. glibenclamide), glucagon-like peptide-1 (GLP-1) receptor agonists (e.g. bydureon), thiazolidinediones (e.g avandia), and sodium–glucose cotransporter 2 (SGLT2) inhibitors (e.g canagliflozin) etc. However, several clinical side effects have been found with these antidiabetic drugs (Moller, 2001, Corathers et al., 2013). Furthermore, difficulty in achieving and/or maintaining tight glycaemic control, incidence of hypoglycaemia and increased mortality are limitations in intensive glycaemic treatment warranting the development of more efficient therapeutics that have additional qualities but less/no side effects (Rotenstein et al., 2012). The total economic costs of managing diabetes keeps increasing making patients' compliance levels especially in low-income countries very low and is partly responsible for the continuous rise in the incidences of diabetes globally (Mendis et al., 2007).

Prevention of diabetes and its complications is a challenging health problem and The World Health Organization (WHO) which predicts that the disease will become the 7th leading cause of deaths by 2030 (Mathers and Loncar, 2006), has also emphasized the safe, effective, rational use of traditional medicines as part of the healthcare systems to limit the rise of chronic non-communicable chronic diseases (World Health Organization (WHO), 2013). Traditionally, plant products and decoctions from plant parts have been shown to possess significant antidiabetic activity (Okyar et al., 2001). Studies have shown that the effectiveness of these plants/herbs is as a result of the presence of nutraceuticals. Some of these active compounds include flavonoids, terpenes, nitrogen-containing compounds and proanthocyanidins etc (Mariod et al., 2008, Ezuruike and Prieto, 2014).

Furthermore, an estimated 27 million consumers of traditional medicine have been reported in South Africa alone (Mulholland and Drewes, 2004). The value of trade of ethno-botanical plants was worth R60 million in 1998 in KwaZulu-Natal province alone with an estimated 20,000 – 30,000 people drawing an income from trading indigenous plants (Mulholland and Drewes, 2004). This confirms the importance of indigenous medicinal plants in the South African economy. Therefore, it has become very necessary to search, screen, select and test South African indigenous medicinal plants with profound antidiabetic properties.

A study of literature reveals that *Centella asiatica* leaves are rich sources of medicinal compounds such as flavonoids triterpene acids, alkaloids, glycosides, oils etc (Maulidiani et al., 2014). *Centella asiatica* is a traditional medicinal plant extensively used in South African folklore in the management of DM (Orhan, 2012). Although the anti-hyperglycaemic and antioxidant properties of *Centella asiatica* have been reported (Pittella et al., 2009, Kabir et al., 2014, Nur-Hidayah et al., 2015), there is paucity of pertinent information in scientific literature on the effect of the plant on inflammation, oxidative stress, and insulin signaling mechanisms related specifically to type II DM. Data from this study will also provide information on possible mechanism(s) of action of *Centella asiatica* in type II DM with a view to discovering and isolating new compounds that could serve as an alternative and/ or complementary therapy against this disease. It could also become the basis for further investigations for use in clinical setting and probably qualify for clinical trials in humans in the future.

1.2 CLASSIFICATION OF *DIABETES MELLITUS*

Diabetes mellitus can be broadly divided into 3 main categories based on etiology and clinical presentation. These include; type I *diabetes mellitus,* type II *diabetes mellitus* and gestational *diabetes mellitus.*

1.2.1 Type I *diabetes mellitus*

Type I *diabetes mellitus* (TIDM) results primarily from an irreversible destruction of pancreatic β-cells leading to the inability of the cell to secrete insulin. This is due to auto-immune processes triggered mostly in genetically susceptible individuals by some viruses such as German measles or mumps, enteroviruses and other environmental factors (Knip, 2003, van Belle et al., 2011). The specific self-destruction of pancreatic β-cell by the immune system

involves several mediators such as β-cell autoantigens, macrophages, dendritic cells, B-lymphocytes, and T-lymphocytes (Yoon and Jun, 2005). The exact basis and mechanisms of pancreatic β-cell destruction in type I DM are not yet fully understood, but oxidative stress, apoptosis and inflammation processes have been implicated in recent times (Pirot et al., 2008). The consequence of lack of insulin secretion in the pancreas is a persistent hyperglycaemia that can only be controlled by the administration of exogenous insulin hence the total dependence on insulin by type I diabetic patients to prevent them from dying. Although the disease can affect all age groups, it has been shown to be more endemic in children and adolescents (Atkinson et al., 2014, Chiang et al., 2014).

To accurately screen and detect individuals at risk of developing type I DM, careful attention should be given to persons with a family history of the disease so as to get a report of their immunity and genetic biomarkers. The presence of pancreatic islet autoantibodies, glutamic acid decarboxylase antibodies (GADAs) and islet antigen-2 antibodies (IA-2As) in the blood of children were reported to indicate pancreatic β-cell destruction and predict the risk of developing type I DM in future (Bingley, 1996, Knip et al., 2010, Goldenberg and Punthakee, 2013).

1.2.2 Type II *diabetes mellitus*

The unrelenting prevalence of diabetes is actually due to type II DM as type I only affects approximately half a million children of the total population of diabetic patients in the world (Oputa and Chinenye, 2012). It has been proposed that type II DM mainly occur in adults although children and adolescents are now increasingly diagnosed with the disease (Copeland et al., 2013, Demmer et al., 2013). Type II DM is caused by a reduced sensitivity to the insulin produced by the pancreatic β-cells which over time can also significantly reduce its secretion leading to hyperglycaemia. Insulin resistance is caused by the combined efforts of genetic and environmental factors. Several gene polymorphisms of insulin receptor, insulin receptor substrate (IRS)-1, β adrenergic receptor and uncoupling protein (UCP) have been reported to influence the development of insulin resistance. Also, xenobiotic-catalyzed inhibition of one or more proteins of the insulin signaling pathways can trigger the accumulation of intermediates in the signaling cascade ultimately resulting in loss of sensitivity. Furthermore, a gradual decline in pancreatic β-cell population has been reported to be responsible at least in part for the impaired insulin secretion in type II DM. Destructions of pancreatic β-cells have

been mediated by excess glucose and lipids which promote pro-inflammation and apoptosis in the pancreas (Donath et al., 2005, Robertson and Harmon, 2006, Kohei, 2010).

Upon the onset of chronic or intermittent hyperglycaemia, clinical manifestations of the disease such as polydipsia, polyuria, polyphagia, weight loss, impaired vision can be seen among patients (International Diabetes Federation, 2015). Type II DM can remain undiagnosed in patients because the symptoms are not easily noticeable and can be easily confused with symptoms of other diseases, allowing the progression to life-threatening complications. As a result, newly-diagnosed type II diabetic patients are often diagnosed with one or more other complications already which contribute to the increasing mortality rates globally (Clark et al., 2007). Despite recent progress in the understanding and management of type II DM, the exact causes are largely unknown. However, key risk factors include lack of exercise, unhealthy eating habits, excess body mass index, and tobacco/alcohol abuse, race/ethnicity, family history of diabetes, previous record of gestational DM and old age (Ekoe et al., 2013). People with an impaired glucose tolerance condition are also highly susceptible to developing type II DM (Nathan et al., 2007).

Unfortunately, type II DM cases have not been properly controlled as only less than 50% of patients meets normal glycaemic, lipid and blood pressure endpoints. This is worrisome because new cases continue to increase while morbidity and mortality rates among patients continue to rise. In South Africa, approximately 70% of type II diabetic patients have a glycated haemoglobin (HbA1c) level above the widely recommended target of 7% (Amod, 2012). Most therapeutic strategies in the management of type II DM involve stimulating insulin sensitivity so as to decrease resistance to insulin. Controlling hyperglycaemia in type II DM is equally very important. Insulin or insulin analogues and various anti-hyperglycaemic agents have been used to maintain normal blood glucose levels in patients. Major breakthroughs have been achieved by the adaptation of patients to important lifestyle changes. Consumption of healthy, low-sugar, low-fat foods or nutrients, cessation of smoking and a regular exercise routine have been proven to be effective in the management of type II DM (Nelson et al., 2002, Steyn et al., 2004). In addition, obese individuals at high risk of developing type II DM are advised to reduce fat and sugar intake to attain and maintain a normal body weight (Salas-Salvado et al., 2011). A combination of all these strategies seems logical and may pave way to finding a permanent solution to the disease.

1.2.3 Gestational *diabetes mellitus*

Gestational diabetes mellitus (GDM) refers to a loss of glucose homeostasis resulting in hyperglycaemia or glucose intolerance that is first diagnosed during pregnancy (IDF, 2015). GDM occurs in approximately 7% of all pregnancies, resulting in more than 200,000 cases each year (American Diabetes Association, 2015, International Diabetes Federation, 2015). Therefore, it is advisable to screen pregnant women with diabetes risk factors at their first pre-natal visit. GDM is mostly diagnosed in 24-28 weeks of pregnancy with fasting plasma glucose $\geq$ 5.8 mmol/L by a two-step method. The first step involves measuring non-fasting plasma glucose level 1 hour following a 50 g oral glucose load. If the plasma glucose level exceeds 7.8 mmol/L, then an oral glucose tolerance test (OGTT) is performed after a 100 g glucose load. A plasma glucose levels between 10.0-10.6 mmol/L at 1 hour and 8.6-9.2 mmol/L at 2 hours after glucose load usually confirms GDM (American Diabetes Association, 2015).

Although the high blood glucose levels seen in women with GDM often revert back to normal levels after delivery, this condition increases the risk of developing type II DM or diabetic complications later in life (Bellamy et al., 2009). The occurrence of pancreatic β-cell-specific autoantibodies in women with GDM have also been shown to predispose to developing type I DM in future (Nilsson et al., 2007). GDM has been associated with maternal complications such as hypertension, preeclampsia, postpartum bleeding and increased risk of caesarean deliveries (Aberg et al., 2001, Schmidt et al., 2001). Uncontrolled or untreated GDM also has negative consequences on the baby; it could result in foetal macrosomia, neonatal hypoglycemia, perinatal deaths, congenital deformity, hyperbilirubinemia, and respiratory distress syndrome (Kjos and Buchanan, 1999, Wood et al., 2000, Setji et al., 2005, Meek et al., 2015). The major causes of GDM are not completely understood. However, the secretion of hormones during pregnancy plays major roles. The placental secretion of progesterone, cortisol, lactogen, prolactin, and growth hormones have been shown to contribute to insulin resistance in pregnancy, which may be important for sufficient foetal glucose supply by favouring maternal energy metabolism through lipids rather than carbohydrates (Di Cianni et al., 2003, Setji et al., 2005). The increase in pancreatic β-cell secretion of insulin to counteract the insulin resistance may also be partly responsible for the hyperinsulinaemia in pregnancy (Setji et al., 2005).

GDM has been generally managed in pregnant women by accurate monitoring of blood glucose levels, healthy nutrition and appropriate exercise. At times, insulin and other oral anti-

hyperglycaemic agents such as metformin are also employed to control high blood glucose levels (Lautatzis et al., 2013, International Diabetes Federation, 2015).

1.2.4 Symptoms of *diabetes mellitus*

DM is usually characterized by an elevated rise in glucose in the blood. It is believed that all other clinical manifestations of the disease may arise as a consequence the effects of excess glucose. Type I DM mostly occur suddenly with easily noticeable symptoms. Type II DM however, may continue for a long time without any signs probably until after complications have arisen. It is also difficult to categorically distinguish between the symptoms of both types of DM as they usually overlap. The following symptoms of DM are generally accepted worldwide:

- Frequent urination and dehydration
- Abnormal excessive thirst
- Overeating and constant hunger/appetite
- Unexplainable or sudden loss of weight
- Blurred vision
- Increased fatigue and tiredness
- Slow healing of wounds and persistent infections
- Irritability
- Dry mouth and fruity breath
- *Acanthoses nigricans* and other skin infections (Clark et al., 2007, International Diabetes Federation, 2015).

1.2.5 Diagnostic criteria

An early diagnosis of DM in patients is extremely important as therapeutic interventions employed early can significantly halt the progression of the disease and possibly prevent the advancement to deleterious complications (Stolar, 2010). Although classifying DM into type I and type II is needed so as to specifically guide therapy accordingly, it may be problematic at the point of diagnosis at times. Therefore, general diagnostic criteria for diabetes have been agreed among the majority of stakeholders.

Diabetes is diagnosed if one or more of the following is present in a patient:

- Fasting plasma glucose ≥ 7.0 mmol/L (fasting means no caloric intake for at least 8 hours before test is administered)
- Glycated haemoglobin (HbA1c) ≥ 6.5% (in adults) using a standardized, validated assay in the absence of factors that affect the accuracy of the HbA1c. Diagnosis based on only HbA1c could sometimes be misleading as several diseases such as haemolytic anaemias, kidney ailments and iron deficiency mirrors the HbA1c pattern seen in DM. Therefore, the HbA1c test should be performed on an individual basis after adequate screening of the patient.
- 2 hour plasma glucose ≥ 11.1 mmol/L following a 75 g oral glucose load
- Random plasma glucose ≥ 11.1 mmol/L (random means any time of the day, without regard to the interval since the last meal). Usually, a positive random plasma glucose test should be confirmed by an alternate test before diagnosing diabetes (Goldenberg and Punthakee, 2013, American Diabetes Association, 2015, International Diabetes Federation, 2015).

The choice of test used in diagnosis varies from clinician to clinician, region to region, patient to patient, costs and resources availability. Each test has its own benefits and limitations. To quickly discover non-symptomatic DM, it is advised that screening should be performed from time to time in individuals who have a family history of diabetes or come from an endemic indigenous population e.g. African, African-American, Asian, Hispanic, or Native American groups. The flow chart summarizing the steps involved in the diagnosis of type II DM is illustrated in Figure 1.2.

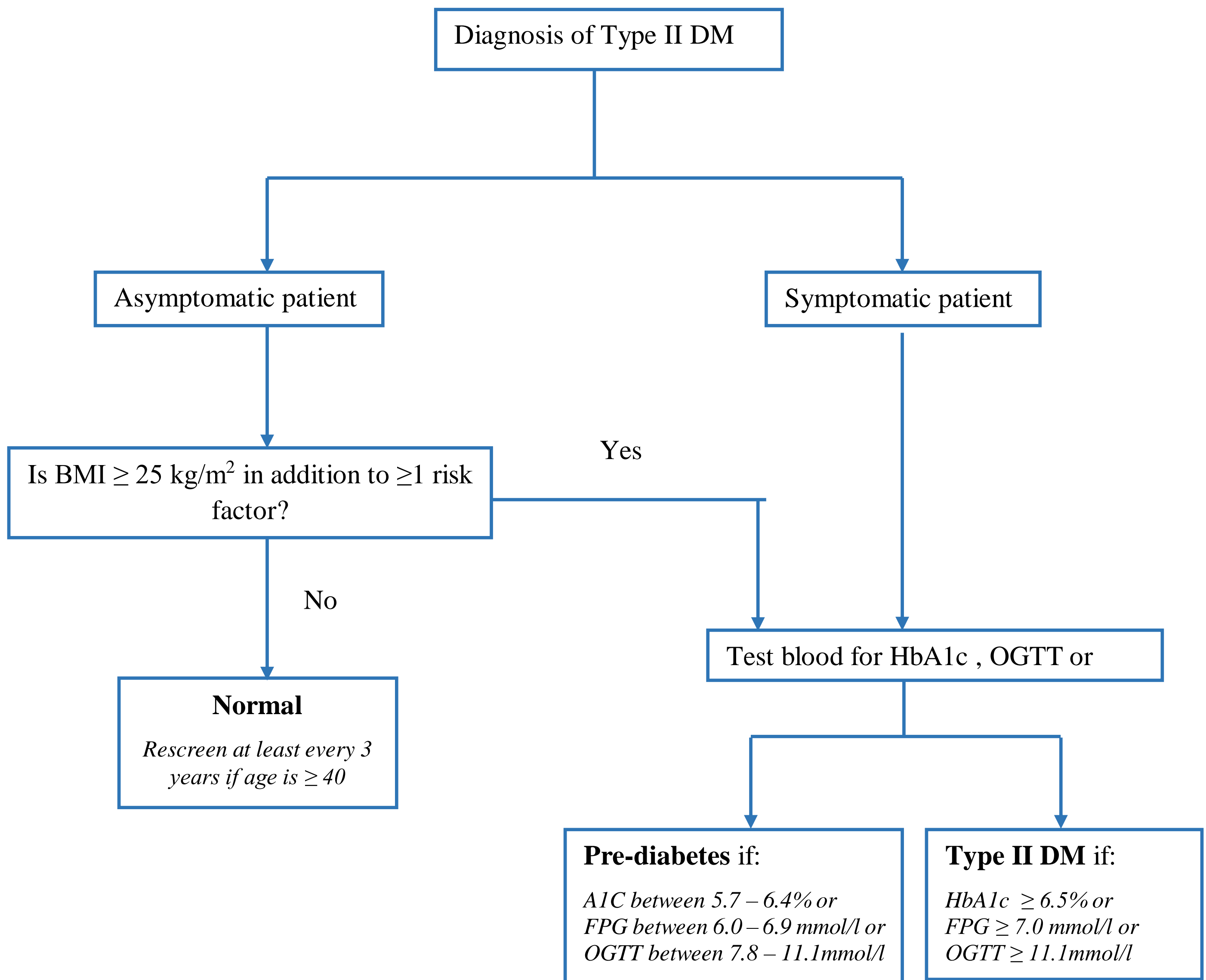


Figure 1.2: Flow chart for the diagnosis of type II DM. BMI – body mass index; HbA1c – glycated haemoglobin, OGTT – oral glucose tolerance tests, FPG – fasting plasma glucose. Adapted from Redmon et al. (2014).

1.2.6 Risk factors

It has been estimated that the number of undiagnosed DM may reach figures above 50% of the population already diagnosed. Therefore, it has become very important to continually screen people so as to reduce this anomaly. The process of screening varies from country to country depending on the availability of resources and personnel. The majority of the DM patients in low to middle-income countries are not detected for a very long time due to economic

conditions; by the time of their discovery, they usually have come down with one or more disabling complications. DM risk scores can be developed by taking into account several risk factors within a given population and can serve as an easy, cost-effective approach in ascertaining individuals with undiagnosed type II DM or at higher risk of developing it in the future.

The following is a summary of generally accepted risk factors in developing type II DM:

- Body mass index (BMI) ≥ 25 kg/m^2
- Age ≥ 40 years
- Immediate family history of type II diabetes
- Member of high-risk race/ethnicity
- History of prediabetes
- History of GDM
- History of delivery of a macrosomic infant
- Presence of diabetic complications such as retinopathy, neuropathy, nephropathy, coronary, cerebrovascular, peripheral diseases
- Presence of vascular risk factors such as hypercholesterolaemia, hyperlipidaemia/dyslipidaemia, hypertension, obesity/overweight, cardiovascular diseases
- Presence of associated diseases such as: polycystic ovary syndrome, *Acanthosis nigricans*, psychiatric disorders (bipolar disorder, depression, schizophrenia), HIV infections
- Use of drugs associated with diabetes such as Glucocorticoid, Atypical antipsychotics, highly active antiretroviral therapy (HAART) (Dixon et al., 2000, Samaras and Chisholm, 2008, Ekoe et al., 2013, Redmon et al., 2014, American Diabetes Association, 2015).

1.2.7 *In vivo* animal models of *diabetes mellitus*

The devastating contributions of DM to the morbidity and mortality rates in populations of the world has necessitated the need to fully understand the complex nature of the disease therefore rendering the use of relevant animal models imperative. The search for a perfect animal model of DM that accurately resembles human DM has been ongoing since the early outstanding

experiments carried out on dogs that led to the discovery of insulin (Singh and Pathak, 2015). Nowadays, legal and ethical considerations in the use of animals for biomedical experiments have also prompted scientists to think deeper and creatively in developing a better, non-invasive and effective alternative. Despite the increasing agitations by animal rights groups, appropriate animal models are still needed to investigate the fundamental pathophysiology of diabetes and also to test pharmacological agents being developed to treat/manage the disease (Regan, 2001, Jones, 2004, Rees and Alcolado, 2005).

Several experimental animal models of type I and type II DM have been reported and can be generally classified based on their mode of diabetes induction. The choice of an animal model depends on the researcher/scientist, economic costs, type of diabetes, research duration and research endpoints with rodents being by far the most popular animals used in *in vivo* models of diabetes mainly because of their ease of handling and breeding (Chatzigeorgiou et al., 2009). Each animal model has its own benefits and limitations. Commonly used animal models include chemically induced, spontaneously induced or selective inbreeding, food- or diet-induced, genetically manipulated animal models. Others use a combination of any 2 of the above mentioned models.

1.2.7.1 Chemically-induced animal model

Most of the chemicals used to induce DM in rodents rely on their ability to selectively inhibit insulin secretion in the pancreas. Among the chemicals used, Alloxan and Streptozotocin are widely accepted. Others are Vacor, Dithizone, 8-hydroxyquinolone and Phlorizin (Rees and Alcolado, 2005).

Alloxan

Alloxan (2,4,5,6-tetraoxypyrimidine;2,4,5,6- pyrimidinetetrone) is an oxygenated pyrimidine derivative (Figure 1.3) that selectively accumulates into the pancreatic β-cells. The water-soluble nature and shape of alloxan allows easy uptake by glucose transporter (GLUT)-2 into the β-cell (Rohilla and Ali, 2012). Alloxan and its metabolite dialuric acid exert their actions by inactivating important metabolic enzymes such as glucokinase, phosphofructokinase, calmodulin-dependent protein kinase, aconitase and others via the oxidation of thiols groups present in these enzymes thereby generating reactive oxygen species (ROS) like superoxide anions and hydrogen peroxide in the process (Lenzen, 2008). The resulting oxidative stress has been shown to induce the process of necrosis in pancreatic β-cells. The ROS produced by the

metabolism of alloxan have also been shown to damage pancreatic β-cell DNA in addition to allowing excess influx of calcium ions (Szkudelski, 2001, Rohilla and Ali, 2012).

Alloxan has been used to induce mild to severe diabetes depending on the doses. The dose required also depends on the specie of animal, age, route of administration. Usually, a single dose of between 140 to 180 mg/kg dissolved in distilled water injected intravenously, intraperitoneally or subcutaneously in mice and rats yields an established diabetes model after 7 - 12 days (Frode and Medeiros, 2008). The overall effects of alloxan are characterized mainly by hyperglycaemia and insulinopaenia which has been shown to closely mimic the type I DM in humans.

Streptozotocin

Streptozotocin (STZ), a naturally occurring compound, produced by the bacterium *Streptomyces achromogenes* has been used extensively in animal species to induce DM. (Hayden and Tyagi, 2002, Yang and Wright, 2002). Streptozotocin has been shown to cause necrosis of the pancreatic β-cells by mechanisms involving DNA alkylation (Murata et al., 1999, Mythili et al., 2004) and free radical generation leading to DNA and chromosomal damage (Bolzan and Bianchi, 2002). The glucose moiety in the chemical structure of streptozotocin (Figure 1.3) allows preferential uptake of STZ into β-cells, via the glucose transporter (GLUT)-2 leading to its accumulation and toxicity in the cells.

Symptoms resembling human type I or type II DM can be induced after 3 days of parenteral administration of STZ depending on the dose and age of animals used hence justifying its selection as a diabetogenic agent in experimental studies. A single parenteral administration of 60-80 mg/kg dose or multiple low-doses (mostly 40 mg/kg) (Rees and Alcolado, 2005, Patel et al., 2006) has been reported to induce type I DM in adult rats. In addition, STZ has been shown to induce type II DM in neonatal rodents or in adult rodents when combined in low doses with nicotinamide, fructose, or fat diet (Wilson and Islam, 2012, Singh and Pathak, 2015).

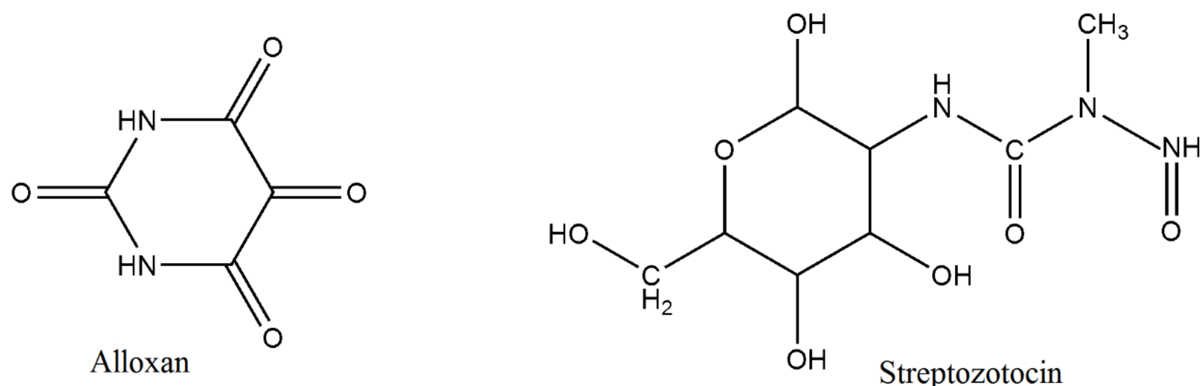


Figure 1.3: Chemical structures of alloxan and streptozotocin

1.2.7.2 Genetic animal model

Genetic animal models of DM comprise spontaneously developed diabetic rats and genetically engineered animals. These models exhibit complex characteristics similar to the heterogeneous nature of human type I and type II DM. The ability to selectively breed animals with a homogeneous genetic origin under strictly-controlled environment is very important in understanding the genetic basis of multifactorial diseases like diabetes (Clee and Attie, 2007). Major risk factors for the development of type II DM are obesity and insulin resistance. These phenomena have also been exploited genetically to develop animal models that show characteristics of type II DM. Commonly used genetic models include Non-obese diabetic (NOD) mouse, diabetic-prone Bio-breeding (BB) rat, *Lep*^{ob} (ob/ob) mouse, *Lepr*^{db} (db/db) mouse and Zucker diabetic fatty (ZDF) rat. Others animal models include LETL (Long Evans Tokushima lean) rat, New Zealand white rabbit, Keeshond dog, Chinese hamster, Celebes black ape (*Macacca nigra*), , Goto Kakizaki rat, KK mouse, NSY mouse, Israeli sand rat etc (Rees and Alcolado, 2005). The major limitations of genetically induced animal model of DM is the expensive cost of breeding and maintenance.

NOD mouse

The NOD mouse is the most commonly used animal model in the study of type I DM. It was initially developed through selective inbreeding of an earlier strain referred to as Jcl:ICR, used in the study of cataract development (Chatzigeorgiou et al., 2009). The NOD mouse closely displays symptoms of type I DM but is less prone to ketoacidosis and therefore can survive for up to 4 weeks without the administration of exogenous insulin. Also, the NOD mice develop

autoantibodies to insulin, GAD , IA-2, Sjögren's syndrome and thyroiditis making it a sought-after model in the study of auto-immune type I DM (Roep et al., 2004).

BB rat

The BB rat was initially developed after selective inbreeding of Wistar rats in the Bio-Breeding Laboratories in Ottawa, Canada (Nakhooda et al., 1977). As an advantage over the NOD mouse, the rapid infiltrations of T lymphocytes into pancreas and T-cell mediated auto-immune type I DM in BB rat is very similar to human type I DM although it is more prone to ketoacidosis. Diabetes prone BB rat shows symptoms of diabetes including weight loss, polyuria and polydipsia around 12 weeks of age but has limitations because it exhibits symptoms of lymphopenia and is highly susceptible to subclinical thyroiditis and sialitis (Greiner et al., 2001, Chatzigeorgiou et al., 2009).

Lep^{ob} mouse

The obese *Lep^{ob}* mouse (also known as the ob/ob mouse) was developed from the C57BL/6J mouse strain by inheriting the monogenic recessive mutation on chromosome 6 in the leptin gene. This results in the lack of the hormone leptin leading to a rapid increase in body weight up to 3X the wild type body weight (Chen and Wang, 2005). The *Lep^{ob}* has been used severally as a model of type II DM to especially understand the roles of obesity and insulin resistance. The model is characterised by excessive craving for food, mild hyperglycaemia hyperinsulinemia, obesity and insulin resistance beginning from 3-4weeks of age (Chen and Wang, 2005). The *Lep^{ob}* mouse has been shown to probably keep up euglycemia because of its compensating excess insulin production (Frode and Medeiros, 2008). Insulin resistance in this model has been reported to be a consequence of a reduction in insulin binding to receptors, impaired insulin receptor (IR), and decreased signal transduction (Srinivasan and Ramarao, 2007). The over-production of neuropeptide Y (NPY) in the hypothalamus of diabetic *Lep^{ob}* mouse has been linked with leptin deficiency, obesity and type II DM (Sainsbury et al., 2002).

$Lepr^{db}$ mouse

The *$Lepr^{db}$* also known as db/db mouse was developed from the C57BL/KsJ strain by an autosomal recessive mutation on chromosome 4 of *db* leptin receptor gene. The *$Lepr^{db}$* mouse is more hyperglycaemic than the *Lep^{ob}* mouse because its pancreatic β-cells cannot sustain the secretion of elevated levels of insulin required to live and therefore dies after 8-10 months of birth. Other symptoms exhibited by *$Lepr^{db}$* include hyperphagia, obesity, hyperinsulinaemia

and insulin resistance. This animal model has been widely used to study type II DM (Reed and Scribner, 1999, Srinivasan and Ramarao, 2007).

Zucker diabetic fatty rat

The Zucker diabetic fatty (ZDF) rat model of DM was developed from the inbreeding of a sub-strain of leptin receptor-deficient Lep^{fa} (also referred to as Zucker fa/fa) rats. This model only shows symptoms of diabetes in males beginning from 7-8 weeks after birth. The ZDF rat has been reported to show symptoms of hyperglycaemia, obesity and insulin resistance as a result of the apoptosis of pancreatic β-cells (Pick et al., 1998, Kahn, 2000). Hyperglycaemia in ZDF rats have been shown to be triggered by the loss of insulin and pancreatic duodenal homeobox (PDX)-1 mRNAs (Chen and Wang, 2005) and the downregulation of GLUT-2 in β-cells (Srinivasan and Ramarao, 2007). This model has been important in understanding the mechanisms of type II DM.

1.2.7.3 Diet or nutrient induced animal model

Type II DM is a chronic metabolic disorder with accompanying disruptions in carbohydrate, fat and lipid metabolism. The influences of unhealthy eating habits, obesity and insulin resistance cannot be overemphasized. Based on this background, it has been hypothesized that the excessive consumption of high-calorie, high-fat and high-carbohydrate rich diets could trigger the onset of insulin resistance, obesity and type II DM (Angelova and Boyadjiev, 2013). Thus, several animal models have been developed to confirm these assumptions. Chronic intake of diet containing excess high-fat, sucrose, fructose and their mixtures have been used extensively as animal models of type II DM (Angelova and Boyadjiev, 2013). Diets containing more than 40-50% saturated fats have been described to progress the development of obesity (Flanagan et al., 2008); hypertriglyceridemia (Fraulob et al., 2010) and type II DM (Barbosa-da-Silva et al., 2014) in animals. Similarly, high-sucrose diet has been shown to induce insulin resistance via the dysregulation of IRS-1 and 2 phosphorylations and phosphatidylinositol 3-kinase (PI3K), and therefore insulin signaling in rodents (Pagliassotti and Prach, 1995, Pagliassotti et al., 2002). In a similar way, fructose has been used to induce insulin resistance in animals (Ackerman et al., 2005, Basciano et al., 2005, Reuter, 2007, Hininger-Favier et al., 2009) and was recently shown to reduce insulin sensitivity in a clinical trial (Malik and Hu, 2012). Schultz and others reported the deleterious effects of a high fructose diet such as hyperinsulinaemia, impaired glucose tolerance, and hypertriglyceridemia in animals (Schultz et al., 2013).

The Israeli sand (*Psammomys obesus*) rat, C57BL/6J mouse and *Acomys calirinus* (spiny mouse) were reported to develop diabetes when fed on high energy, high fat and high calorie diets respectively (Srinivasan and Ramarao, 2007, Barbosa-da-Silva et al., 2014). These rodent models have been shown to display diabetes characteristics such as hyperphagia, obesity, hyperinsulinaemia, glucose intolerance, insulin resistance hence justifying their extensive use in the study of the pathogenesis of type II DM.

1.2.7.4 Diet-fed plus chemical-induced animal model

The failure of pancreatic β-cells to secrete more insulin that can make up for insulin resistance under a chronic hyperglycaemic environment is the major hallmark of type II DM. Therefore, a perfect animal model of type II DM should be able to mimic these conditions as seen in humans. The unavailability of the genetic animal models due to economic costs to researchers especially in low-middle income countries has popularised the non-genetic animal models mostly in these regions (Singh and Pathak, 2015). Unfortunately, the difficulty in attaining insulin resistance as well as pancreatic β-cell dysfunction in either the diet-induced or chemically-induced models of type II DM has limited their use in the aetiology of the disease (Srinivasan and Ramarao, 2007). Therefore, it is believed that a combination of diet (such as fat, carbohydrates) and a chemical (such as alloxan, STZ) might give rise to a better, cost-effective animal model of type II DM (Reed et al., 2000).

The nicotinamide (NAD) plus STZ animal model has been developed and used extensively (Masiello et al., 1998, Pellegrino et al., 1998). Also, the fat-fed and STZ-treated animal model have yielded commendable results (Reed et al., 2000, Srinivasan et al., 2005, Mansor et al., 2013). Recently, the fructose-fed, STZ-injected animal model of type II DM was developed by administering 10% fructose in drinking water for 2 weeks followed by a single intraperitoneal 40 mg/kg STZ injection in rats (Wilson and Islam, 2012). This model has been reported to show symptoms in rats very close to humans and have been confirmed by relevant biochemical investigations to evaluate insulin resistance and partial pancreatic β-cell dysfunction, two key manifestations of type II DM (Ibrahim and Islam, 2014, Mohammed et al., 2015).

1.3 PATHOGENESIS OF TYPE II *DIABETES MELLITUS*

Type II DM represents metabolic disorders of multifactorial origins encompassing defects in glucose, fat and carbohydrate metabolism; insulin secretion by pancreatic β-cells; and insulin actions in insulin-sensitive tissues (Kahn et al., 2014). Normally, the cell seeks to achieve homeostasis through the control of glucose production, utilization and storage by signaling the production of insulin, glucagon and other important enzymes. However, when the secretion of insulin is not sufficient to counteract the effects of insulin resistance usually present in pre-disposed high-risk individuals, hyperglycaemia ensues which promotes the development of type II DM (Boada and Martinez-Moreno, 2013, Fu et al., 2013). A feedback cycle has been identified between pancreatic β-cells and tissues sensitive to insulin to ensure that normal glucose levels are maintained in addition to the regulation of glucose metabolism. How these events occur and in what order they happen is still not entirely understood although signaling between the nervous and circulatory systems has been implicated (Kahn et al., 2014). Genetic and environmental factors also play important roles in the pathogenesis of type II DM (Leahy, 2005).

Acute or chronic hyperglycaemia has been reported extensively in literature to activate oxidative stress, inflammation and other signaling mechanisms that progress the development of type II DM and its complications (Folli et al., 2011, Navale and Paranjape, 2013, Vinagre et al., 2014, Sottero et al., 2015). Reactive oxygen species (ROS), reactive nitrogen species (RNS), pro-inflammatory proteins have been shown to mediate the transcription of genes and expression of proteins involved in the development of type II DM (Fatehi-Hassanabad et al., 2010, Rains and Jain, 2011, Banerjee and Vats, 2013). The participation of these pathways in pancreatic β-cell dysfunction and insulin resistance have been confirmed in diabetic patients and several animal models of DM (Lin and Sun, 2010).

1.3.1 Insulin signaling and downstream effects

Insulin signaling is initiated by the activation of a specific insulin receptor, which belongs to a subfamily of receptor tyrosine kinases (Rhodes and White, 2002). The insulin receptor (IR) is composed of two extracellular α-subunits and two transmembrane β-subunits linked together by disulphide bonds. Upon binding of the insulin molecule to the alpha subunit of the receptor, the inhibition of tyrosine auto-phosphorylation by the β subunit is removed (Saltiel and Kahn, 2001, Bloch-Damti and Bashan, 2005, Rains and Jain, 2011) and the receptor is auto-phosphorylated at distinct tyrosine residues. In contrast to most tyrosine kinase receptors, the

activated IR directly phosphorylates insulin receptor substrates (IRS-1-4) on multiple tyrosine residues. IRS-1 and 2 are the most important for glucose transport (Kriauciunas et al., 2000, Rains and Jain, 2011). Tyrosine phosphorylated IRS proteins then act as a binding site for signaling molecules containing SH-2 (Src-homology-2) domains such as phosphatidylinositol-3-kinase (PI-3K), growth factor receptor-bound protein 2 (GRB-2/mSos), and Src homology 2-containing tyrosine phosphatase (SHP-2). These molecules bind the phosphorylated tyrosine residues of IRS proteins, forming a signaling complex to mediate downstream signaling. PI-3K binds IRS-1/2 through its p85 regulatory subunit increasing the activity of the p110 catalytic subunit. This leads to the phosphorylation of its substrate, phosphatidylinositol-4,5-diphosphate (PI-4,5DP), on the 3rd position of the inositol ring to generate phosphatidylinositol -3,4,5-triphosphate (PI-3,4,5TP) which in turn recruits the serine kinases phosphoinositide-dependent kinase 1 (PDK-1), protein kinase B (PKB/Akt), and protein kinase C (PKC) to the plasma membrane via their pleckstrin homology (PH) domains. The activation of these kinases results in several of insulin's metabolic and mitogenic actions, such as GLUT4 translocation to the membrane, glycogen synthesis by phosphorylation of PKA or GSK-3, and lipogenesis by up-regulating synthesis of the fatty acid synthase gene (Bloch-Damti and Bashan, 2005, Rains and Jain, 2011). The insulin signaling pathways are illustrated in Figure 1.4. Alterations in any protein involved in this pathway may result in insulin resistance and consequently type II DM. For example, serine phosphorylation of IRS-1 has been shown to cause inhibition of transduction of insulin signals leading to insulin resistance (Aguirre et al., 2002, Zhande et al., 2002, Zick, 2004). Also, IRS-2 knockout mice have been demonstrated to develop type II DM and other complications (Withers et al., 1998).

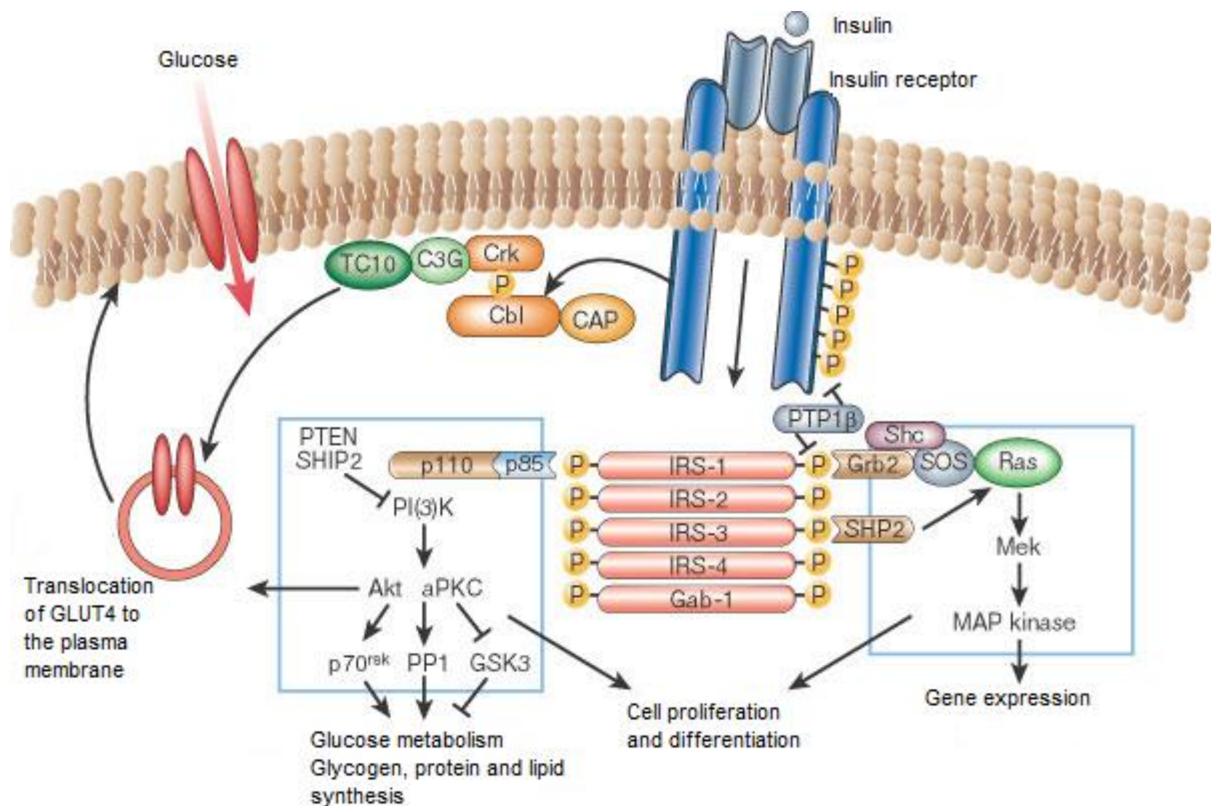


Figure 1.4: The insulin signaling pathways Adapted from Saltiel and Kahn (2001).

The second pathway of insulin signaling involves the activation of the mitogen-activated protein kinase (MAPK), which leads to gene expression of various cellular proliferation or differentiation components. After phosphorylation of IRS-1 and 2, the adaptor proteins GRB-2 and Son of Sevenless (SOS) are recruited and work in concert with a stimulated tyrosine phosphatase, SHP-2, to activate membrane bound Ras. Activated Ras leads to a kinase cascade, allowing MAPK to translocate to the nucleus for gene expression (Saltiel and Kahn, 2001, Rains and Jain, 2011). The third pathway requires the tyrosine phosphorylation of the Cbl proto-oncogene. Cbl is associated with the adaptor protein c-Cbl-associated protein (CAP), which contains three Src-homology-3 (SH3) domains and a sorbin homology (SoHo) domain. The SoHo domain of the phosphorylated Cbl-CAP complex allows translocation to lipid rafts and association with the protein flotillin. A signaling complex is formed at the site of the lipid raft, resulting in the activation of a small G protein, TC10. TC10 is thought to act as a second signal in recruitment of the GLUT 4 protein to the membrane (Saltiel and Kahn, 2001, Rains and Jain, 2011, Pirola et al., 2004). Proper understanding of the pathways of insulin signaling

may improve current understanding of insulin resistance which may open new frontiers in treatment and management of type II DM (Zick, 2004, Fernandez Mejia, 2006).

The activation of insulin signaling results in downstream effects such as the regulation of glycogen metabolism. Although a vast majority of glycogen needed in humans is synthesized and stored in the skeletal muscle, the glycogen metabolizing mechanisms could be found in other cell types such as liver, brain and adipose tissues (Montori-Grau et al., 2011). Glycogen concentration in tissues is partly dependent on the activation of the anabolic enzyme, glycogen synthase (GS) and the degrading enzyme, glycogen phosphorylase (GP), both regulated by phosphorylations (Figure 1.5) (Nicolau et al., 2005, Gardiner et al., 2015). Insulin has been shown to promote glycogen synthesis by inducing GS and inhibiting GP activities leading to the regulation of blood glucose level (Dimitriadis et al., 2011). High blood glucose level observed in type II DM has been shown to be a consequence of loss/reduction of conversion to glycogen due to dysregulation in GS and GP activities in muscle (Shulman, 2000). Reports have also shown a positive correlation between abnormal glycogen metabolism and diabetes in humans (Nikoulina et al., 2001, Solini et al., 2001) and animal models (Ansarullah et al., 2012, Shanmuga and Subramanian, 2012). Therefore, it has been proposed that key proteins involved in insulin signaling and downstream enzymes could be possible targets for the development of useful therapeutics in the management of type II DM (Agius, 2007, Docsa et al., 2011, Nagy et al., 2013).

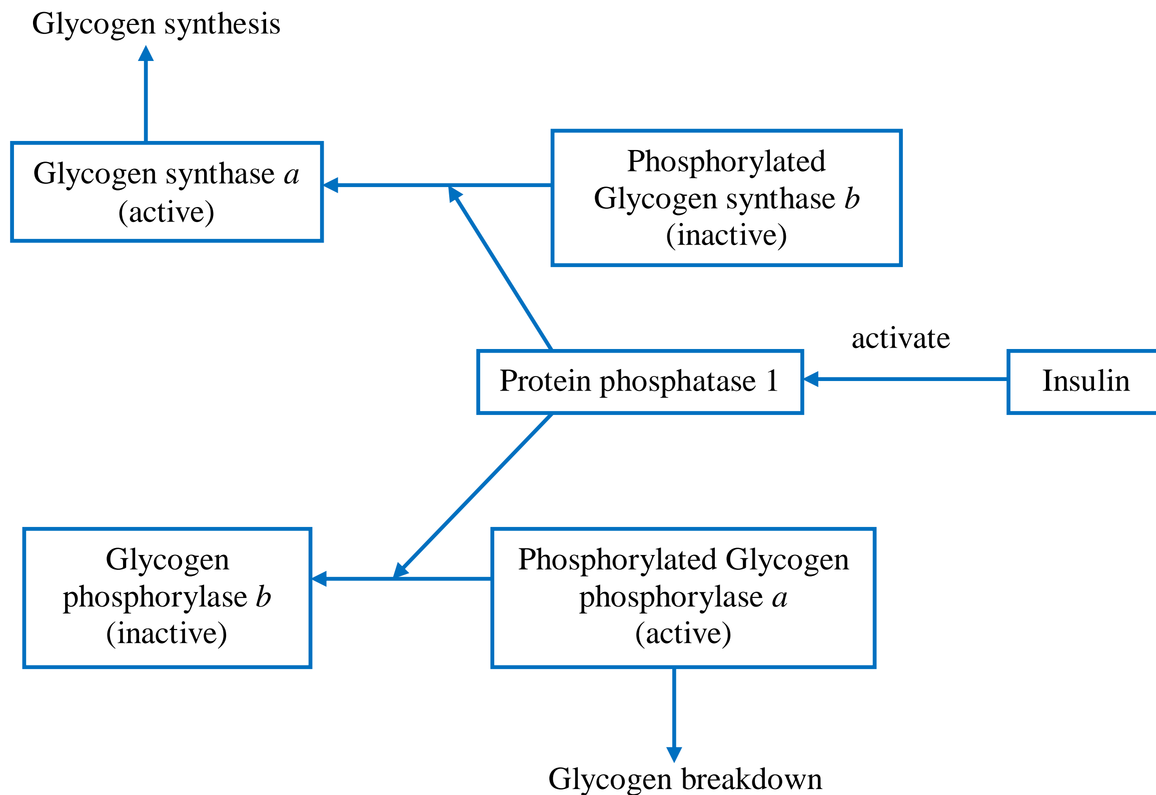


Figure 1.5: A simple scheme showing insulin regulation of glycogen synthase and glycogen phosphorylase enzymes through activation of protein phosphatase 1 enzyme

1.3.2 Insulin resistance and Obesity

Insulin resistance occurs when insulin-sensitive tissues such as the muscle and adipose tissue becomes insensitive to insulin produced by the pancreatic β-cells in response to genetic and environmental changes in predisposed individuals (DeFronzo et al., 2014). The occurrence of insulin resistance has been postulated to be one of the first key events leading to a full-blown type II DM. The skeletal muscle in particular has been shown to be highly susceptible to insulin resistance and has been confirmed in majority of type II DM patients (Bajaj and Defronzo, 2003). This is possible because the activation of insulin promotes protein synthesis by increasing amino acid transport and stimulating ribosomal activity in the skeletal muscle thereby preventing hyperglycaemia. It also promotes glycogen synthesis via the induction of glycogen synthase and inhibition of glycogen phosphorylase activities. Insulin has also been shown to act upon glucose transporters (GLUT) - 2 and 4. Defects in any of the insulin-mediated activities in the muscle therefore lead to insulin resistance and hence type II DM (Pendergrass et al., 2007, Lin and Sun, 2010). Impairment in any of the components of insulin

signaling such as dysregulation of insulin receptor substrate (IRS)-1 and phosphoinositide 3-kinase (PI-3K) in the skeletal muscle also leads to insulin resistance (Zierath et al., 2000). Loss of sensitivity to insulin in the adipose tissue has been reported to increase circulating free fatty acids and reduce triglycerides storage which further hastens the development of type II DM (Nolte and Karam, 2004). Impairment of the activities of glycogen synthase and glycogen phosphorylase enzymes as well as depletion of glycogen stores have also been observed in insulin resistance (Shulman, 2000, Fernandez Mejia, 2006). In addition, when the rate of hepatic glucose production far exceeds its rate of uptake or utilization as a result of lack of sensitivity to insulin, it may partly contribute to hyperglycaemia in type II DM (Fernandez Mejia, 2006).

Obesity is a heterogeneous condition resulting from a combination of genetic and xenobiotic factors to produce phenotypes which have been linked to an increased risk of developing type II DM (Comuzzie et al., 2001, Boada and Martinez-Moreno, 2013). Although obesity has been shown to be an independent risk factor for the development of insulin resistance and type II DM, how this occurs is still not fully understood. A major characteristic in obese individuals is the presence of elevated circulating free fatty acids which has been shown to reduce insulin secretion and decrease glucose uptake in peripheral tissues causing insulin resistance (McKenney and Short, 2011). A reduction in adiponectin levels with increases in levels of tumor necrosis factor (TNF)-α, plasminogen activator inhibitor 1, retinol-binding protein 4, and resistin released from adipose tissues have also been shown to cause insulin resistance and type II DM (Steppan et al., 2001, Mantzoros et al., 2005, Graham et al., 2006, Kanaya et al., 2006, McKenney and Short, 2011).

Genetic factors such as inherited deficiencies as well as environmental factors such as inadequate exercise and consumption of unhealthy diet play major roles in the development of insulin resistance and obesity. The loss of leptin, a hormone involved in the control of appetite has been reported to results in hyperglycaemia and insulin resistance in mice (Chen et al., 1996) and humans (Montague et al., 1997). Genetic experiments have since identified about 20 common genetic variants related to type II DM (Ridderstrale and Groop, 2009). Some genes coding for proteins involved in retinitis (Romao and Roth, 2008), processing of prohormones (Jackson et al., 1997) and stimulation of melanocytes (Kennedy et al., 1997) have been implicated in the development of obesity and type II DM. A sedentary lifestyle or lack of sufficient physical activity contributes to obesity, insulin resistance and type II DM through an

increased fatty acid uptake/oxidation ratio in the skeletal muscle (Venables and Jeukendrup, 2009). The resulting excess triacylglycerol, long-chain acyl-CoAs, diacylglycerols and ceramides have been reported to inhibit insulin signaling via the activation of protein kinase C and phosphorylation of IRS1 (Itani et al., 2002, Yu et al., 2002, Belfort et al., 2005).

1.3.3 Insulin secretion

Type II DM occurs when insulin secretion is not enough to meet the increase in demand caused by inherent insulin resistance observed in high-risk individuals (Cernea and Dobreanu, 2013, Kahn et al., 2014). The pancreatic β-cells of the islets of Langerhans in the human pancreas are responsible for the secretion of insulin whenever needed. The pancreas is composed basically of 2 types of secretory cells- exocrine (98%) and endocrine (2%) cells. The small endocrine secretory cells are of four main types namely α, β, delta and pancreatic polypeptide cells distributed throughout the pancreas within the islets of Langerhans. The β-cells synthesize the 51 amino acid peptide insulin which is the most important hormone involved in maintaining glucose homeostasis (Tan, 2008).

The involvement of pancreatic β-cell dysfunction in type II DM is still a topic under debate among researchers. Controversies surround the precise mechanisms of pancreatic β-cell failure, with some researchers opining that reduction of β-cell mass rather than β-cell dysfunction occur in type II DM (Rhodes, 2005, Rahier et al., 2008, Talchai et al., 2012). Current advances in the pathogenesis of type II DM have revealed that both the number and functions of pancreatic β-cells are impaired, albeit in a gradual manner as the disease progresses (Leibowitz et al., 2011, Talchai et al., 2012, Puri and Hebrok, 2012, DeFronzo et al., 2014). Structural deterioration of the pancreatic islets and β-cell death via apoptosis have been well described in type II DM (Butler et al., 2003, Hayden, 2007, Jurgens et al., 2011). Pancreatic β-cell dysfunction and destruction in type II DM have been reported to be triggered by a number of processes including hyperglycaemia and glucolipotoxicity (Poitout and Robertson, 2008), excess amyloid deposition (Hoppener et al., 1999, Jurgens et al., 2011), dysregulation of insulin signaling (Hennige et al., 2003, Lin et al., 2004), oxidative stress (Kaneto et al., 2006, Cernea and Dobreanu, 2013) and recruitment of inflammatory mediators (Donath et al., 2005).

1.3.4 Hyperglycaemia and Glucotoxicity

The involvement of hyperglycaemia in the pathogenesis of type II DM has been well established in humans and animal models representing one of the most studied characteristic

of the disease (Guillausseau et al., 2008, Defronzo, 2009, Inzucchi et al., 2015). High blood glucose levels have been reported to be a consequence of the failure of insulin production by the pancreatic β-cells to meet the elevated requirement caused by insulin resistance in highly susceptible individuals (Campos, 2012). Hyperglycaemia results when the rate of plasma glucose inflow far exceeds its outflow leading to accumulation within the plasma compartment (Inzucchi et al., 2012). Increased uptake of the excess glucose by glucose transporters (GLUTs) in insulin-sensitive tissues like skeletal muscle and adipose tissue have been shown to increase insulin resistance in these tissues. The pancreatic β-cells are highly sensitive to the perturbations in blood glucose levels and are most affected by hyperglycaemia. Chronic hyperglycaemia has been reported to further promote pancreatic β-cell failure and apoptosis ensuring the progression to type II DM and vascular complications (Campos, 2012). Also, the elevated secretion of glucagon by pancreatic α cells in type II DM have been shown to also increase hepatic glucose production which contribute significantly to hyperglycaemia (Lee et al., 2014).

Persistent or prolonged high plasma level of glucose leads to the condition referred to as glucotoxicity. Glucotoxicity occurs when increased intracellular glucose concentrations damage glucose-sensitive tissues resulting in the impairment of important cellular functions and consequently disabling complications (Kawahito, 2009). Several mechanisms have been implicated in hyperglycaemia-mediated toxicity including generation of reactive oxygen species and oxidative stress via glucose auto-oxidation, advanced glycation end-products (AGEs) over-production, increased hexosamine flux, activation of protein kinase C and polyol pathways (Campos, 2012, Oyenihi et al., 2015). Other mechanisms of hyperglycaemia-induced insufficient insulin secretion have been reported such as impairment of pancreatic β-cell functions and mass (Kim and Yoon, 2011); loss of insulin gene expression (Poitout et al., 1996, Poitout and Robertson, 2008, Cernea and Dobreanu, 2013); impairment of mitochondria morphology and function (Lu et al., 2010, Ma et al., 2012); and over-activation of inflammation (Maedler et al., 2002, van Raalte and Diamant, 2011).

Furthermore, the effects of 2 incretins namely glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide 1 (GLP-1) in type II DM have been demonstrated (Holst et al., 2009, Boada and Martinez-Moreno, 2013, Nauck and Meier, 2016). GIP is a peptide secreted in the intestine and binds to receptors in pancreatic islets, gut, adipose tissue, heart and brain activating adenyl cyclase leading to a subsequent elevation of intracellular cyclic

AMP concentrations. This leads to the activation of several cellular processes such as increased release of insulin-containing granules, elevated intracellular calcium ions levels, anti-apoptotic actions etc. (Baggio and Drucker, 2007, Nauck, 2011, Campbell and Drucker, 2013). Non-responsiveness to GIP leading to loss of the incretin effects have been observed in type II diabetic patients (Baggio and Drucker, 2007, Nauck and Meier, 2016). GLP-1, a gut hormone has been reported to regulate hyperglycaemia by promoting insulin secretion, inhibiting apoptosis, inhibiting glucagon release, and decreasing polyphagia (Farilla et al., 2002, Drucker, 2006). Levels of this hormone were shown to decrease in type II diabetic patients (Toft-Nielsen et al., 2001).

1.3.5 Dyslipidaemia and Lipotoxicity

Dyslipidaemia and hyperlipidaemia represent all disorders arising from abnormalities in the number and type of lipid or lipoprotein parameters and have been identified as major risk factors in the development of diabetes-associated atherosclerosis and cardiovascular diseases (Solano and Goldberg, 2006, Qi et al., 2012). Cardiovascular disease is still one of the most important macrovascular complication responsible for the elevated morbidity and mortality rates among type II diabetic patients (Taskinen and Boren, 2015). Insulin resistance, a prominent feature in type II DM, has been associated with dyslipidaemia which further exacerbates the disease (Gadi and Samaha, 2007, Adiels et al., 2008, Stolar, 2010). Elevated blood concentration of cholesterol, triglycerides, low density lipoprotein cholesterol (LDL-c) and apolipoprotein B (apoB) accompanied by the reduction in level of high density lipoprotein cholesterol (HDL-c) are common features among type II diabetic patients (Isomaa et al., 2001, Eriksson et al., 2011, Scott et al., 2011).

Hyperlipidaemia is characterised by an abnormal elevation of plasma free fatty acids (FFA) which promotes the increased production of triglyceride (TG), apoB and very low-density lipoprotein (VLDL) in the liver which then stimulates the formation of LDL-c (Krentz, 2003, Vijayaraghavan, 2010). Elevated level of LDL-c has been shown to result in accumulation of this lipoprotein within the vascular arterial walls forming plaques implicated in the development of atherosclerosis and coronary heart diseases (Gadi and Samaha, 2007, Mahamuni et al., 2012). Several reports have indicated that the inability to inhibit hepatic production of large TG-rich VLDL (VLDL-TGs) in patients with type II diabetes may be responsible for the increase in plasma TG levels (Adiels et al., 2008, Vijayaraghavan, 2010). The term lipotoxicity has been used to describe the deleterious effects caused by excess

circulating FFAs on pancreatic β-cells, hepatocytes, adipocytes and muscle cells (Cernea and Dobreanu, 2013). The long chain fatty acids are usually converted to their fatty acyl-CoA derivatives in the β-cell which increases the release of insulin. However, the over-production of long fatty acyl CoA have been shown to inhibit insulin secretion through activation of the Randle cycle (Boada and Martinez-Moreno, 2013), alteration of G protein-coupled receptor-40 and overexpression of uncoupling protein (UCP)-2 (Poitout and Robertson, 2008). Lipotoxicity has also been reported to stimulate β-cell apoptosis and dysfunction via induction of inducible nitric oxide synthase (iNOS), interleukin (IL)-1 and tumor necrosis factor (TNF)-α (Boada and Martinez-Moreno, 2013). In addition, studies have suggested that excess fatty acids inhibit insulin-activated glucose uptake into muscle cells by obstructing the translocation of GLUT-4 to the plasma membrane (McKenney and Short, 2011). Furthermore, increased fatty acids have been demonstrated to encourage glycogenolysis and gluconeogenesis in the liver (Boden and Shulman, 2002, McKenney and Short, 2011).

1.3.6 Oxidative stress

Oxidative stress occurs as a result of excessive formation of ROS and RNS, all collectively known as oxidants. When the rate of production of ROS or RNS in a cell far exceeds their rate of utilization and conversion to more stable products, oxidative stress ensues, leading to cellular and tissue damage. The imbalance of pro-oxidants/antioxidant ratio favoring the former causes an alteration in the normal redox signaling of the cell triggering impairment in several pathways of the cell's metabolism- a critical feature in type II DM (Calabrese et al., 2012). Examples of ROS include free radicals such as superoxide ($^{\bullet}O_2^-$), hydroxyl ($^{\bullet}OH$), peroxyl ($^{\bullet}RO_2^-$), hydroperoxyl ($^{\bullet}HRO_2$) and non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). RNS include free radicals like nitric oxide ($^{\bullet}NO^-$), nitrogen dioxide ($^{\bullet}NO_2^-$), and non-radical species such as peroxynitrite (ONOO), nitrous oxide (HNO_2), and alkyl peroxynitrates (RONOO) (Valko et al., 2007). These reactive species have been reported to abstract electrons from carbohydrates, protein, lipid and DNA forming adducts which affect proper cellular signaling, gene regulation and function of cells (Pop-Busui et al., 2006). Naturally, the human system has mechanisms to deal with oxidative damage and free radical formation through endogenous antioxidants (which act as scavengers of free radicals and oxidants) and those derived externally from food sources. Antioxidants are either enzymatic e.g. superoxide dismutase (SOD), catalase (CAT), glutathione S-transferases (GST), and glutathione peroxidase (GPx) or non-enzymatic e.g. reduced glutathione (GSH), vitamins A, C and E etc. However, when the rate of formation of these reactive species overwhelms the

detoxifying ability of the antioxidants, oxidative stress occurs (Ridnour et al., 2004, Halliwell, 2011).

Chronic hyperglycaemia results in oxidative stress via auto-oxidation of glucose in the presence of transition metals (Wolff et al., 1988); decreased activities of antioxidant enzymes such as SOD, CAT, GST and GPx (Banerjee and Vats, 2013); increased oxidative phosphorylation (Nishikawa et al., 2000), glycosylation of proteins (Negre-Salvayre et al., 2008); activation of the hexosamine pathway (Kaneto et al., 2001) and formation of excess advanced glycated end products (AGEs) (Motawi et al., 2013, Nowotny et al., 2015). It has also been established that hyperglycaemia increases mitochondrial ROS, which could represent a key event in the development of diabetes complications (Nishikawa et al., 2000, Kiritoshi et al., 2003, Mackenzie et al., 2013). Increases in biomarkers of oxidative stress related to lipid (thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), isoprostane); protein (protein carbonyls, nitrosylated proteins); carbohydrate (AGEs); and DNA (8-hydroxy-deoxyguanine (8-OHdG)), together with inhibition of the synthesis of endogenous antioxidants, have been observed in several *in vitro* and *in vivo* experimental models of diabetes (Inoguchi et al., 2000, Brownlee, 2001, Nowotny et al., 2015).

Hyperglycaemia-induced oxidative stress has been reported to inhibit the secretion of insulin in pancreatic β-cell through the activation of an uncoupling protein-2 (UCP-2) which lowers the ATP/ADP ratio by leaking protons in the β-cell (Brownlee, 2003). ROS has been shown to leak into cell membranes and damage pancreatic β-cells (Lepore et al., 2004, Chen et al., 2006). Over-production of free radicals like superoxide anion in β-cells can also lead to the activation of stress-signaling pathways that can induce downstream effectors like NF-κB leading to β-cell apoptosis and dysfunction ultimately reducing insulin secretion (Rhodes, 2005).

Oxidative stress has also been implicated in the alteration of insulin signaling leading to insulin resistance. Under conditions of increased oxidative stress, stress-responsive signaling cascades are activated leading to the modification of IRS proteins by increased serine/threonine phosphorylation which are subsequently degraded contributing to insulin resistance (Pitocco et al., 2010). High concentration of H_2O_2 has been shown to directly induce insulin signaling (PI3K-dependent pathway) leading to insulin resistance prior to the onset of diabetes (Higaki et al., 2008, Pitocco et al., 2010). Similarly, studies have confirmed the association between

excess production of ROS and insulin resistance in type II diabetes and obesity experimental models (Atabek et al., 2004, Styskal et al., 2012).

Mitochondria play a critical role in regulating the metabolic imbalance seen in diabetes-induced oxidative stress since it is the organelle responsible for maintaining the transfer of electrons through the electron transport chain to molecular oxygen during aerobic respiration in cells (Mackenzie et al., 2013). This becomes a potential site for the over-production of reactive species like H_2O_2 and $ONOO^-$ which can cross mitochondria membranes and damage macromolecules in other cellular regions (Rachek et al., 2006). The excess formation of $^{\bullet}O_2^-$ from the mitochondrial electron transport chain (ETC) may trigger a cascade of oxidative reactions including lipid peroxidation that ultimately culminates in the formation of advanced lipid peroxidation end-products (ALEs) such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which have the potential to cause DNA damage by forming adducts with DNA bases (Figure 1.6) (Valko et al., 2007). Also, $^{\bullet}O_2^-$ levels have been reported to increase in the mitochondrial ETC as a result of hyperglycaemia during diabetes leading to increase in oxidative stress (Ceriello and Testa, 2009). Hyperlipidaemia (abnormal increase in lipid levels) in the presence of hyperglycaemia generates additional ROS that are also implicated in β-cell dysfunction (Furukawa et al., 2004). Excess free fatty acids have previously been shown to cause ROS over-production leading to mitochondrial DNA damage and pancreatic β-cell malfunctioning (Poitout and Robertson, 2008). Hyperglyacemia leads to increased levels of ROS and D-glucose which has been shown to be capable of inducing apoptosis through the activation of Bax-caspase pathway (Green and Reed, 1998). Caspases are a family of cysteine proteases known to be the effectors of apoptosis. Upon activation of Bax by free radicals, caspases are activated which alter mitochondrial function by reducing the electrochemical gradient across the mitochondrial membrane leading to the release of mitochondrial cytochrome c to cytoplasm (Liu et al., 1996, Green and Reed, 1998). Reports have shown that movement of Bax into the mitochondrial membrane is accompanied by a significant increase in the activities of caspase-3 and caspase-9 (Fraser and Evan, 1996, Yang et al., 1997, Nakagami et al., 2002). Other pathways like synthesis of metabolites (through xanthine oxidase pathway), production of neurotransmitters and serotonin, and detoxification of xenobiotics via cytochrome P450 system and NADPH oxidase utilize oxygen molecules with the possibility of ROS formation and these could add to the burden of oxidative stress in diabetes (Johansen et al., 2005).

Other mechanisms by which hyperglycaemia increases oxidative insult are through the activation of transcription factor such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and c-Jun N-terminal kinase (JNK)- stress-associated kinases (Mohamed et al., 1999, Ho et al., 2000).

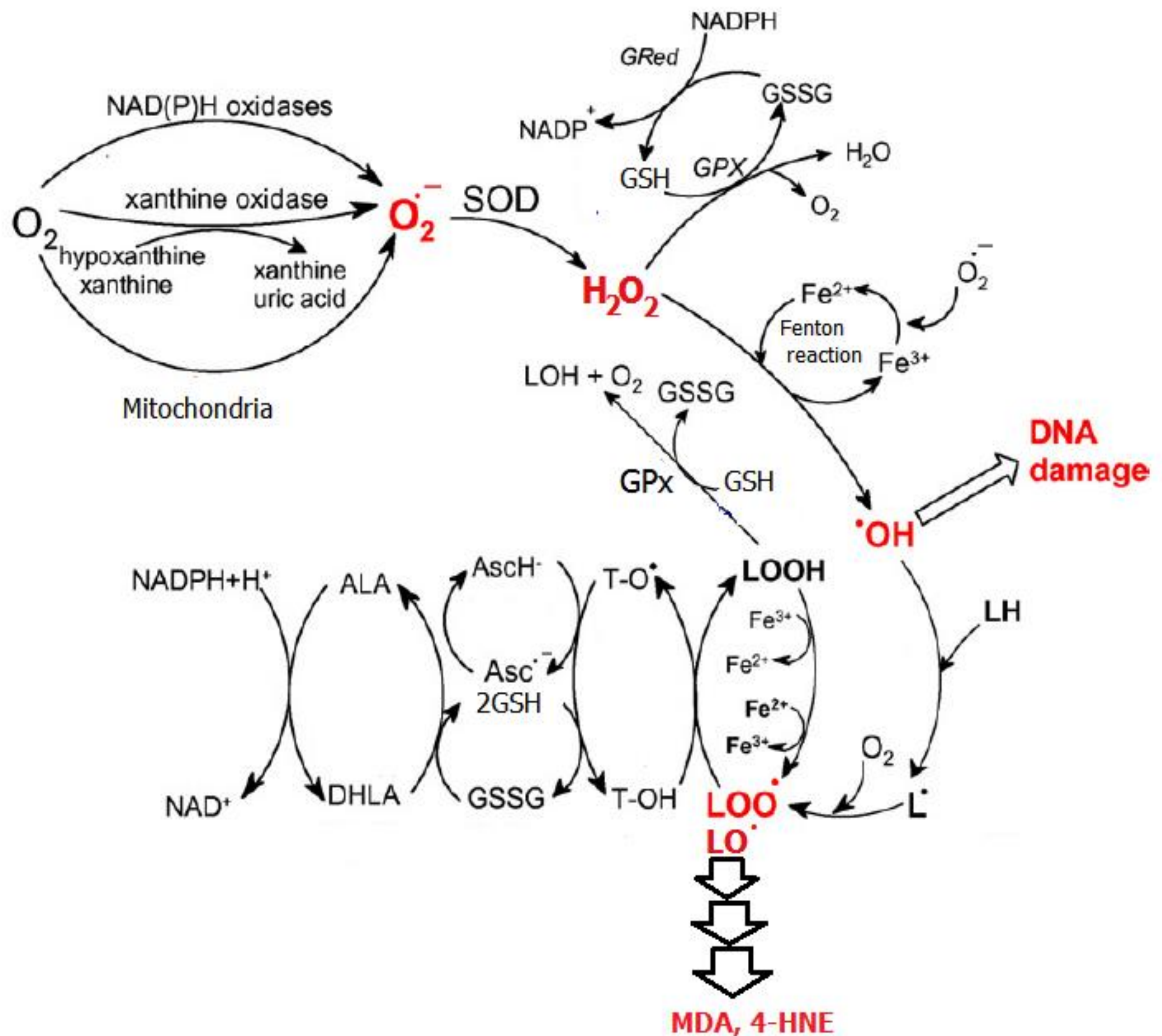


Figure 1.6: Pathways of production of reactive oxygen species, lipid peroxidation and the role of antioxidants. $^{\bullet}O_2^-$ - Superoxide anion; H_2O_2 - Hydrogen peroxide; $^{\bullet}OH$ – Hydroxyl radical; SOD – Superoxide dismutase; GSH – Reduced glutathione; GSSG – Oxidized glutathione; GPx – Glutathione peroxidase; GRed – Glutathione reductase; LH- Polyunsaturated fatty acid; $L^{\bullet}$ - Carbon-centred lipid radical; $LO^{\bullet}$ – Lipid alkoxyl radical; $LOO^{\bullet}$ - Lipid peroxyl radical; LOOH – Lipid hydroperoxide; LOH – Lipid alcohol; MDA – Malondialdehyde; 4-HNE – 4-Hydroxynonenal; $T\text{-}O^{\bullet}$ - Vitamin E radical; T-OH – Vitamin E; $AscH^-$ - Ascorbate monoanion; $Asc^{\bullet-}$ - Ascorbyl radical; ALA – Alpha-lipoic acid; DHLA - Dihydrolipoic acid. (Adapted from Valko et al. (2007)

1.3.7 Inflammation

The importance of inflammatory processes such as recruitment of B cells and T cells; expression of pro-inflammatory chemokines, cytokines and cell adhesion molecules in insulin resistance and type II DM have been extensively described (Cruz et al., 2013, DeFuria et al., 2013, Esser et al., 2014). Inflammation has been shown to be triggered by metabolic and haemodynamic factors observed in the pathophysiology of type II DM (Garcia et al., 2010, Vinagre et al., 2014). Increases in macrophages, leucocytes and monocytes infiltration into cells together with an elevation in pro-inflammatory cytokines has been positively correlated with the progression of the disease (Calle and Fernandez, 2012, Ma et al., 2014b). Elevated levels of pro-inflammatory cytokines and chemokines such as IL-1, IL-6, IL-18, C-reactive protein (CRP), TNF-α, MCP-1, and IFN-γ have been reported in type II DM (Zozulinska and Wierusz-Wysocka, 2006, Jagannathan-Bogdan et al., 2011, Damanhouri, 2012, Duran-Salgado and Rubio-Guerra, 2014). Decreased levels of anti-inflammatory mediators such as IL-4 and IL-10 that counteract the effects of pro-inflammatory mediators have also been observed (Opal and DePalo, 2000, van Exel et al., 2002). TNF-α has been shown to alter insulin sensitivity through dysregulation of insulin signaling; reduction of GLUT-4 in adipose tissue and activation of other pro-inflammatory cytokines such as IL-6 and MCP-1 (Fasshauer and Paschke, 2003, Calle and Fernandez, 2012). MCP-1, a chemokine produced by adipocytes, endothelial cells and vascular smooth muscle cells, activates monocyte recruitment into visceral fat with a corresponding increase in expression in insulin resistance, obesity and type II DM (Sartipy and Loskutoff, 2003, Takahashi et al., 2003, Kamei et al., 2006). IL-1β has been reported to inhibit insulin secretion by impairing pancreatic β-cell function partly through the activation of nuclear factor kappa B (NF-κB)-dependent apoptosis (Donath et al., 2008, Kacheva et al., 2011, Banerjee and Saxena, 2012). The activation of NF-κB is one of the most understood mechanisms implicating reactive oxygen species (ROS) and inflammation in the pathogenesis of DM. Once NF-κB is activated, it translocates to the nucleus where it signals the transcription of pro-inflammatory cytokines. An abnormal activation of NF-κB has been observed in diabetic patients and animal models of DM (Goldfine et al., 2011, Locke and Anderson, 2011).

Increases in the mass of adipose tissue coupled with the elevated production of metabolic products (leptins, toll-like receptors (TLRs)) have been suggested as possible pathways of inflammation-mediated development of insulin resistance, obesity and type II DM (Greenberg and McDaniel, 2002, Williams and Nadler, 2007). Abnormally high levels of the hormone

leptin promote T cell responses; activate monocytes and neutrophils; and further induce pro-inflammatory cytokines such as TNF-α and IL-6 ultimately leading to insulin resistance (Maya-Monteiro and Bozza, 2008, Cruz et al., 2013). Recently, Toll-like receptors (TLR) have been implicated in the development of inflammation in DM as shown by a positive correlation between the expression of TLR4 and the degree of inflammation in diabetic rats (Liu et al., 2013). In addition, elevated FFAs also activates TLR and inhibits IRS-1, contributing to insulin resistance (Hotamisligil, 2008). The levels of the anti-inflammatory adipokine-adiponectin have been shown to decrease in type II diabetic patients (Bastard et al., 2006).

The activation of inflammatory processes in type II DM can also be triggered by an unabated production of free radicals, lipid peroxidation products, and advanced glycated end-products which partially inhibit pancreatic β-cells functions exacerbating the symptoms of the disease (Akash et al., 2013). The interplay between hyperglycaemia, and inflammatory mediators in the development of insulin resistance and pancreatic β-cell destruction in type II DM is illustrated in Figure 1.7. Inflammation and oxidative stress are inseparable partners since they exert their cellular effects through interconnected pathways designed to propagate mutual signal cascades especially under diseased conditions like type II DM (Ambade and Mandrekar, 2012). Upon activation, immune-sensitive cells are massively recruited and secrete pro-inflammatory mediators that are capable of inducing the production of several reactive species (Costa and Garlid, 2008). Reports have shown that pro-inflammatory cytokines directly activate macrophages which in turn, defend the cell via the generation of ROS and RNS (Fialkow et al., 2007). Therefore, chronic inflammation has been shown to be facilitated through the continuous over-production of oxidative products and reduction of antioxidants resulting in tissue destruction and fibrosis (Hold and El-Omar, 2008). In the same vein, oxidative stress increases inflammation by stimulating stress signals notably extracellular signal-regulated kinase (ERK), JNK and p38 pathways, as well as inducing the translocation of NF-κB into the nucleus to increase the expression of pro-inflammatory cytokines (Ho et al., 2000, Goldfine et al., 2011).

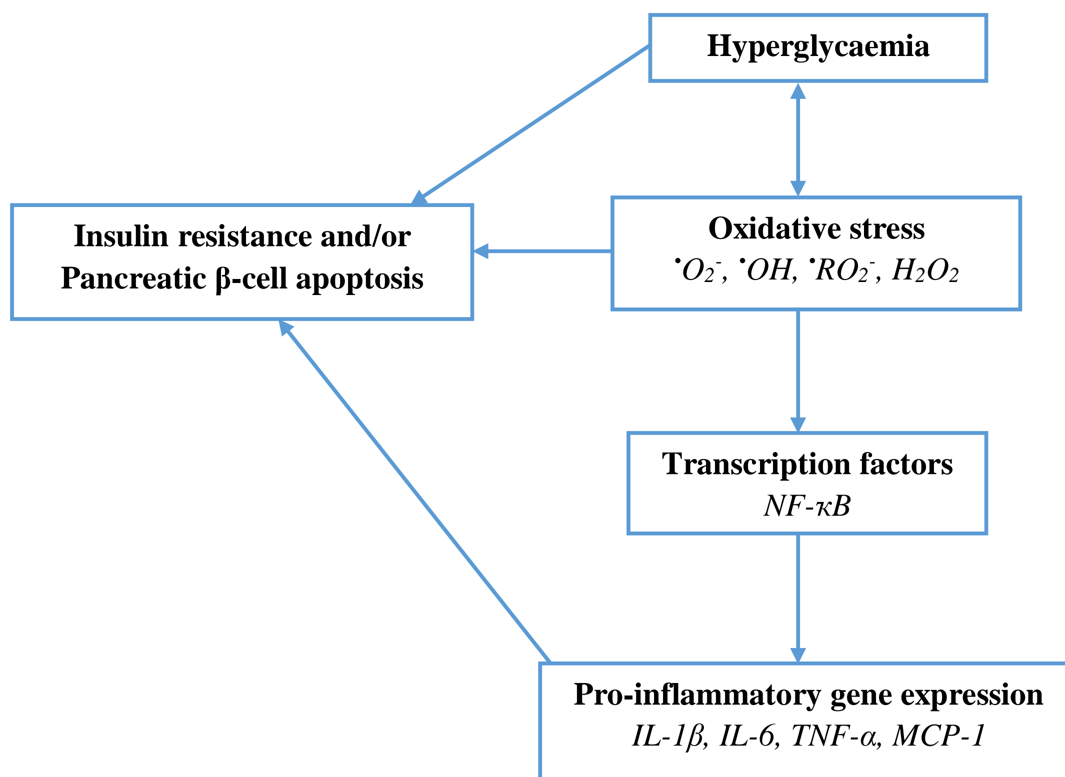


Figure 1.7: The interplay between hyperglycaemia, oxidative stress and inflammation in the development of insulin resistance and pancreatic β-cell apoptosis. $^{\bullet}O_2^-$ (superoxide anion), $^{\bullet}OH$ (hydroxyl radical), $^{\bullet}RO_2^-$(lipid peroxyl radical), H_2O_2 (hydrogen peroxide), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), IL (interleukin)-1β, IL-6, TNF (tumor necrosis factor)-α, MCP (monocyte chemoattractant protein-1).

1.4 COMPLICATIONS OF TYPE II *DIABETES MELLITUS*

The occurrence of disabling and life-threatening complications increases the morbidity and mortality in type II diabetic patients (Litwak et al., 2013, Deepa et al., 2014). Globally, DM is believed to be one of the most prominent causes of cardiovascular diseases, blindness, kidney failure and lower-limb amputation (International Diabetes Federation, 2015). Microvascular complications may result from long term damage, dysfunction and failure of organs like liver (hepatopathy), kidneys (nephropathy), nerves (neuropathy), eyes (retinopathy), and testes (reproductive toxicity). Macrovascular complications are caused by chronic damage to the arterial blood vessels becoming a major risk factor for atherosclerosis, coronary heart, cardiovascular and cerebrovascular diseases (Fowler, 2008, Forbes and Cooper, 2013). These complications arises from impairment in haemodynamic and metabolic cellular processes in

genetically susceptible individuals that ultimately culminate in cell dysfunction or death via over-production of oxidative stress and pro-inflammatory mediators (Forbes and Cooper, 2013).

1.4.1 Pathways leading to diabetic complications

A relationship has been established between hyperglycaemia, oxidative stress, inflammation and other numerous pathways which can lead to the development of diabetic complications. Some of these pathways include: activation of protein kinase C isoforms, increased hexosamine pathway flux, increased advanced glycation end product formation, and increased aldose-reductase pathway flux (Rolo and Palmeira, 2006). Oxidative stress has been implicated to play a central role in these pathways as shown in Figure 1.8. The individual pathways, their inter-relationship and how they lead to diabetic neuropathy, as an example of diabetic complication, have been extensively reviewed by Oyenihi and others (Oyenihi et al., 2015).

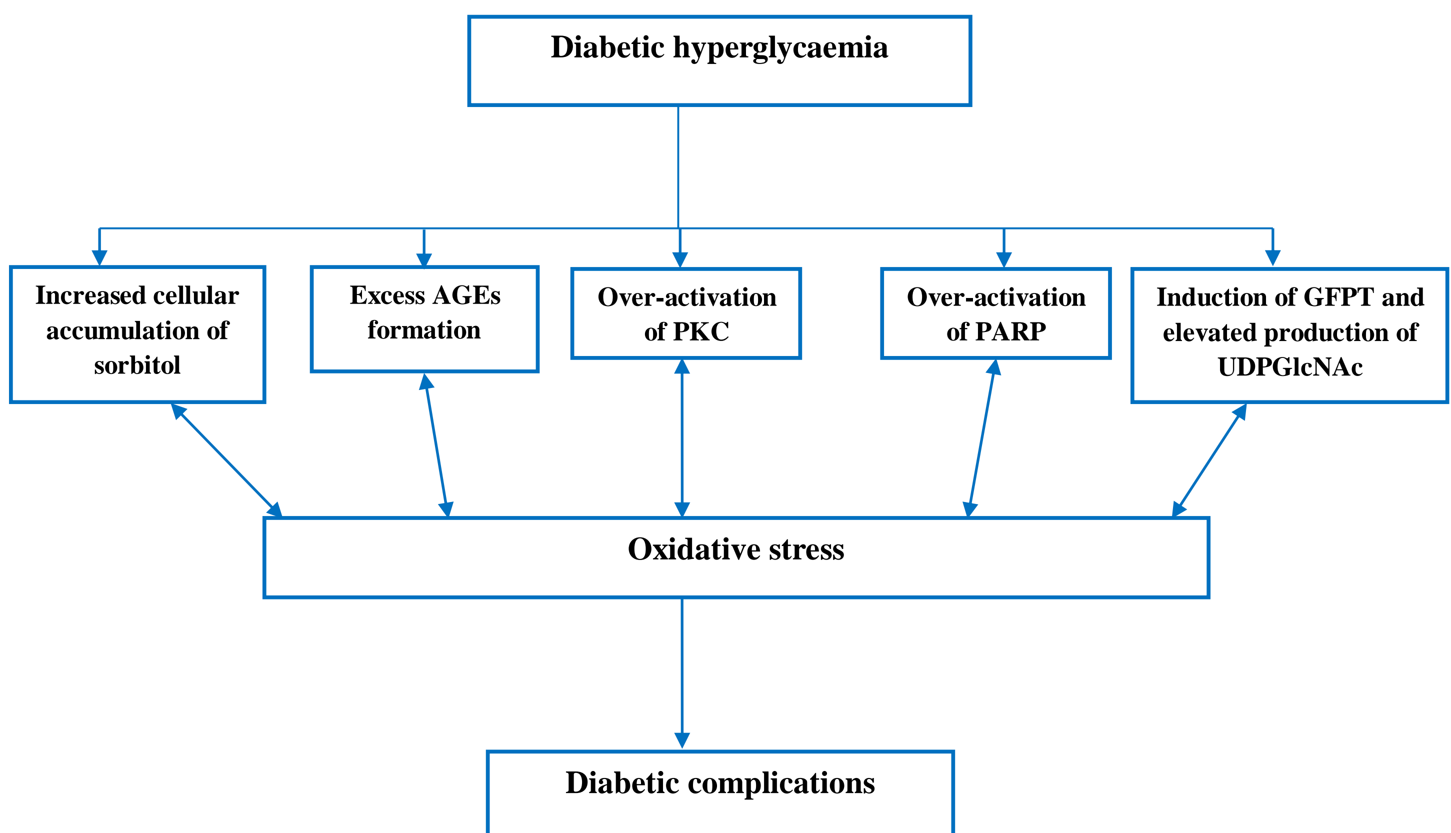


Figure 1.8: A simplified scheme showing the common pathways of hyperglycaemia-induced oxidative stress leading to the development of diabetic complications. AGEs - Advanced glucose end-products; PKC - Protein kinase C; PARP - Poly-ADP ribose polymerase; UDPGlcNAc - Uridine diphosphate-N-acetylglucosamine; GFPT - Glutamine-fructose-6-phosphate transaminase.

1.4.2 Diabetic hepatopathy

The liver is the most important organ in the metabolism of macromolecules central to the pathogenesis of type II DM. It is highly sensitive to the insulin produced in the pancreas. Once insulin has entered the circulation, it is bound by specialized receptors found on the membrane of most cells. This binding of insulin to its receptor is highly specific and triggers a number of biological responses especially in liver, muscle and adipose tissues. Insulin promotes the storage of glucose and fat within these target cells and influences cell growth and the metabolism of a wide variety of tissues (Nolte and Karam, 2004). In the liver, insulin acts to increase the storage of glucose as glycogen and resets the liver to the fed state by reversing mechanisms such as glycogenolysis, gluconeogenesis and ketogenesis. This is achieved by insulin-induced phosphorylations, which activate glucokinase, phosphofructokinase and pyruvate kinase, while inactivating glucose-6-phosphatase, fructose bisphosphatase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase (Nolte and Karam, 2004, Staehr et al., 2004).

Due to the pivotal role of the liver in glucose and lipid homeostasis, it is severely affected during DM with major biochemical and functional abnormalities that are instrumental to the development of a variety of liver diseases, acute liver failure or death (Harrison, 2006, Hickman and Macdonald, 2007, Hamed, 2014). There have been several reports associating type II DM and the severity of liver diseases including non-alcoholic steatohepatitis (NASH) or nonalcoholic fatty liver disease (NAFLD) (Trombetta et al., 2005, Kashanian and Fuchs, 2015, Saponaro et al., 2015), chronic viral hepatitis (Mason et al., 1999, Mehta et al., 2003, Moscatiello et al., 2007), hemochromatosis (Conte et al., 1998, Kwan et al., 1998), alcoholic liver disease (Wei et al., 2000), cirrhosis (Holstein et al., 2002, Kashanian and Fuchs, 2015) and hepatocellular carcinoma (Lai et al., 2006, Amarapurkar et al., 2008). NAFLD is probably the most common diabetes-related chronic liver condition in developed countries occurring in more than 55% of individuals with obesity and type II DM (Del Gaudio et al., 2002, Chalasani et al., 2012, Anstee et al., 2013). Cirrhosis has also been reported to account for approximately 4-12% of diabetes-related deaths (de Marco et al., 1999, Tolman et al., 2007).

Several mechanisms suggested to be involved in the development of different liver diseases in type II DM (or vice versa) have been thought to share common pathways which can be summarized thus: persistent hyperglycaemia; over-production of ROS/RNS; excess induction of pro-inflammatory cytokines; insulin resistance; increased hepatocyte and pancreatic β-cell

apoptosis; elevated collagen synthesis and fibrosis; obesity and genetic predisposition (Tolman et al., 2007, Hickman and Macdonald, 2007, Frances et al., 2010, Hsieh and Hsieh, 2011, Kumashiro et al., 2011).

1.4.3 Diabetic nephropathy

Diabetic nephropathy (DN) is a life-threatening microvascular complication of DM described as a progressive decline in glomerular filtration rate and commonly characterized by glomerular hyperfiltration, glomerular and tubular epithelial hypertrophy, increased urinary albumin excretion, increased basement membrane thickness and mesangial expansion with the accumulation of extracellular matrix proteins (ECM) (Jain, 2012). Despite recent advances in the control of glycaemia and successes in the use of rennin-angiotensin-aldosterone system inhibitors in clinical trials, the number of chronic kidney disease (CKD) patients keeps increasing. DN remains the most common cause of CKD and end stage renal disease (ESRD) affecting approximately 20-40% of adult diabetic patients worldwide (Hakim and Pflueger, 2010, Reddy et al., 2012, Velez and Bhalla, 2012). Also, the mortality rate among type II diabetic patients with ESRD is on the rise mainly as a result of the susceptibility to cardiovascular diseases such as hypertension, stroke, and atherosclerosis (Vinod, 2012). The most important risk factors in the development and progression of DN are obesity, persistent hyperglycaemia, negative lifestyle, environmental and hereditary factors (Elmarakby and Sullivan, 2012). Macroalbuminuria and microalbuminuria have been the most sensitive clinical manifestation of DN (Singh et al., 2013).

CKD in diabetes has severally been linked to hyperglycaemia, oxidative stress, inflammation, haemodynamic and genetic factors. Lack of control of these processes may lead to glomerular endothelial dysfunction, glomerular fibrosis and sclerosis, mesangial cells inflammation and expansion that ultimately contribute to the reduced glomerular filtration rate (GFR) found in DN (Vinod, 2012, Arora and Singh, 2013). Inflammation is now known to be triggered by metabolic and heamodynamic events such as those found in the diabetic kidney (Lim and Tesch, 2012). An abnormal activation of NF-κB which translocates to the nucleus, where it signals the transcription of pro-inflammatory cytokines has been observed among patients with DN and experimental animal model (Mezzano et al., 2004, Lu et al., 2014). Increases in macrophages, leucocytes and monocytes infiltration together with an elevation in pro-inflammatory cytokines (IL-1, TNF-α, INF-γ), chemokines (MCP-1, CCL5, CX3CL1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-

1)), C reactive protein (CRP1) and adipokines that occurs via a step-by-step process have been shown to contribute to the pathologic changes in DN patients (Duran-Salgado and Rubio-Guerra, 2014, Vinagre et al., 2014) and animal model (Ma et al., 2014a).

Furthermore, persistent high glucose level in the blood has been shown to trigger the production of oxidative stress in the mesangial and tubular epithelial cells in diabetic kidney. Elevation of $OH^{\cdot}$, H_2O_2, AGEs, and sorbitol concentrations in the kidney; uncoupling of endothelial nitric oxide synthase (eNOS) leading to production of $^{\bullet}O_2^-$; in addition to increased activities of xanthine oxidase, NAD(P)H oxidase and aldose reductase are common features observed in human patients and animal models of DN (Dunlop, 2000, Prabhakar et al., 2007, Forbes et al., 2008, Dellamea et al., 2014). Over-activation of PKCα and PKCβ has been shown to cause renal fibrosis and dysfunction through the upregulation of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β expressions in hyperglycaemic microenvironment (Yao et al., 2006, Ohshiro et al., 2006, Arora and Singh, 2013). Studies have suggested the involvement of the stress-activated protein kinases (SAPK) and JAK/STAT pathway in the development of DN (Marrero et al., 2006, Arora and Singh, 2013). Significant elevations of both p38 and JNK activities leads to renal inflammation and injury as observed in the kidneys of *in vivo* and *in vitro* diabetic models (Lim and Tesch, 2012).

1.4.4 Diabetic neuropathy

Diabetic neuropathy (DNeuro) seems to be the most common yet least understood complication of diabetes being present in over 50% of chronic diabetics (Callaghan et al., 2012, Kasznicki et al., 2012). In the United States, DNeuro is the leading cause of diabetes-related hospital admissions and non-traumatic amputation (Boulton et al., 2005). It can be found late in type I diabetes but early in type II diabetes and the cause of this occurrence is still not clear (Shaikh and Somani, 2010). DNeuro encompasses all diabetes-related neuropathies resulting from peripheral nerve dysfunctions involving different parts of the somatic and autonomic nervous systems which are the basis for the many classifications of the disease (Casellini and Vinik, 2006, Hosseini and Abdollahi, 2013).

Oxidative stress has been implicated in causing nerve damage in several experimental animal models of diabetes and diabetic patients (Feldman and Vincent, 2004, Babizhayev et al., 2014). The mechanisms involved in oxidative stress-induced nerve dysfunctions include generation of ROS/RNS, lipid peroxidation, DNA damage and reduction in cellular antioxidants (Ho et

al., 2006, Niti et al., 2016). Increased reactive species are capable of damaging lipids present in the myelinated structures of nerves resulting in the loss of axons and disruption of the microvasculature in the peripheral nervous system (Casellini and Vinik, 2006, Aziza et al., 2014). Experimental studies have revealed that high glucose induces apoptosis via a mitochondria-dependent route in embryonic sensory neurons (Vincent et al., 2004). Oxidative damage to peripheral nerves causes hyper-excitability in the afferent nociceptors and central neurons leading to the generation of spontaneous impulses within the axons and dorsal root ganglions of the nerves contributing to the neuropathic pain associated with DNeuro. Recent findings implicate free radicals in the development of DNeuro in addition to the impairment of antioxidant defense system in type II diabetes mellitus patients (Ziegler et al., 2004, Kasznicki et al., 2012, Ko and Cha, 2012).

Hyperglycaemia has been postulated to induce oxidative stress *via* several well-studied, interconnected pathways (including glucose auto-oxidation, AGEs over-production, increased hexosamine flux, activation of diacylglycerol and protein kinase C, activation of polyol pathway) which ultimately lead to nerve dysfunction through activation of downstream signaling pathways involving NF-κB, MAPK, pro-inflammatory cytokines and gene transcriptions (Oyenihi et al., 2015). The different pathways all seem to have a central recurring effect of oxidative stress due to diabetes. Increased ROS and RNS together with significant reductions in the antioxidant defense mechanisms within the neurons contribute to the manifestations of DNeuro which include nerve blood flow impairment, endoneural hypoxia, motor and sensory nerve conduction impairment, peripheral nerve degeneration, increased vibration and thermal perception, sensory loss, axonal atrophy of large myelinated fibers and neuropathic pain (Massaad et al., 2016).

The involvement of inflammatory processes and mechanisms in the pathogenesis of DNeuro have been extensively described (Sandireddy et al., 2014, Zhou and Zhou, 2014). The roles of pro-inflammatory mediators such as IL-1 (Liao et al., 2011, Ren et al., 2012), IL-6 (Cotter et al., 2010), TNF-α (Leung and Cahill, 2010, Yamakawa et al., 2011), INF-γ (Bour-Jordan et al., 2005), CRP (Azenabor et al., 2011) in DNeuro have been highlighted in several animal models of diabetes and diabetic patients. Also, the clinical manifestations of DNeuro are probably propagated via the activation of NF-κB and JNK molecular pathways (Donath and Shoelson, 2011, Kumar et al., 2011, Negi et al., 2011).

1.4.5 Diabetic retinopathy

Diabetic retinopathy (DR) represents the progressive development of morphological abnormalities within the retina, multicellular or light-sensitive tissue at the back of the eye of type II diabetic patients ranging from non-proliferative and proliferative diabetic retinopathy to diabetic macular oedema (DME) and vision-threatening diabetic retinopathy (VTDR) (Safi et al., 2014). DR has been shown to be the leading cause of the loss of vision and blindness among adults aged 20–74 years and has been associated with an increased probability of developing cardiovascular diseases (Lee et al., 2015, Rodriguez-Poncelas et al., 2015). The global prevalence of the disease has been estimated to be 93 million people with non-proliferative DR, 17 million with proliferative DR, 21 million with DME, and 28 million with VTDR (Yau et al., 2012). DR is mainly characterised by loss/death of mural cells, haemorrhages, basement membrane thickening, small dilations of the retinal capillaries, formation of new blood vessels (angiogenesis) within the retina and the breakdown of blood-retinal barrier (Fong et al., 2003, Chistiakov, 2011). Modifiable (such as hyperglycaemia, hypertension, dyslipidaemia, obesity) and non-modifiable (such as diabetes duration, genetic predisposition, age, and ethnicity/race) risk factors have been identified with the occurrence of DR (Scanlon, 2010, Cheung et al., 2010, Ding and Wong, 2012, Lee et al., 2015).

Chronic hyperglycaemia is a major inducer of several mechanisms involved in the pathogenesis of DR including polyol, hexosamine, PKC, and AGE pathways discussed earlier. Also, increased hypoxia, upregulation of the renin-angiotensin system and induction of VEGF have been implicated in the development of DR (El-Asrar et al., 1998, Cheung et al., 2010, Falcão et al., 2010). The activation of these pathways culminates in excess production of ROS/RNS and NF-κB -activated production of pro-inflammatory mediators that damages the microvasculature within the eyes of diabetic patients (Safi et al., 2014). Elevated levels of sorbitol resulting from an increase in the activity of polyol pathway in the retina of diabetic rats have been reported (Asnaghi et al., 2003, Obrosova et al., 2003). Similarly, accumulation of carboxymethyllysine (CML) and other AGEs (Stitt, 2001) as well as increased N-acetylglucosamine (GlcNAc) levels (Wang et al., 2012a) in the retinal blood vessels of diabetic patients have been associated with the severity of DR. Furthermore, overactivation of PARP has been reported to add at least in part to the formation of pericyte ghosts and acellular capillaries, induction of VEGF and retinal angiogenesis (Zheng et al., 2004, Drel et al., 2009). Inflammatory processes such as migration of monocytes and leukocytes; activation of ICAM1 and VCAM1; upregulation of cytokines/chemokines (IL-1β, TNF-α, MCP-1); induction of pro-

inflammatory enzymes (inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, 5-lipoxygenase); and tissue oedema are common characteristics observed in DR (Joussen et al., 2002, Falcão et al., 2010, Tang and Kern, 2011).

1.4.6 Macrovascular diabetic complications

Diabetes-related macrovascular complications include cardiovascular diseases (CVD) encompassing atherosclerosis, coronary heart diseases (CHD), myocardial infarction (MI) and stroke. Type II DM is a major risk factor for CVD with patients having 2-5 times higher chance of developing atherosclerosis (Forbes and Cooper, 2013). Greater than 70% of mortality cases in type II diabetic patients are as a result of CVD (Laakso, 2010). Type II diabetic patients have been reported to exhibit significant increased CHD morbidity and mortality rates when compared to their non-diabetic counterparts (de Grauw et al., 1995, Ali et al., 2010).

The onset of atherosclerosis is thought to be a major pathological event in the development of macrovascular complications in type II DM. The process of atheroma formation begins with the narrowing or blockage of the body's arterial walls as a result of chronic inflammation and oxidative onslaught. The oxidation of LDL particles leads to the formation of foam cells or fatty streaks that stimulate the recruitment of immune cells notably monocyte-derived macrophages and T-lymphocytes. The accumulation of T-lymphocytes has been shown to promote collagen and fibrous lesions formation. Then, the multiplication of smooth muscle cells and matrix deposition leads to the development of atherosclerotic plaques (Beckman et al., 2002, Boyle, 2007). The disruption of these plaques results in myocardial infarction (Forbes and Cooper, 2013). Excess generation of ROS/RNS and dysregulation of calcium have also been shown to stimulate platelets aggregation and hyper-coagulation that contribute to cardiovascular conditions in type II DM (Fowler, 2008).

Hyperglycaemia accelerates the process of atherosclerosis mainly by non-enzymatic glycosylation of proteins and lipids producing highly reactive AGEs and ALEs which aggregate within arterial walls. Interactions of AGE with its receptor AGE, further complicates this process (Aronson and Rayfield, 2002). In addition, hyperglycaemia-mediated inhibition of endothelial production of nitric oxide (NO) probably by decreasing tetrahydrobiopterin (BH_4) cofactor leading to excess generation of $^{\bullet}O_2^-$ has been postulated as a major pathway for the development of CHD (Quagliaro et al., 2007, Wang et al., 2012b). Diabetes-induced dyslipidaemia mainly characterised by decreased HDL-cholesterol levels, increased

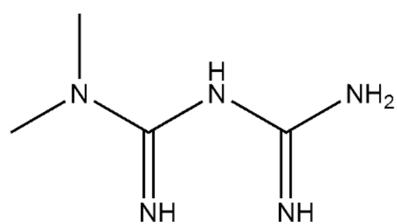
triglyceride-rich lipoprotein concentrations, and irregularities in the composition of HDL, LDL, and triglyceride-rich lipoprotein particles contribute to the development of CVD by triggering pro-inflammatory and oxidative processes that damage endothelial cells and vessel walls (Mazzone et al., 2008). Inflammation has been shown to contribute to the development of diabetic complications such as atherosclerosis via a number of mechanisms (Williams and Nadler, 2007). Increases in levels of IL-1β, interferon-γ, TNF-α and VEGF have been shown to upregulate the expression of cell surface adhesion molecules, such as VCAM-1 and ICAM-1 that are associated with the development of atherosclerosis (Min et al., 2005). Insulin resistance, a major component of type II DM, has also been linked to the development of CVD through the impairment of vasodilatations, elevation of oxidative stress and inflammation, and increased formation of thrombosis (Laakso, 2010, Roever et al., 2015).

1.5 TREATMENT AND MANAGEMENT OF TYPE II *DIABETES MELLITUS*

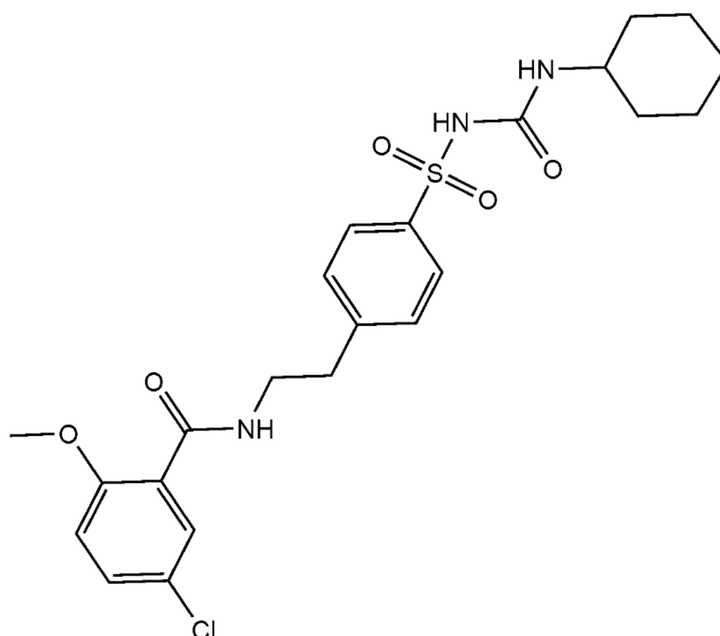
Despite recent advances in the search for potent therapeutic agents and proliferation of various classes of drugs with different modes of action in the management of type II DM, the number of individuals diagnosed each year keeps increasing unabatedly. The management of glycaemia in type II diabetic patients remains the major target of most therapies. Several anti-hyperglycaemic agents have been designed to specifically attenuate insulin deficiency, insulin resistance, oxidative stress, inflammation and other processes involved in the pathogenesis of type II DM through the inhibition of one or more enzymes in the pathway. Apart from insulin, other common oral therapeutic agents such as biguanides, thiazolidinediones, sulfonylureas, meglitinides, sodium-glucose transporter inhibitors, amylin analogues, glucagon-like peptide-1 agonists and α-glucosidase inhibitors are currently used to control glycaemia in diabetic patients although with varying degrees of efficacy (Bastaki, 2005, Inzucchi et al., 2015). The chemical structures of commonly used antidiabetic drugs are shown in Figure 1.9. However, none can be termed as an ideal one, due to their side effects and sometimes diminution in response after prolonged use (Moller, 2001, Corathers et al., 2013). A combination of two different classes of drugs with different mechanism of actions have been reported to be more effective even though it might increase the total cost of therapy and make patient compliance difficult.

Lifestyle modifications also play major role in the outcome of diabetes treatment regimen. Lack of adequate exercise or physical activity in addition to being obese or overweight represent key events in the development of type II DM (Pratley, 2013). Recent studies have revealed that consumption of healthy foods low in fat and sugar together with regular exercise mitigated symptoms of type II DM in humans and animal models.

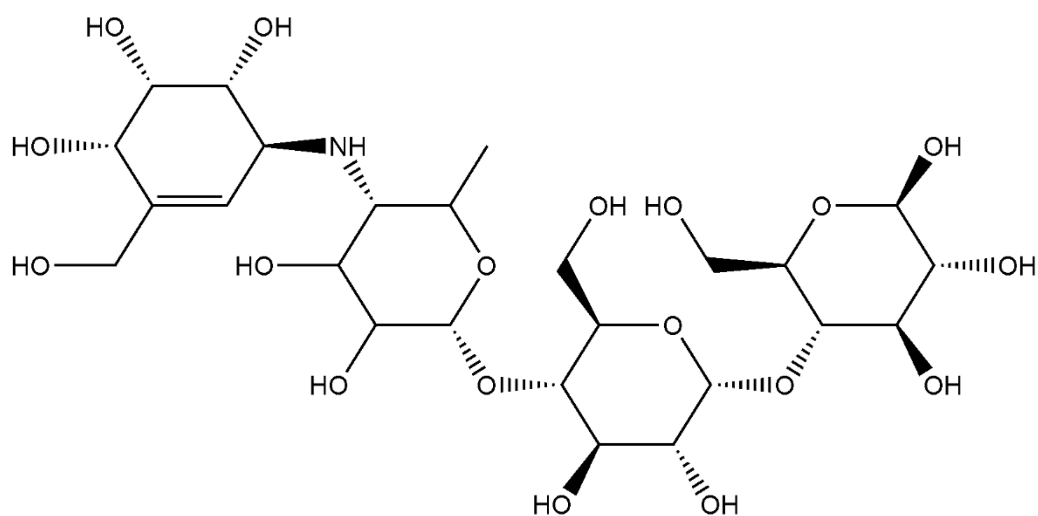
The limitations and side effects associated with existing synthetic oral hypoglycaemic agents necessitate the need to search for newer drugs. As a result, natural agents from plants and plant products have been the alternative or complementary target to source for new antioxidant and antidiabetic agents based on their use traditionally since time immemorial. The presence of polyphenolic compounds such as flavonoids, phenols, flavonols, and proanthocyanidins in plants is associated with this potential (Mariod et al., 2008). The antidiabetic effect of most plants has been attributed to their components such as flavonoids, alkaloids, glycosides and terpenoids. Some of the mechanisms of action of these herbal medicines include increasing insulin secretion, enhancement of glucose uptake by adipose or muscle tissues and inhibition of glucose absorption from intestine and glucose production from liver (Hui et al., 2009). The involvement of hyperglycaemia-mediated oxidative damage in diabetes has led to the hypothesis that drugs that improve glycaemic control and/or limit oxidative stress will be beneficial in the treatment of this disease and its associated complications.



Metformin



Glibenclamide



Acarbose

Figure 1.9: Chemical structures of commonly used antidiabetic drugs

1.5.1 Conventional orthodox medicines

Currently, there are several conventional antidiabetic drugs with different mechanisms of actions and varying degrees of efficacy. Some of these drugs have been targeted at improving insulin secretion and sensitivity or reducing gastrointestinal tract glucose absorption. Others have been designed to reduce the occurrence or deleterious effects of diabetes-induced excess oxidative stress and inflammation processes.

1.5.1.1 Therapeutics targeted at improving insulin secretion and sensitivity

Drugs have been designed to specifically improve insulin secretion, sensitivity or both and consequently lower blood glucose levels. Biguanides (such as metformin), thiazolidinediones (such as rosiglitazone) and sulphonylureas (such as glibenclamide) are the most commonly used in the management of type II DM. Metformin is the first-line oral drug approved for the treatment of type II DM (Inzucchi et al., 2012, Rena et al., 2013, Ferrannini, 2014). Although the exact mechanisms of its action are not fully elucidated, several studies have shown that metformin decreases fasting plasma glucose concentrations through activities that eventually reduce hepatic glucose production, reduce intestinal glucose absorption, improve insulin sensitivity, inhibit gluconeogenesis, induce AMP-activated protein kinase and modulate incretin actions (Natali and Ferrannini, 2006, Maida et al., 2011, Madiraju et al., 2014). Despite the low-cost and efficacy of metformin especially in reducing incidences of cardiovascular complication in type II DM, there have been concerns on its side effects. The occurrence of gastrointestinal disturbances, lactic acidosis and multiple contraindications have necessitated the search for a more efficient drug (Corathers et al., 2013, Inzucchi et al., 2015).

1.5.1.2 Therapeutics targeted at the gastrointestinal tract

Alpha-glucosidase inhibitors (AGIs) such as acarbose, have direct inhibitory properties on intestinal α-glucosidase and pancreatic α-amylase thereby allowing delayed absorption of glucose in the gastrointestinal tract and reduction in post-prandial blood glucose concentrations. AGIs also slightly reduce glycated haemoglobin levels and elicit no hypoglycaemic events. However, they have to be administered 3 times daily due to shorter half-lives making it difficult for patients' compliance. Gastrointestinal side effects are also observed (Corathers et al., 2013). GLP receptor agonists such as Exenatide have longer half-lives than GLP-1 receptors and act to stimulate glucose-dependent inhibition of glucagon in addition to reducing gastric emptying leading to decrease in postprandial blood glucose levels. Dipeptidyl peptidase (DPP)-4 inhibitors (like Sitagliptin) on the other hand, increase the

concentrations of activated GLP-1 and GIP (Drucker et al., 2010, Kahn et al., 2014). These drugs are however more expensive and may also elicit gastrointestinal disturbances. In addition, drugs whose mechanisms are incretin-based have been suggested to predispose acute pancreatitis in patients (Garg et al., 2010, Elashoff et al., 2011, Singh et al., 2013)

1.5.1.3 Therapeutics targeted against inflammation

Inflammation has been demonstrated to play a big role in the development of insulin resistance, obesity and type II DM. Therefore, it is believed that the inhibition of pro-inflammatory processes by therapeutic agents may be beneficial to diabetic patients (Goldfine et al., 2011). In animal models of diabetes, high-dose of the non-steroidal anti-inflammatory drug- salicylate has been reported to inhibit the transcription of NF-κB, thereby enhancing muscle insulin resistance and lowering the blood glucose concentration (Yuan et al., 2001). These pre-clinical results have been translated to human clinical trials. In type II diabetic obese patients, aspirin normalizes excess glucose concentrations and improve insulin sensitivity (Hundal et al., 2002). Also, salsalate, a member of salicylates, was shown to decrease the blood concentrations of glucose, triglycerides, and free fatty acids in type II diabetic or obese patients (Goldfine et al., 2011, Fleischman et al., 2008). Recently, the TINSAL-T2D (Targeting Inflammation Using Salsalate in Type II Diabetes) study indicates that salsalate given to type II diabetic patients for 14 weeks in addition to their former medications reduced glycated haemoglobin levels, fasting blood glucose and triglyceride concentrations and elevated adiponectin concentrations (Goldfine et al., 2010). Nevertheless, the small sample size of patients in the study and the short period of study are not enough to start prescribing salsalate for the treatment of type II DM. However, the encouraging success achieved in these studies has surely paved the way for novel anti-inflammatory-based strategies for the management of the disease. Furthermore, the side effects of chronic use of salicylates such as tinnitus and gastrointestinal bleeding still remain a limitation to their therapeutic use thus necessitating the search for a safer, more efficacious drug (Goldfine et al., 2011).

1.5.1.4 Therapeutics targeted against oxidative stress

The involvement of hyperglycaemia-mediated oxidative damage in type II DM has led to the hypothesis that drugs that ameliorate hyperglycaemia and/or oxidative stress will be the panacea of the disease and its associated complications (Erejuwa, 2012). Antioxidants are endogenous or exogenous substances utilized by the human defense system to counteract the effects of excess oxidative stress induced by diseases such as type II DM. Examples include

enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx) and non-enzymatic antioxidants like reduced glutathione (GSH), uric acid, carotenoids, flavonoids, lipoic acid and vitamins A, C and E. Other antioxidants include minerals (copper, zinc, manganese, and selenium), cofactors (folic acid, vitamins B1, B2, B6 and B12) and specialized proteins (such as peroxiredoxins, thioredoxins and glutaredoxins) (Oyenihi et al., 2015). These antioxidants offer protection from oxidative stress either by inhibiting the production or scavenging ROS/RNS; or boosting the natural defense systems by inducing the activities of other antioxidants (Maritim et al., 2003). Comprehensive studies have been carried out to evaluate the beneficial effects of antioxidants in insulin resistance, obesity and type II DM (Ruhe and McDonald, 2001, Rahimi et al., 2005, Evans, 2007). Results obtained from experiments using cultured vascular cells, animal models of diabetes, and diabetic patients indicate that the administration of antioxidants or antioxidant-mimetics such as vitamins A, C, D and E, alpha lipoic acid, *N*-acetyl cysteine, glutathione and others protect cells from oxidative damage and is partly responsible for the concomitant reduction in blood glucose concentrations and reduced risk of developing diabetic complications (Scott and King, 2004, Yildirim, 2009, Pazdro and Burgess, 2010, Dakhale et al., 2011, Shinde et al., 2011, Salum et al., 2013, Saraswathi and Devaraj, 2013).

Some drugs currently used in the treatment of DM have been shown to possess antioxidant activities in addition to their primary pharmacological properties. For example, aminoguanidine has been shown to exhibit free radical scavenging properties and inhibit AGEs and ALEs (El Shazly et al., 2009, Berdal and Jenssen, 2014, Sadowska-Bartosz and Bartosz, 2015) although clinical trials were discontinued in Europe and in the US due to its long term toxicity. Troglitazone was reported to reduce ROS generation, lower hydroperoxides and decrease SOD activity in type II diabetic rats (Fukui et al., 2000, Garg et al., 2000). Glibenclamide, in addition to its glucose lowering effects, possesses antioxidant properties due to its ability to restore antioxidant capacity in diabetic patients and rats (Signorini et al., 2002, Elmali et al., 2004). Also, repaglinide commonly employed in the treatment of type II DM exhibited antioxidant properties and inhibited lipid peroxidation by enhancing glutathione reductase activity and glutathione levels in diabetic mammals in addition to its insulin releasing effects (Gumieniczek, 2005, Obi et al., 2016).

1.5.2 Traditional and complementary/alternative medicines

The use of traditional medicines (TM) or complementary/alternative medicines (CAM) in the management of type II DM has been going on since time immemorial. Traditional beliefs and customs among indigenous populations of the world play tremendous roles in the day-day decision making, value principles and health management choices of individuals living in these countries. The Chinese, Ayurvedic, Native American and African traditional medicines are well-established and widely accepted non-conventional forms of healthcare delivery (Forouhar and Sack, 2012). The World Health Organization (WHO) has accorded TM its rightful place in the fight to achieve a global healthier society by encouraging the integration of individual-based, safe, rational and effective use of TMs into the national healthcare systems of countries (World Health Organization (WHO), 2013). In South Africa, significant progress has been made to integrate TM into the national health policy with the establishment of two different regulatory bodies; Traditional Healers Council (THC) for TM and Allied Health Professions Council of South Africa (AHPCSA) for CAM as well as the passing of Traditional Health Practitioners' Act of 2007 into law (Chitindingu et al., 2014). Almost 4,000 CAM practitioners are already registered with AHPCSA and over 200,000 traditional healers registered with different organizations (Gqaleni et al., 2007).

Studies have shown that about 48% of diabetic patients use CAMs and are 1.6 times more prone to use CAMs than their non-diabetic counterparts (Egede et al., 2002, Garrow and Egede, 2006). In fact, it is believed that as much as 80% of people living with DM in Africa employ the use of CAMs either as an adjuvant to conventional orthodox medicines or as a replacement therapy (Awah, 2006, Chang et al., 2007). TM or CAM includes therapies using crude plant extracts, plant-derived nutraceuticals or nutritional supplements, standardized herb concoctions or mixtures, spiritual healing acupuncture, hypnosis, functional foods and other homeopathic techniques. The choice of a particular TM to treat DM in Africa depends on traditional folklore, culture, religious beliefs, age of patient, duration of DM, and presence of complications (Coulter and Willis, 2004, Matheka and Demaio, 2013).

Plants and plant extracts/concoctions are major component of all TMs in the world. Several natural occurring plants and herbal-based products have been reported to possess anti-hyperglycaemic and antidiabetic qualities and have been used in traditional folklore to treat DM. Examples include *Centella asiatica*, *Prosposis glandulosa, Sclerocarya birrea, Momordica charantia, Artemesia afra, Aloe vera*, *Allium sativum, Camellia sinensis*, *Ocimum*

sanctum Tamarindus indica and *Tinospora cordifolia* (Dham et al., 2006, Noor et al., 2013, Oyenihi et al., 2014). The presence of phytocompounds such as alkaloids, flavonoids, phenols, flavonols, and pro-anthocyanidins in plants has been suggested to be responsible for their antioxidant and antidiabetic potentials (Mariod et al., 2008). Notable plant-derived compounds that have been used in the treatment of DM in several models and diabetic patients include allicin, quercetin, curcumin, rutin, diosmin, luteolin, lycopene, catechins, cinnamic acids and gallic acid (Forouhar and Sack, 2012, Noor et al., 2013, Oyenihi et al., 2014).

The rise in the variety and use of TM and CAMs in the management of type II DM in developing nations stems from the unavailability, high costs and side effects which have been associated with conventional, oral, anti-hyperglycaemic therapeutics. These orthodox medicines have been found to be limited by their mode of actions since they are mostly designed to target only the diabetic symptoms instead of the fundamental causes. In addition, combinations of two or more conventional medicines with different mechanisms of action which have been proven to be more efficacious are mostly administered by the health practitioners when monotherapy fails. By that time, the disease might have worsened (Brown et al., 2004, Ho et al., 2006). TMs on the other hand, usually contain several phyto-constituents that act synergistically using different mechanisms to achieve improved outcomes since the multifactorial pathogenicity of DM may require multi-dimensional therapeutic approaches (Tiwari and Rao, 2002). Although, CAMs are believed to be more affordable and exhibit lower toxicity profiles compared to conventional medicines because they are mostly derived from naturally-occurring substances, there have been several concerns on their interactions with drugs. Lack of standard reproducible dosing, inadequate scientific validation of traditional claims and unavailability of proper, research-based toxicity screening in CAMs raise further questions in the mind of most health practitioners (Ezuruike and Prieto, 2014, Chikezie and Ojiakor, 2015). To overcome these linitations, research being conducted to search, select, screen and investigate indigenous plants and plant-derived compounds using modern biomedical techniques in order to discover a potent remedy for the menace of type II DM in developing countries should continually be encouraged and adequately funded.

1.5.3 *Centella asiatica (L.) Urban*

Centella asiatica (L.) Urban (CA) is an herbaceous creeping plant (Family; Apiaceae) also known as ‘Gotu kola’, ‘Pennywort’ or ‘Brahmi’ that has been used in African, Ayurvedic and Chinese medicines to treat a variety of diseases for generations (Gohil et al., 2010). CA is an

aromatic herb with stem about 2 m long and leaves usually growing 1-3 from each node of the stem (Figure 1.10). The petioles are between 2 to 3cm long and 2 to 5 cm wide (Alfarra and Omar, 2013).

Figure 1.10: Leaves of *Centella asiatica (L.) Urban*

The antioxidant property of CA is well documented in animal models of diseases (Siddique et al., 2014, Nur-Hidayah et al., 2015). Recently, the neuroprotective action of CA against aluminum-induced oxidative damage and cognitive dysfunction in rats was reported based on its antioxidant effects (Amjad and Umesalma, 2015). CA plant is widely known for its intrinsic wound healing effects (Howes and Houghton, 2003) and memory improving qualities (Orhan, 2012). CA has also demonstrated wide spectrum of pharmacological actions such as anti-hyperglycaemic (Kabir et al., 2014), anti-proliferative (Mutua et al., 2013), anti-microbial (Norrapoke et al., 2014) anti-inflammatory, analgesic effects (Qureshi et al., 2015), cytotoxic, antitumor (Bunpo et al., 2004), and hepatoprotective actions (Sasikala et al., 2015).

CA has been reported to contain a plethora of compounds. Notable classes of important phytochemicals from CA include triterpene acids, alkaloids, glycosides, flavonoids, oils etc. The major compounds which have been isolated from CA are asiatic acid, madecassic acid, asiaticoside, madecassoside, and madasiatic acid (James and Dubery, 2011, Orhan, 2012, Maulidiani et al., 2014). The presence of a variety of compounds has been proposed to be responsible for the medicinal properties of the plant. Despite the many reported medicinal

properties of CA, there is paucity of pertinent information in scientific literature on the effects of the plant on oxidative stress, antioxidant capacity, inflammation, and insulin downstream signaling mechanisms related specifically to type II DM.

1.6 AIMS AND OBJECTIVES

The specific aim of this research is to investigate the antidiabetic properties of *Centella asiatica (L.) Urban* leaves in a type II diabetic rat model. The present study aims to ascertain whether the methanol extract of *Centella asiatica (L.) Urban* could reduce diabetes-induced oxidative stress and ameliorate diabetes-mediated changes in inflammatory processes, glucose metabolism and insulin signaling mechanisms in type II diabetic Sprague-Dawley rats. These effects may lead to the protection of organs like liver, kidney, brain, pancreas and muscle from diabetes-related organ damage. To achieve this aim, methanol extract of the plant was administered to fructose:streptozotocin induced diabetic rats for 90 minutes at 30 minutes interval in the acute study and daily for period of 14 days in the sub-chronic study. The goal is to scientifically validate the traditional use of the plant in the management of type II DM and also possibly isolate and characterize new compounds from *Centella asiatica (L.) Urban* that may serve as basis for the development of new types of therapeutics.

The step-by-step objectives are as follows:

1. To prepare methanol extract from the leaves of the medicinal plant.
2. To induce a type II DM model in adult male Sprague-Dawley rats using 10% fructose in drinking water followed by a single intraperitoneal administration of streptozotocin (40mg/kg body weight).
3. Assess the effects of extract on hyperglycaemia by monitoring blood glucose levels in diabetic rats after oral administration of extract.
4. Investigate the effects of the extract on serum lipid parameters; insulin signaling; glycogen metabolism; oxidant/antioxidant status and inflammatory cytokines in diabetic rats.
5. Characterize the crude methanol extract using Gas Chromatography-Mass Spectroscopy.

CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The following chemicals used were of analytical grade and purchased from Sigma-Aldrich through Capital Labs, New Germany, South Africa: 1-chloro-2, 4-dinitrobenzene (CDNB); 2-(N-morpholino) ethanesulfonic acid (MES); 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH); 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ); 3-(N-morpholino) propanesulfonic acid (MOPS); 5′, 5′-Dithiobis- (2-nitrobenzoate) (DTNB); 6-hydroxy-2,5,7,8-tetra-methylchroman-2 carboxylic acid (Trolox); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS); acrylamide; ammonium persulphate (APS); benzamidine hydrochloride hydrate; bovine serum albumin (BSA); bromophenol blue; butanol; Coomasie blue; dipotassium hydrogen orthophosphate (K_2HPO_4); dithiothreitol (DTT); ethanol; ethylene diaminetetraacetic acid (EDTA); fluorescein sodium salt; Folin-Ciocalteu reagent; glacial acetic acid; glucose-6-phosphate disodium salt; glycerol; glycine; glycogen; L-ascorbic acid; leupeptin; metformin; methanol; N, N'-bismethyleneacrylamide; Nonidet P-40; phenol; phenylmethanesulfonyl fluoride (PMSF); potassium chloride (KCl); potassium dihydrogen phosphate (KH_2PO_4); reduced glutathione (GSH); sodium acetate; sodium azide (NaN_3); sodium chloride (NaCl); sodium fluoride (NaF); sodium orthovanadate (Na_3VO_4); sodium pyrophosphate ($Na_4P_2O_7$); streptozotocin (STZ); thiobarbituric acid (TBA); Triton-X 100; Tween-20; uridine diphosphate glucose (UDPG); α-D-Glucose 1-phosphate disodium salt hydrate and β-mercaptoethanol.

The chemicals procured from Merck, Modderfontein, South Africa include: acetone; citric acid; copper sulphate ($CuSO_4.H_2O$); disodium hydrogen phosphate (Na_2HPO_4); hexane; hydrochloric acid (HCl); perchloric acid (PCA); potassium hydroxide (KOH); potassium iodide (KI); potassium peroxodisulphate ($K_2S_2O_8$); potassium sodium tartrate ($KNaC_4H_4O_4.H_2O$); sodium carbonate; sodium citrate; sodium sulfate (Na_2SO_4); sulfuric acid; sulphursalicyclic acid ($C_7H_6S.2H_2O$); trichloroacetic acid (CCl_3COOH). Tetramethylethylenediamine (TEMED) was obtained from Fluka Chemie, Buchs, Switzerland. Sodium hydroxide (NaOH) and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were products of Wako Pure Chemical Industry, Osaka, Japan and Saarchem, Gauteng, South Africa respectively. Primary antibodies including Rabbit anti-phospho-Akt (p-Akt), Rabbit anti-

glycogen synthase (GS) and Rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Cell Signaling Technology, Danvers, MA, USA through Laboratory Specialist Services, Cape Town, South Africa while Rabbit anti-glycogen phosphorylase (GP) was purchased from Abcam, Cambridge, UK through Biocom Biotech, Centurion, South Africa. The secondary antibodies, chemiluminesence Immuno-Star Goat anti-Rabbit IgG alkaline phosphatase conjugate system and Goat anti-Rabbit IgG alkaline phosphatase conjugate; Tris; 10X Tris/Glycine/SDS buffer and polyvinylidene fluoride (PVDF) were purchased from Bio-Rad, Johannesburg, South Africa. The radionuclides, [U-^{14}C]-UDPG and α-D-[U-^{14}C]-glucose-1-phosphate were purchased from BioTrend Chemikalien GmbH, Koln, Germany.

2.1.2 Plant material

Fresh leaves of *Centella asiatica (L.) Urban* (CA) were collected within the lush vegetation around the Sports Centre premises in Westville Campus of University of KwaZulu-Natal, South Africa and immediately authenticated by Prof. A. Nicholas of the Discipline of Biological Sciences in the same University. A voucher specimen (Dladla 02) was deposited in the Ward Herbarium of the University of KwaZulu-Natal, Westville Campus.

2.1.3 Animals and ethical statement

Upon approval from the University of KwaZulu-Natal Animal Ethics Committee (References 120/13/Animal and 024/15/Animal), 70 male Sprague-Dawley rats (150-180 g) were procured from the Biomedical Resource Unit (BRU) at Westville campus of the University of KwaZulu-Natal, South Africa. The animals were housed two rats per cage in a temperature and humidity controlled room (23 ± 1°C, 40–60% humidity) with a set 12 h light-dark cycle and fed with commercially available rat chow diet (Meadows, Pietermaritzburg, South Africa) and drinking water *ad libitum* throughout the experimental period. All animals received humane care according to the principles of Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publication no. 80-23, revised 1978).

2.2 METHODS

2.2.1 Preparation of plant extract

Leaves of *Centella asiatica (L.) Urban* were dried at room temperature and made into powder using an electric grinder. Approximately 100 g of the powder was subsequently extracted with 1 L of absolute methanol by continuous stirring for 72 h. Thereafter, the mixture was first filtered using a clean muslin cloth then Whatman filter paper (Number 2). The filtrate was subsequently concentrated to dryness in a water bath. This gave a crude extract yield of 19% dry weight which was stored at -20°C until needed. Figure 2.1 illustrates the step-by-step process of crude extract preparation.

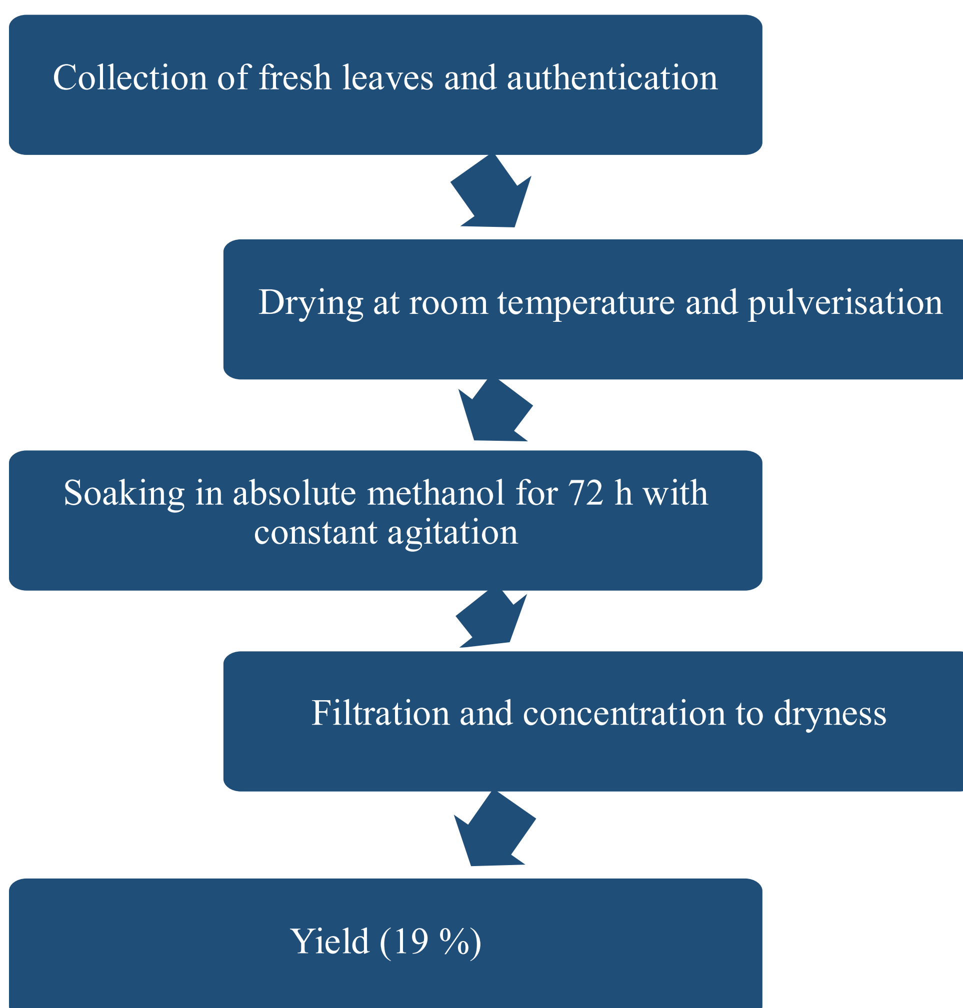


Figure 2.1: Scheme for the preparation of crude extract of *Centella asiatica (L.) Urban*

2.2.2 Induction of Type II *diabetes mellitus*

After 7 days of acclimatization, type II DM was induced in rats by having them exposed to 10% fructose solution *ad libitum* for 14 days followed by a single intraperitoneal (*ip*) injection of low dose streptozotocin (STZ, 40 mg/kg b.wt) in freshly prepared 0.1M citrate buffer (pH 4.5) after an overnight fast as shown in figure 2.2. This animal model has been reported to induce type II DM symptoms that closely resemble human subjects because it causes delayed insulin sensitivity as well as partially destroying the pancreatic β-cells population (Wilson and Islam, 2012). Blood was obtained by once-off tail prick and glucose levels determined using Accu-Chek Glucometer (Roche, Germany) a week after STZ injection. Animals having fasting blood glucose values within 7mM to 18 mM at day 21 that is 7 days after the STZ injection were considered as having stable diabetes. However, animals used for the study had glucose values of 12-18 mM to avoid those animals that might revert to normal glucose status. Normal rats were given drinking water *ad libitum* for 14 days prior to a single *ip* injection of freshly prepared citrate buffer to serve as experimental controls.

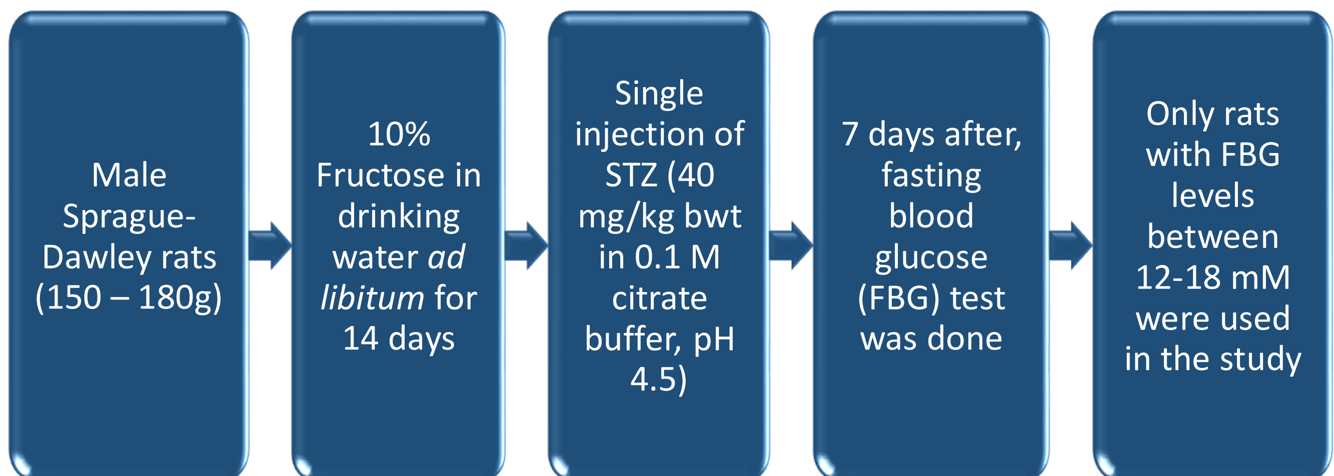


Figure 2.2: Scheme for the induction of type II *diabetes mellitus* in rats. STZ – streptozotocin; bwt – body weight.

2.2.3 Experimental design

2.2.3.1 Acute study

For the time-dependent acute study, 30 rats were divided randomly into 5 groups of 6 rats each as follows:

NC: Normal control rats orally treated distilled water

DC: Diabetic control rats orally treated with distilled water

D500: Diabetic rats orally treated with CA extract (500 mg/kg b.wt) dissolved in distilled water

D1000: Diabetic rats orally treated with CA extract (1000 mg/kg b.wt) dissolved in distilled water;

DME: Diabetic rats orally treated with the standard antidiabetic drug, metformin (300 mg/kg b.wt) dissolved in distilled water.

Two (2) rats from each group were treated at 30 minutes interval and euthanized 30, 60, and 90 minutes after drug and extract administration through overdose of gas inhalant with Isofor (Safeline Pharmaceuticals, Roodepoort, South Africa) in the anaesthetic chamber. The skeletal muscle from the hindquarter of each rat was carefully removed, rinsed in physiological saline, blotted dry and weighed before it was put in appropriately labelled sterile sample container and snap-frozen in liquid nitrogen. The samples were then kept in -20ºC until use.

2.2.3.2 Sub-chronic study

For the sub-chronic study, 40 rats were divided into 5 groups of 8 rats each as illustrated in Figure 2.3. All treatment regimens started 8 days after injection with STZ and continued daily for 14 days. NC and DC rats were orally treated with distilled water which was the vehicle for dissolving the CA extract and metformin.

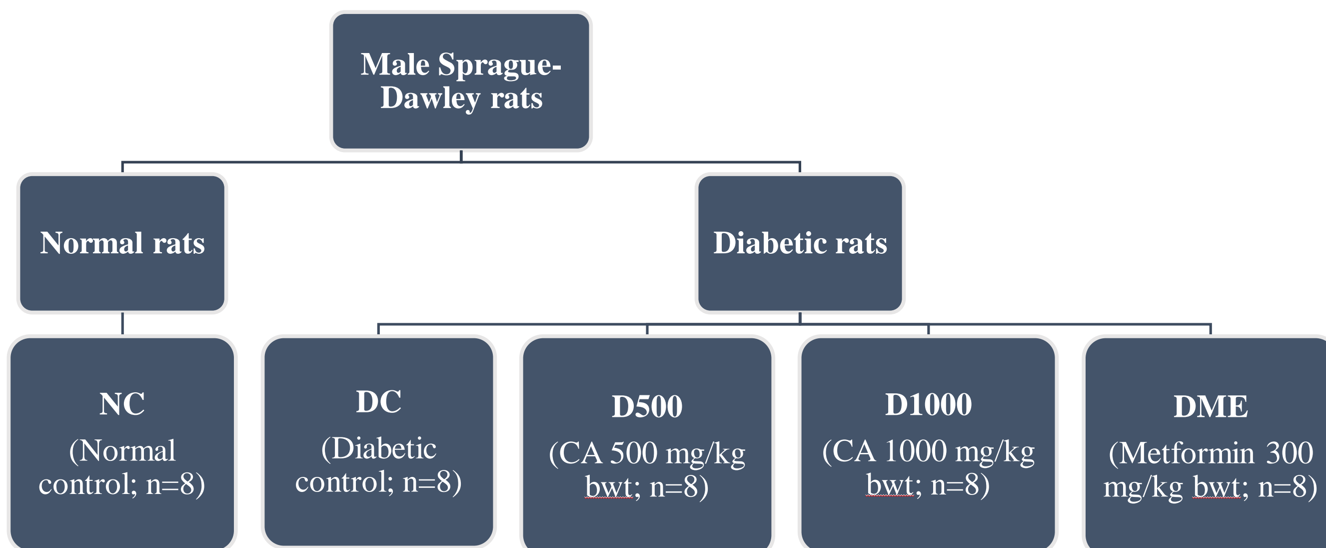


Figure 2.3: Scheme for animal grouping and design for sub-chronic study. CA – *Centella asiatica* extract; bwt – body weight.

After the treatment period, all rats were fasted overnight and euthanized through overdose of gas inhalant with Isofor (Safeline Pharmaceuticals, Roodepoort, South Africa) in the anaesthetic chamber followed by immediate cardiac puncture for final blood collection into uncoated tubes. The blood sample from each animal was allowed to stand for about 1 h and then centrifuged at 4,000 x *g* for 10 mins using a Heraeus Labofuge 200 Centrifuge (Thermo Scientific, MA, USA) to separate the serum. Whole liver, kidney, brain and hind-quarter skeletal muscle from each rat were excised, rinsed in physiological saline, blotted dry, weighed, put in appropriately labelled sterile sample containers and snap-frozen in liquid nitrogen. Both serum and organ samples were subsequently stored at -20°C until needed. Pancreatic samples from all animal groups were immediately fixed in 10% formalin for histopathological examination.

2.2.4 Determination of malondialdehyde (MDA) - a product of lipid peroxidation

The tissue samples (liver, kidney and brain) were homogenized (1:5) in 0.05 M Tris–HCl buffer (pH 7.4) containing 1.15% KCl using a Glas-Col tissue homogenizer (Glas-Col, Indiana, USA)

and centrifuged at 10,000 x *g* for 15 mins at 4ºC using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA).

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) formed in the tissue samples according to the method described by Varshney and Kale (Varshney and Kale, 1990) with slight modifications. This method is based on the reaction between 2-thiobarbituric acid (TBA) and MDA (Figure 2.4). On heating in acidic pH, the coloured complex formed absorbs maximally at 532 nm. The supernatant (200 µl) was mixed with 1.8 ml of Tris-HCl buffer before 500 µl of 30% trichloroacetic acid (TCA) was added. Then, 500 µl of 0.75% thiobarbituric acid (TBA) was added followed by heating the mixture in a water bath for 45 mins at 80ºC. After cooling in ice, the mixture was centrifuged at 4,000 rpm for 5 mins using a Heraeus Labofuge 200 Centrifuge (Thermo Scientific, MA, USA). Absorbance of the resulting supernatant was read at 532 nm in a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan) against reference blank containing distilled water. Lipid peroxidation in µmol MDA formed per mg protein was calculated using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

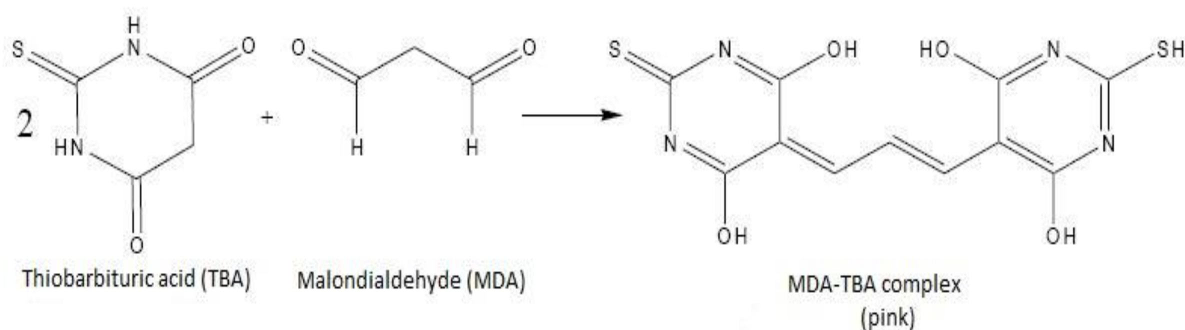


Figure 2.4: Reaction principle of lipid peroxidation assay

2.2.5 Evaluation of antioxidant status

Tissue samples (liver, kidney and brain) were homogenized (1:10) in 10 mM phosphate buffered saline (PBS, pH 7.2) using a Glas-Col tissue homogenizer (Glas-Col, Indiana, USA) and centrifuged at 15,000 x *g* for 15 mins at 4ºC using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The supernatants obtained were purified further by repeating the centrifugation process before being de-proteinized by the addition of 5% perchloric acid

(PCA). The resulting mixture were thereafter centrifuged at 20,000 rpm for 1 min using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The resulting supernatants were used for the determination of ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and trolox equivalent absorbance capacity (TEAC) values.

For the determination of reduced glutathione (GSH) concentration, glutathione S-transferase (GST) and glutathione peroxidase (GPx) activities, tissue samples (liver, kidney and brain) were homogenized (1:5) in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% KCl using a Glas-Col tissue homogenizer (Glas-Col, Indiana, USA). The homogenates were centrifuged at 10,000 x *g* for 15 mins at 4ºC using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA) to obtain the supernatants that were used for the assays.

2.2.5.1 Determination of FRAP

The FRAP assay was performed using a modified method of Benzie and Strain (Benzie and Strain, 1996) that utilizes the electron-donating antioxidants reduction of ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous (Fe^{2+}) form at low pH, monitored by measuring the change in absorption at 593 nm in a Multiskan Spectrum plate reader (ThermoFischer Scientific, MA, USA). Tissue samples (10 µl) were added into each designated well in a clear 96-microwell plate and 300 µl of the FRAP reagent (containing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3$) was added. The mixture was incubated in an oven at 37ºC for 30 mins following which absorbance was read at 593 nm. Ascorbic acid (0-1000 µM range) was used as a standard. The FRAP values of samples were then extrapolated from the standard graph and expressed as µmole /g tissue.

2.2.5.2 Determination of ORAC

The ORAC value of tissue samples was determined by slightly modifying the method of Prior and colleagues (Prior et al., 2003). This method is based on the observation that the fluorescence of a fluorescein salt (or β-phycoerythrin) changes with respect to time upon damage caused by peroxyl or hydroxyl radical attack. These changes are then followed in a fluorescence spectrophotometer until zero fluorescence is obtained. Briefly, 12 µl of tissue samples was mixed with 138 µl of 1 mM fluorescein solution in respective wells in a 96-microwell plate. A 50 µl sample of 25 mg/ml AAPH (2,2'-Azobis (2-methylpropionamidine) dihydrochloride) in phosphate buffer (75mM, pH 7.4) was then added and the plate was

immediately inserted into the Fluoroskan Ascent Spectrophotometer (ThermoFischer Scientific, MA, USA) with excitation wavelength set at 485 nm and the emission wavelength set at 530 nm. A Trolox standard curve was prepared within 0-25 µM range. The ORAC value was calculated by dividing the area under the sample curve by the area under the Trolox curve with both areas being corrected by subtracting the area under the blank curve. One ORAC unit was assigned as being the net protection area provided by 1 µM Trolox in final concentration. ORAC values are expressed as µmole trolox equivalents /g tissue.

2.2.5.3 Determination of TEAC

The TEAC assay measures antioxidants ability to scavenge ABTS (2,2'-azino-di-3-ethylbenzthialozine sulphonate) radical cation and was performed as described by Miller and others (Miller et al., 1993). To 10 µl of tissue sample, 250 µl of TEAC reagent (containing 7 mM ABTS and 140 mM potassium persulfate mixture diluted 1:20 with ethanol) was added to each well of a 96-microwell plate. The plate was then incubated at room temperature for 30 mins before reading absorbance at 732 nm in a Multiskan Spectrum plate reader (ThermoFischer Scientific, MA, USA). TEAC values are expressed as µmol trolox equivalents /g tissue.

2.2.5.4 Determination of GSH concentration

GSH concentration in tissue samples was measured according to the method described by Jollow and others (Jollow et al., 1974). Since GSH comprises in most instances the bulk of cellular non-protein sulfhydryl groups, the principle of this method is therefore based upon the development of a relatively stable chromophoric product when 5′, 5′–dithiobis-(2-nitrobenzoic acid) (DTNB) is added to sulfhydryl compounds as shown in figure 2.5. In brief, tissue sample was de-proteinized by the addition of an equal volume of 4% sulfosalicyclic acid. The mixture was then centrifuged at 3,000 x *g* for 10 mins using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of 10 mM DTNB dissolved in 0.1 M phosphate buffer (pH 7.4). Absorbance of sample tubes was read at 412 nm using a Varian Cary 50 UV/Vis Spectrophotometer, CA, USA. A GSH standard curve was prepared within 0-200 µM range. GSH concentration in tissue samples was then extrapolated from the GSH standard graph and expressed as µmol /g tissue.

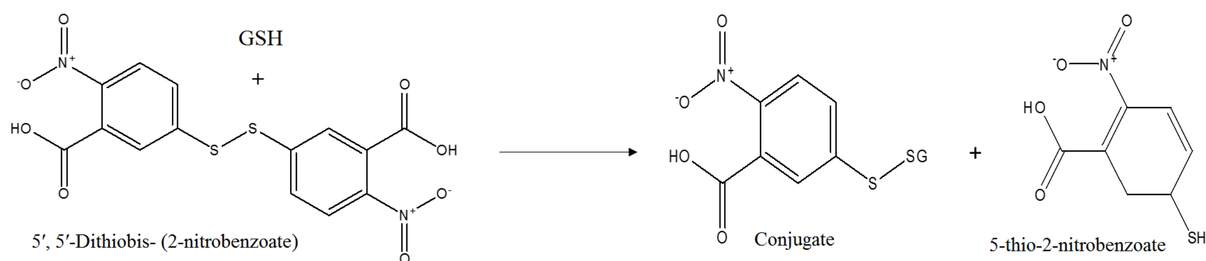


Figure 2.5: Reaction principle of GSH assay

2.2.5.5 Assay of GST activity

GST activity in tissue samples was measured by the method described by Habig et al. (1974). The principle is based on the conjugation reaction of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB) that is rapidly catalyzed by GST. When CDNB is used as a substrate, GSH becomes conjugated to CDNB shifting the absorption maximum to a longer wavelength. The increase in absorption at the new wavelength of 340 nm provides a direct measurement of the enzymatic reaction catalyzed by GST. The reaction mixture was prepared by adding 30 µl of 0.1 M GSH and 150 µl of 20 mM CDNB to 2.79 ml of 0.1 M phosphate buffer (pH 7.4). After addition of 30 µl tissue sample to the mixture, the reaction was allowed to run for 3 mins with absorbance read at 340 nm every 60 s interval in a Varian Cary 50 UV/Vis Spectrophotometer, CA, USA. GST activity in µmol GSH–DNB complex formed per minute per milligram protein was calculated using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

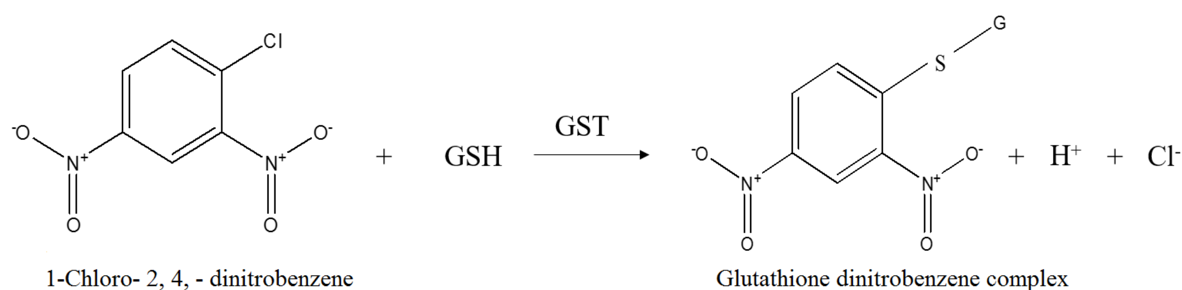


Figure 2.6: Reaction principle of GST activity assay

2.2.5.6 Assay of GPx activity

GPx activity in tissue samples was determined by the method of Rotruck et al. (1973) with slight modifications. Tissue sample was allowed to react with H_2O_2 in the presence of GSH for a specific time period according to the equation below. The GSH concentration remaining after the reaction was then measured.

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

A reaction mixture was prepared containing 500 µl of 0.3 M phosphate buffer (pH 7.4), 100 µl of 10 mM sodium azide and 500 µl of tissue sample. 200 µl of 4 mM GSH and 100 µl of 2.5 mM H_2O_2 were added to the mixture and incubated at 37°C for 5 mins. The reaction was stopped by the addition of 500 µl of 10% TCA and the samples were centrifuged at 4,000 rpm for 5 mins using a Heraeus Labofuge 200 Centrifuge (Thermo Scientific, MA, USA). The supernatants obtained were assayed for remaining GSH concentration by mixing 1 ml of the supernatant with 2 ml 0.3 M phosphate buffer (pH 7.4) and 1 ml of 10 mM DTNB. Absorbance of sample tubes were then read at 412 nm using a Varian Cary 50 UV/Vis Spectrophotometer, CA, USA. The activity of GPx in tissue samples was expressed as µmol of GSH utilized per mg protein.

2.2.6 Estimation of the concentration of pro-inflammatory and anti- inflammatory cytokines

Tissue samples (liver, kidney and brain) were homogenized (1:10) in 10 mM phosphate buffered saline (PBS, pH 7.2) using a Glas-Col tissue homogenizer (Glas-Col, Indiana, USA) and centrifuged at 15,000 x *g* for 15 mins at 4ºC using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The supernatant obtained was purified further by repeating the centrifugation process. The concentrations of IL- 1α, IL-1β, IL-4, IL-6, IL-10, IFN-γ, MCP-1, MIP-1α and TNF-α in tissue samples were analysed using a Bio-plex Pro-magnetic bead-based Luminex kit (Bio-Rad Laboratories, California, USA) on the Bio-plex platform. Briefly, each undiluted supernatant was reacted with a mixture of internally dyed magnetic beads bound with specific anti-cytokine primary antibodies. Each specific cytokine binds to the bead with its corresponding antibody forming a complex that in turn binds to biotinylated anti-cytokine secondary antibody. The addition of fluorescent phycoerythrin-conjugated streptavidin allows

visualization and subsequent bead acquisition and analysis by Bio-Plex Manager software version 6.1. The concentrations of cytokines in tissue samples were expressed as pg/ g tissue.

2.2.7 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Muscle tissue samples were homogenized (1:5) in a 20 mM Tris-HCl (pH 7.5) buffer containing 5 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 2 mM sodium vanadate, 1% Nonidet P-40, 7 µmol/L leupeptin, 3 mmol/L benzamidine, and 1 mmol/L phenylmethylsulphonyl fluoride using an OMNI TH2 tissue homogenizer (Georgia, USA) at a speed of 30-35,000 rpm for 1-2 mins and then centrifuged at 14,000 x *g* for 20 mins at 4 ºC using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The supernatants were then used for SDS-PAGE and Western blot analysis.

SDS-PAGE was performed according to the method of Laemmli (1970) using electrophoresis systems from Vacutec, Johannesburg, South Africa. A 15% stacking gel (10 ml) was prepared as follows; 5 ml of 30% acrylamide/Bis, 2.5 ml of 0.5 M Tris (pH 6.8), 100 µl of 10% SDS, 2.4 ml distilled water, 50 µl of 10% APS and 10 µl of 100% TEMED. The resolving gel was also prepared in the same manner using 1.5 M Tris (pH 8.8) and 5 µl of 100% TEMED instead of the concentration and volume for the stacking gel respectively. Tissue samples were prepared 1:1 in sample buffer containing 0.5 M Tris (pH 6.8), glycerol, 10% SDS, 0.5% bromophenol blue and β-mercaptoethanol. The sample mixture was vortexed, heated at 95 ºC for 4 mins and cooled. Equal concentration of denatured protein samples was loaded into individual wells of BG-VerMINI Marine Electrophoresis cell (Vacutec, Johannesburg, South Africa) and electrophoresed in Tris/Glycine/SDS running buffer, pH 8.3 at 110 V. After complete protein separation on the gels, they were immediately transferred to PVDF membranes in a Tris/glycine/methanol transfer buffer (pH 8.3), using a Trans-Blot Electrophoretic Transfer Cell (Vacutec, Johannesburg, South Africa) run at 100 V for 2 hr. The membranes were then blocked with the blocking buffer (tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20 and 3% non-fat dried milk), at 4°C overnight. After blocking, membranes were incubated in the blocking buffer for 1 hr at room temperature with anti-p-Akt, anti-GS, anti-GP and anti-GAPDH. All the primary antibodies were diluted 1:1500 with the blocking buffer. Membranes were then probed further with the secondary antibody, Bio-Rad chemiluminesence Immuno-Star Goat anti-Rabbit IgG Alkaline phosphatase conjugate system

or Goat anti-Rabbit IgG Alkaline phosphatase conjugate diluted 1:1500 in blocking buffer for 1 hr at room temperature. Subsequently, protein bands were visualized using a Syngene (Cambridge, UK) G-BOX Chem XR5 (supplied by Vacutec, Johannesburg, South Africa). Images were captured using Syngene GeneSys software and bands quantified by Syngene GeneTools analysis software.

2.2.8 Determination of glycogen synthase (GS) activity

GS activity was determined by the radiochemical assay adapted from the method described by Mandarino and others (Mandarino et al., 1987). Tissue samples were homogenized (1:5) in a 50 mM phosphate buffer (pH 7.4) containing 20 mM EDTA, 2 mM DTT, and 20 mM NaF using an OMNI TH2 tissue homogenizer (Georgia, USA) at a speed of 30-35,000 rpm for 1-2 mins. The homogenates were immediately centrifuged at 20,000 x *g* for 20 mins at 4°C using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). The reaction mixture consisted of 50 mM Tris-HCl (pH 7.8), 20 mM EDTA, 25 mM NaF, 1% glycogen, 10 mM glucose-6-phosphate (G-6-P), 5 mM uridine diphosphate glucose (UDPG) and 1 µCi/ml [U-^{14}C]-UDPG. The reaction was started by the addition of 50 µL of tissue sample (diluted 1:5 in buffer containing 50 mM Tris-HCl (pH 7.8), 20 mM EDTA and 25 mM NaF) to 50 µL of reaction mixture and incubating for 20 min at 30 °C. The reaction was stopped when 50 µL of the incubating mixture was added onto a 2 x 2 cm sterile filter paper which was immediately immersed in 70% ethanol in order to precipitate glycogen. Then, the filter paper was washed for 30 mins twice in 70% ethanol, dried and inserted into scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C was determined using a Packard Tri-Carb Liquid Scintillation Counter 1900 TR (PerkinElmer, MA, USA). For each set of assays, 3 sample blanks were prepared using distilled water to determine background counts, which were subtracted from the tissue samples counts. GS activity was expressed as nmoles of UDPG converted to glycogen per min per mg protein.

2.2.9 Determination of glycogen phosphorylase (GP) activity

The GP activity radiochemical assay was slightly modified from the method described by Taylor et al. (2006). Tissue samples were homogenized 1:10 in a 10 mM MOPS buffer (pH 7.0) containing 5 mM EDTA, 1 mM DTT, and 50 mM NaF using an OMNI TH2 tissue homogenizer (Georgia, USA) at a speed of 30-35,000 rpm for 1-2 mins. The homogenates were immediately centrifuged at 9,000 x *g* for 10 mins at 4°C using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA). GP activity in tissue samples was assayed in the reverse

direction by the incorporation of radiolabelled D-glucose-1-phosphate (G-1-P) into glycogen. The reaction mixture consisted of 33 mM MES, 0.34% glycogen, 22.3 mM G-1-P and 1 µCi/ml D-[$U^{14}C$]-G-1-P. The reaction was started by the addition of 50 µL of tissue sample to 50 µL of reaction mixture and incubating for 20 min at 30 °C. The reaction was stopped when 50 µL of the incubating mixture was added onto a 2 x 2 cm sterile filter paper which was immediately immersed in 70% ethanol in order to precipitate glycogen. Then, the filter paper was washed for 30 mins twice in 70% ethanol, dried and inserted into scintillation vials containing 3 ml FLUKA scintillation cocktail and the amount of ^{14}C determined using a Packard Tri-Carb Liquid Scintillation Counter 1900 TR (PerkinElmer, MA, USA). For each set of assays, 3 sample blanks were prepared using distilled water to determine background counts, which were subtracted from the tissue samples counts. GP activity was expressed as nmoles of G-1-P converted to glycogen per min per mg protein.

2.2.10 Determination of glycogen concentration

The glycogen concentration in tissue samples (liver and muscle) were quantified by the method of Nader and Esser (2001) with slight modifications. In brief, tissue samples (100-500 mg) were digested by boiling for 30 min in 1 ml of 30% potassium hydroxide (KOH) saturated with sodium sulfate ($NaSO_4$). Four (4) ml of 95% ice-cold ethanol was then added to the mixture and placed on ice for 30 mins to precipitate glycogen. The samples were then centrifuged at 4ºC for 30 mins at 20,000 x *g* using an Avanti J26-XPI centrifuge (Beckman Coulter, California, USA) and the pellets re-suspended in 1 ml distilled water, followed by the addition of 1 ml of 5% phenol. A colorimetric reaction was started by the addition of 5 ml concentrated sulfuric acid followed by incubation on ice for 30 mins. Absorbance was measured at 490 nm using a UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). A standard glycogen curve was prepared within the range 0-100 µg/ml and the concentration of glycogen in samples were extrapolated from the standard curve. Glycogen concentrations are expressed as mg/ g tissue.

2.2.11 Determination of protein concentration

The protein concentration in tissues was determined by the Biuret method as described by Gornall and colleagues (Gornall et al., 1949) with some modifications. Potassium iodide (KI) was added to the reagent to prevent precipitation of Cu^{2+} as cuprous oxide. The principle is based on the formation of a coloured complex that can be measured spectrophotometrically when proteins react with Cu^{2+} in an alkaline solution. 3 ml of the Biuret reagent (containing 3

g $CuSO_4.H_2O$, 9 g $KNaC_4H_4O_4.H_2O$, 5 g KI dissolved in 1 L 0.2 M NaOH) was added to 2 ml of each sample and the solution was vortexed and then incubated for 10 mins at 37^0C in a water bath. Upon cooling, the absorbance of sample tubes was read at 540 nm using a Varian Cary 50 UV/Vis Spectrophotometer, CA, USA. A standard BSA curve in the range of 0-10 mg/ml was prepared and the protein concentration in each tissue sample was calculated from the curve. Protein concentrations are expressed as mg/ml.

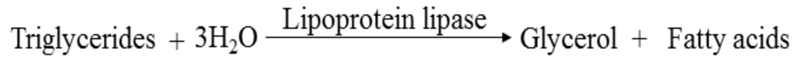
The Lowry method (Lowry et al., 1951) of determining protein concentration was also used because it has a wider sensitivity range. Briefly, fresh alkaline reagent was prepared by mixing alkaline sodium carbonate solution (containing 20 g/L Na_2CO_3 in 0.1 M NaOH) with copper sulphate-sodium potassium tartrate solution (containing 5 g/L $CuSO_4.5H_2O$ in 10 g/L $KNaC_4H_4O_4.H_2O$) in the ratio 50:1. Subsequently, 5 ml of alkaline reagent was added to 0.5 ml of tissue samples and incubated at 40 °C for 15 mins in a water bath. After cooling, 0.5 ml of diluted Folin-Ciocalteu reagent (1:1 with distilled water) was added to each sample then vortexed and incubated at room temperature for 30 mins. Absorbance of sample tubes were read at 600 nm using a Varian Cary 50 UV/Vis Spectrophotometer, CA, USA. A standard BSA curve in the range of 20 - 100 μg range was prepared. The protein concentration of each tissue sample was calculated after extrapolation from the standard curve and expressed as mg/ml.

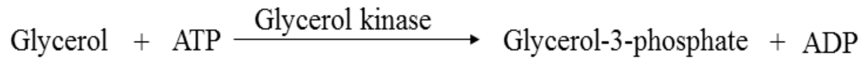
2.2.12 Analysis of serum lipids

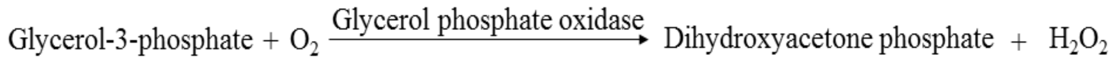
Triglycerides (TG), total cholesterol (Tc) and high-density lipoprotein cholesterol (HDL-c) were analysed by an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) using commercial assay kits obtained from the same company and following manufacturer's instructions for sample preparations. Serum lipid concentrations are expressed as mg/dl.

2.2.12.1 Determination of concentration of TG

The principle of the TG assay is based on a series of enzymatic coupled reactions initiated by the hydrolysis of triglycerides to glycerol (Figure 2.7). The glycerol formed is then oxidized to give hydrogen peroxide as one of the products, which undergoes a peroxidase-catalyzed reaction to produce a coloured product that can be measured quantitatively at 500 nm. The intensity of the colour generated is directly proportional to the concentration of triglycerides present in serum samples.







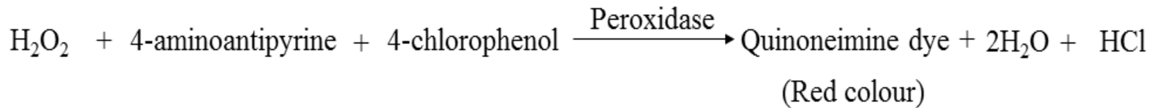
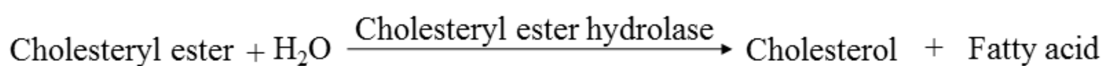
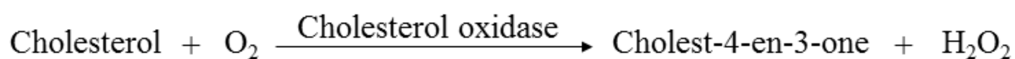


Figure 2.7: Reaction sequence for TG assay

2.2.12.2 Determination of concentration of Tc

The concentration of Tc in serum is measured through a sequence of enzymatic reactions as shown in Figure 2.8. The hydrolysis of cholesteryl ester and oxidation of the 3-OH group of cholesterol yield hydrogen peroxide as a by-product which undergoes a peroxidase-catalyzed reaction to produce a coloured product that is measured quantitatively as described above for TG assay.





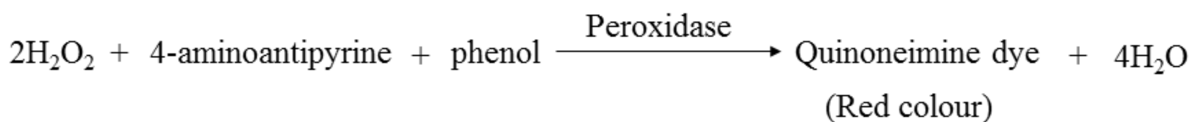


Figure 2.8: Reaction sequence for Tc assay

2.2.12.3 Determination of concentrations of HDL-c and LDL-c

The HDL-c assay principle is based on the precipitating action of polyethylene glycol (PEG) which when added to serum samples, separate HDL from all other lipoproteins. The supernatant obtained after centrifuging the mixture at 2,000 x *g* for 20 mins using a Heraeus Labofuge 200 Centrifuge (Thermo Scientific, MA, USA) was then used for the Tc assay as described previously. LDL-c was calculated from Tc, HDL-c and TG levels using the Friedewald's formula (Friedewald et al., 1972) shown below.

$$\text{LDL-c} = (\text{Tc}) - (\text{HDL-c}) - (\text{TG}/5)$$

2.2.13 Histopathological studies

The standard histology laboratory procedure for tissue processing was followed for the histopathological examination of pancreas. Briefly, pancreatic samples were dehydrated in a stepwise manner in increasing concentrations of 50-100 % ethanol and finally xylene before being embedded in molten wax using HistoStar Embedding Workstation (Thermo Scientific, MA, USA). Later, sections (4 µm) were cut using a microtome, placed on glass slides, dried, de-waxed in xylene and rehydrated in decreasing concentrations of ethanol (100%, 80 %, 70 %, and 50 %) then washed in distilled water. Thereafter, slides were stained in haematoxylin, rinsed with water followed by counterstaining with eosin. After drying, slides were mounted in DPX, cover-slipped and scanned using Leica SCN400F (Leica Microsystems, Wetzlar, Germany).

2.2.14 Gas chromatography-mass spectroscopy (GC-MS) analysis

GC-MS analysis of crude extract of CA was performed using GC–MS 2010 QP-plus (Shimadzu, Japan) equipped with a 30 m × 0.25 mm i.d. 0.25 µm film thickness capillary column containing a stationary phase 5% phenyl and 95% methyl polysiloxane. The injection was carried out in CT split mode at an injector temperature of 250ºC. Helium gas was used as a carrier gas with a flow rate of 0.68 mL/min. The oven temperature was initially set at 60ºC and then increased to 300ºC at a rate of 10ºC/min held for 16 min. The ion source and transfer line temperature were 200ºC and 250ºC, respectively. Mass spectra were taken at 70 eV; a scan interval of 0.3 s and fragments from 50 to 800 m/z. Compounds were identified by comparing

their mass spectra with the National Institute for Standards and Technology (NIST) library installed within the instrument.

2.2.15 Statistical analysis

All quantitative data obtained are expressed as mean ± standard deviation (SD) or standard error of mean (SEM). Data were statistically analysed with InStat version 5 (Graph-Pad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to test for significant differences between experimental groups followed by Tukey's multiple comparison or unpaired Student's *t* test to compare differences between two groups. Differences were considered significant at $P<0.05$.

CHAPTER THREE: RESULTS

The results obtained from this research are presented in the order described below. A brief explanation of the results of each experiment is given with supporting figure(s) or table(s) as the case may be for each section. The effects of daily oral treatment of type II diabetic rats with *Centella asiatica (L) Urban* (CA) extract and metformin for 14 days on food and water intake (Section 3.1); body and organ weights (Section 3.2) and fasting blood glucose (FBG) levels (Section 3.3) are presented first. These are followed by the effects of CA on MDA formation (Section 3.4); antioxidant status (Sections 3.5 - 3.7) and levels of inflammatory cytokines (Section 3.8 – 3.10) in liver, kidney and brain in type II diabetic rats. The acute effects of CA on p-Akt protein expression and sub-chronic effects of CA on GS and GP protein expression in muscle in type II diabetic rats are presented next in Section 3.11. The effects of CA on GS and GP activities in muscle and liver in type II diabetic rats are described in Sections 3.12 and 3.13 respectively. Then, effects of CA on glycogen levels in muscle and liver (Section 3.14) and serum lipids (Section 3.15) are shown. Section 3.16 describe the effects of CA on changes in the architecture of the pancreas following the induction of type II *diabetes mellitus* in rats. Finally, the crude extract of CA used in this study was characterised by Gas Chromatography-Mass Spectroscopy and results are presented in Section 3.17.

3.1 Effects of CA on food and water intake in type II diabetic rats

The induction of diabetes in rats (DC, D500, D1000 and DME groups) resulted in an increased appetite for food (polyphagia), water (polydipsia) and frequent urination (polyuria) in these groups when compared with NC group rats. Oral administration of CA (500 and 1000 mg/kg) and metformin to diabetic rats for 14 days significantly ($P<0.05$) decreased the excess food intake (Figure 3.1) when compared to untreated diabetic rats. Similarly, the daily oral treatment of diabetic rats with CA (500 and 1000 mg/kg) and metformin for 14 days significantly ($P<0.05$) decreased the excess water intake when compared to untreated diabetic rats as shown in Figure 3.2.

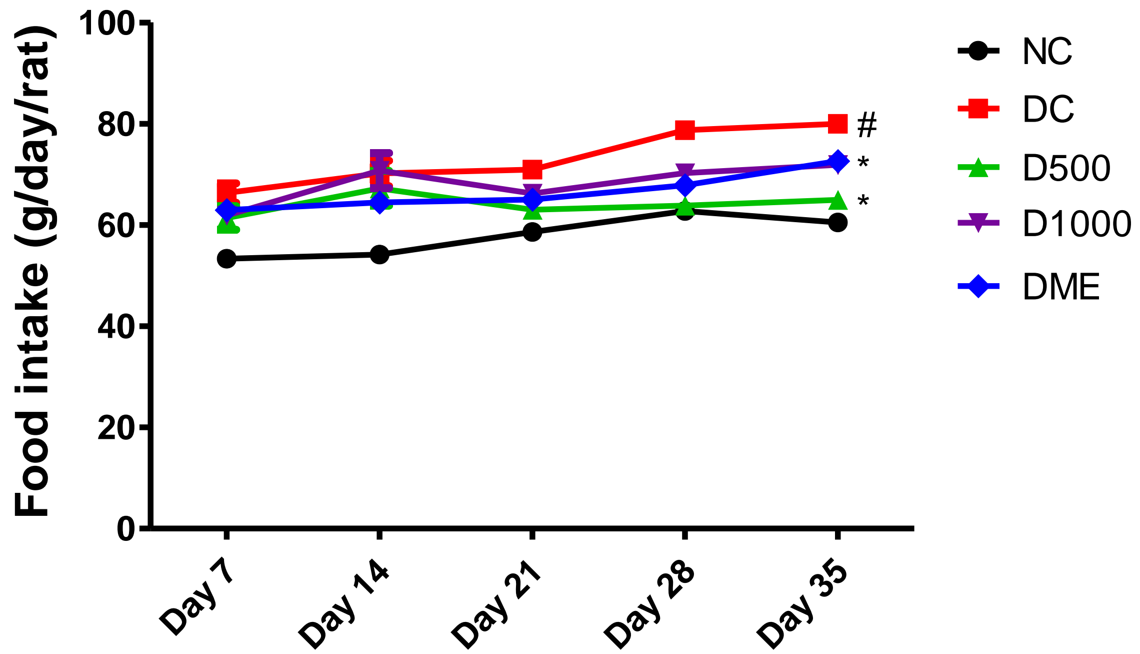


Figure 3.1: Effects of CA on food intake in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban.* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

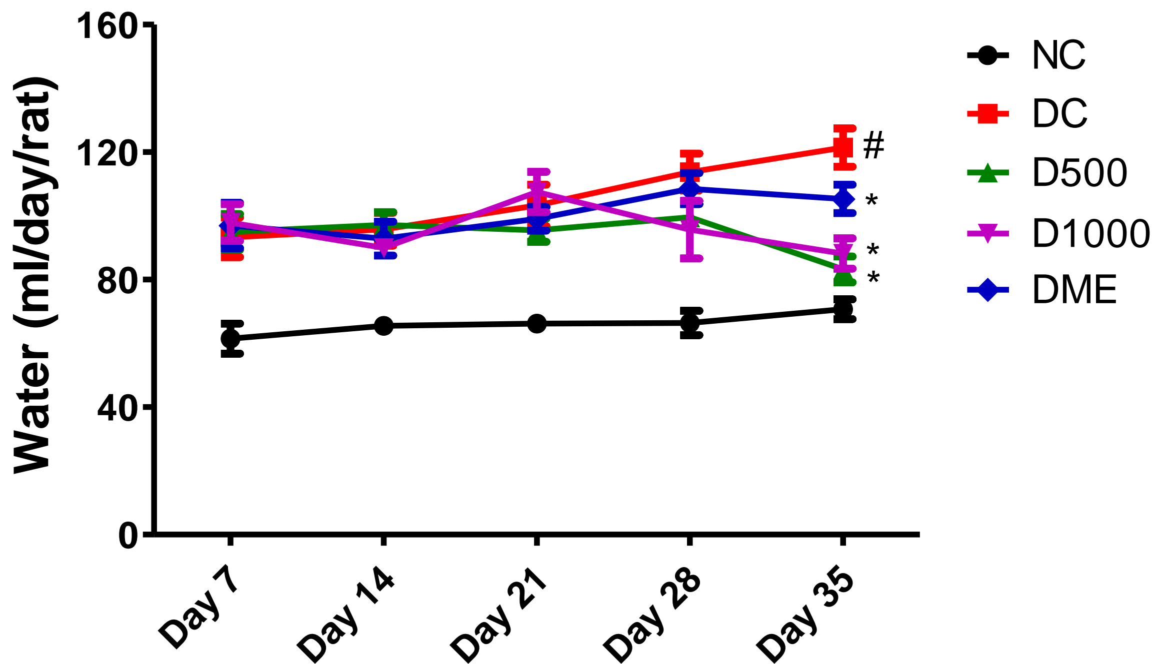


Figure 3.2: Effects of CA on water intake in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.2 Effects of CA on body and organ weights in type II diabetic rats

The results in Table 3.1 show body weights of normal rats and diabetic rats treated with CA and metformin. The induction of diabetes in rats caused a significant (P<0.05) reduction in body weight gain of diabetic control rats compared to normal control rats. Treatment with CA (500 mg/kg) and metformin significantly (P<0.05) increased the body weight gain to 135 % and 133 % of that in the DC group respectively. However, the change in body weight gain in D1000 rats was not statistically different (P>0.05) from the DC rats.

Table 3.1: Effects of CA on body weights in type II diabetic rats

Group	Body weight		
	Initial body weight (g)	**Final body weight (g)**	**Weight gain (%)**
NC	163.38 ± 5.53	281.88 ± 8.97	72.65 ± 6.49
DC	161.00 ± 12.73	241.00 ± 12.41	50.02 ± 6.82 #
D500	170.25 ± 20.00	275.25 ± 13.45	67.74 ± 9.78 *
D1000	166.67 ± 11.01	255.33 ± 16.50	53.52 ± 12.13
DME	151.40 ± 14.22	251.40 ± 21.93	66.40 ± 10.92 *

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

Table 3.2 shows organ weights of normal rats and diabetic rats treated with CA and metformin. Liver weight and liver weight/ body weight ratio were significantly reduced (P<0.05) by 28 % and 19 % respectively in diabetic control rats compared to normal rats. Treatment with CA or metformin did not significantly change this reduction in liver weight. The kidney weight and kidney weight/body weight ratio in diabetic control rats were significantly (P<0.05) increased by 10 % and 24 % respectively when compared to normal control rats while those of CA-treated rats remained unaltered when compared with diabetic control rats. Diabetes induction caused increases in brain weights, difference being significant (P<0.05) for the brain weight/body weight ratio (12 %) when compared to normal control rats. The increases observed in brain weights were returned to below normal in CA-treated rats whereas the reduction in brain weight/body weight ratio was only significant at 500 mg/kg dose (10 %) when compared to the value obtained in diabetic control rats. Treatment with metformin led to significant (P<0.05) 11 % and 10 % decreases in kidney weight and kidney weight/body weight ratio respectively compared to diabetic control rats while the changes in brain weights were not statistically significant in comparison to those in diabetic control rats.

Table 3.2: Effects of CA on organ weights in type II diabetic rats

Group	Organ weights					
	Liver weight (g)	**Liver/bw ratio (%)**	**Kidney weight (g)**	**Kidney/bw ratio (%)**	**Brain weight (g)**	**Brain/bw ratio (%)**
NC	12.66 ± 0.93	4.49 ± 0.26	0.93 ± 0.05	0.33 ± 0.02	1.83 ± 0.05	0.65 ± 0.04
DC	9.12 ± 1.10 #	3.64 ± 0.38 #	1.02 ± 0.12 #	0.41 ± 0.05 #	1.86 ± 0.03	0.73 ± 0.06 #
D500	9.61 ± 0.96	3.72 ± 0.43	1.02 ± 0.12	0.40 ± 0.05	1.81 ± 0.03 *	0.66 ± 0.04 *
D1000	9.79 ± 0.77	3.93 ± 0.25	1.04 ± 0.13	0.42 ± 0.04	1.80 ± 0.02 *	0.70 ± 0.03
DME	9.14 ± 0.63	3.63 ± 0.29	0.91 ± 0.08 *	0.37 ± 0.02 *	1.84 ± 0.06	0.73 ± 0.03

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; bw – body weight. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.3 Effects of CA on hyperglycaemia in type II diabetic rats

Diabetic control rats maintained a significant (P<0.05) elevation in fasting blood glucose (FBG) levels during the duration of the experiment when compared to normal control rats. However, daily treatment with CA extract at 500 and 1000 mg/kg doses as well as metformin for 14 days significantly reduced (P<0.05) the FBG levels by 51 %, 42 % and 50 % respectively when compared to the value seen in diabetic control rats (Figure 3.3).

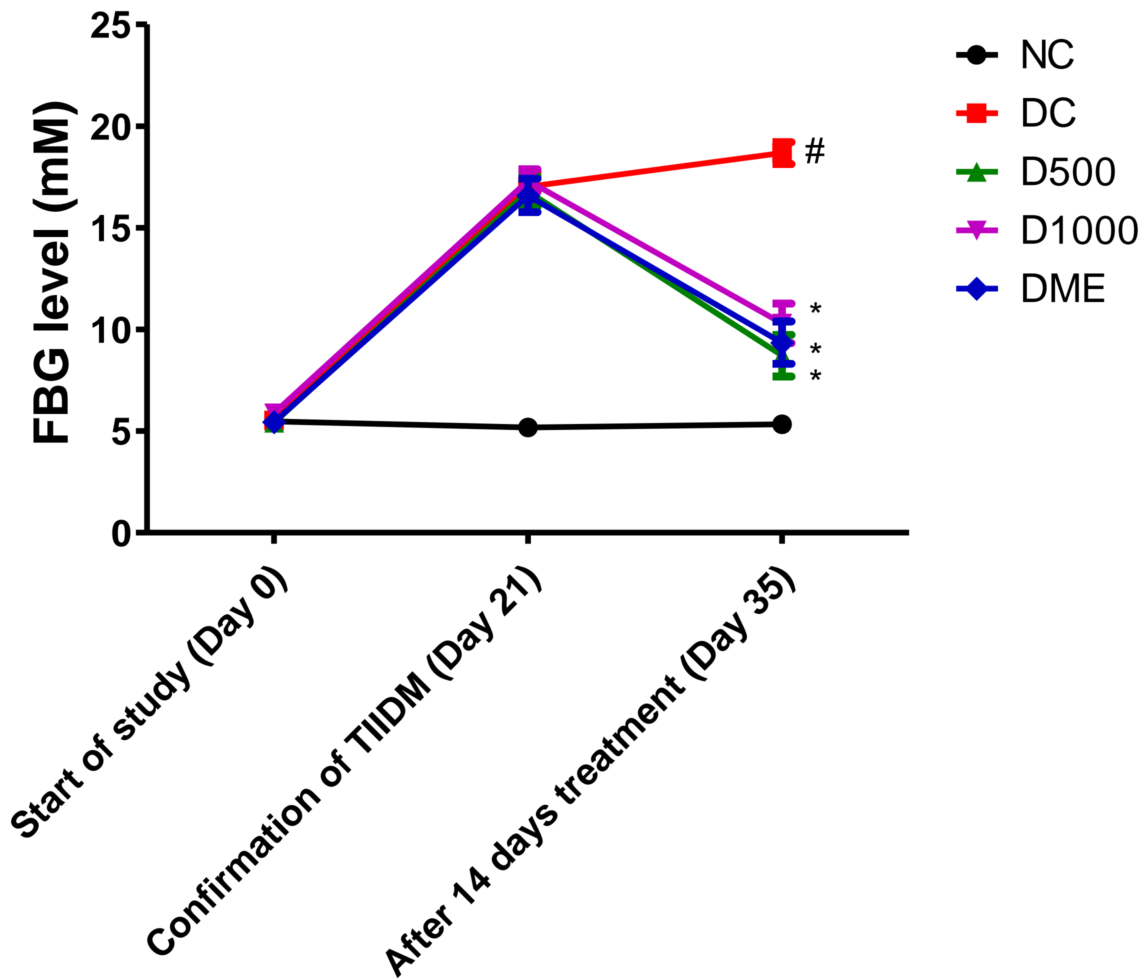


Figure 3.3: Effects of CA on fasting blood glucose (FBG) levels in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight. FBG – fasting blood glucose; TIIDM – type II *diabetes mellitus*. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly ($P<0.05$) from NC group and DC group, respectively.

3.4 Effects of CA on malondialdehyde (MDA) formation in type II diabetic rats

The formation of MDA as a result of lipid peroxidation in rat liver, kidney and brain was significantly (P<0.05) increased by 47 %, 39 % and 182 % respectively following induction of diabetes in comparison to normal rats (Figure 3.4). The elevation of MDA levels in liver and brain were significantly (P<0.05) decreased following administration of CA at both 500 mg/kg and 1000 mg/kg doses in a dose-dependent manner approaching values seen in normal rats. The reduction observed in the kidney of CA-treated rats was only significant at 500 mg/kg dose (33 %) when compared to diabetic control rats. However, decreased MDA levels associated with the administration of metformin was significant (P<0.05) in the liver and brain but not in kidney (Figure 3.4).

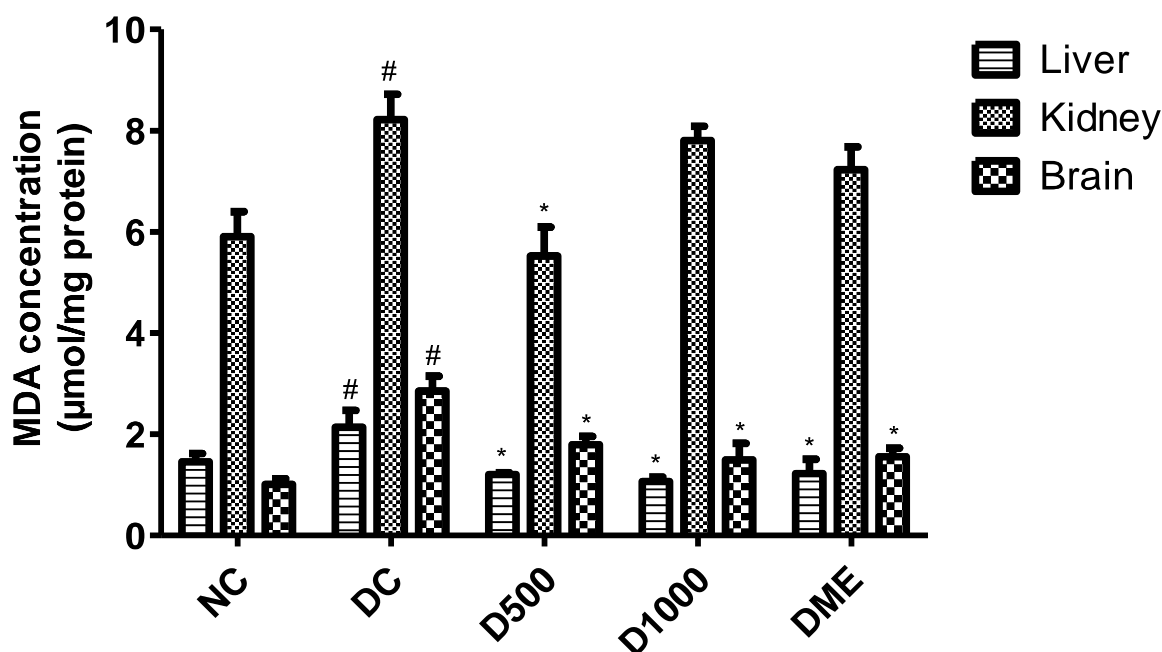


Figure 3.4: Effects of CA on the formation of MDA in liver, kidney and brain in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; MDA – malondialdehyde. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.5 Effects of CA on hepatic antioxidant status in type II diabetic rats

The results of assessment of hepatic FRAP, ORAC and TEAC in type II diabetic rats are presented in Table 3.3. The hepatic values of FRAP and TEAC significantly ($P<0.05$) decreased by 20 % and 14 % respectively following diabetes induction compared to the normal control rats while ORAC concentration remained unchanged between groups. Treatment of diabetic rats with CA extract at 500 and 1000 mg/kg doses as well as metformin significantly ($P<0.05$) increased FRAP levels by 15 %, 18 % and 24 % respectively when compared to diabetic control rats. TEAC level only increased by 13 % ($P<0.05$) in metformin-treated rats while those in rats treated with CA extract remained unchanged when compared to diabetic controls.

Also, this study revealed significant ($P<0.05$) reductions of 32 %, 8 % and 24 % in hepatic GSH levels, GST and GPx activities respectively in DC rats when compared to NC (Table 3.3). The administration of CA increased the activities of GST and GPx in diabetic rats, these increases being significant ($P<0.05$) for CA at 500 mg/kg dose while that of GSH levels significantly ($P<0.05$) increased by 131 % and 153 % at 500 and 1000 mg/kg doses respectively. Treatment of diabetic rats with metformin significantly increased GSH level and GST activity by 75 % and 16 % respectively while the change in GPx activity was not significant. It is worth mentioning that the concentration of GSH and activity of GST in treated diabetic rats were above that in the normal rats especially the GSH levels in D500 and D1000 groups that were 56 % and 71 % higher respectively.

Table 3.3: Effects of CA on hepatic antioxidant status in type II diabetic rats

Group	End points					
	FRAP	**ORAC**	**TEAC**	**GSH**	**GST**	**GPx**
NC	2.56 ± 0.38	8.12 ± 0.94	3.05 ± 0.23	6.82 ± 0.73	108.12 ± 3.45	5.28 ± 0.42
DC	2.05 ± 0.26 #	7.80 ± 0.98	2.61 ± 0.29 #	4.61 ± 0.85 #	99.67 ± 8.94 #	4.00 ± 0.49 #
D500	2.35 ± 0.11 *	8.14 ± 1.29	2.55 ± 0.18	10.66 ± 0.75 #*	115.21 ± 11.98*	4.65 ± 0.47 *
D1000	2.43 ± 0.17 *	8.34 ± 0.75	2.65 ± 0.13	11.68 ± 1.12 #*	109.39 ± 11.92	4.50 ± 0.60
DME	2.54 ± 0.19 *	7.71 ± 0.32	2.96 ± 0.26*	8.05 ± 1.05 *	115.34 ± 9.82 *	4.44 ± 0.60

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; FRAP - ferric reducing antioxidant power in µmole /g tissue; ORAC - oxygen radical absorbance capacity in µmole trolox equivalents /g tissue; TEAC - trolox equivalent antioxidant capacity in µmol trolox equivalents /g tissue; GSH - reduced glutathione level in µmole /g tissue; GST - glutathione S-transferase activity in µmole GSH-DNB complex formed per minute per mg protein; GPx - glutathione peroxidase activity in µmole GSH utilized per mg protein. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.6 Effects of CA on renal antioxidant status in type II diabetic rats

Table 3.4 shows the effects of CA on antioxidant status in kidney in type II diabetic rats after 14 days oral administration. Diabetes induction caused significant (P<0.05) reductions in all the antioxidant indices evaluated in the kidney in rats except TEAC value and GST activity where changes were not statistically significant when compared with the normal control rats. The administration of CA at 500 and 1000 mg/kg doses resulted in significant (P<0.05) increases in the levels of FRAP, ORAC, GSH and activity of GPx when compared with diabetic controls. Although the administration of CA at 500 mg/kg dose also resulted in significant (P<0.05) 40 % increase in GST activity when compared to diabetic contol rats, and was 142 % of the value of normal control rats, the elevation observed at 1000 mg/kg dose was not statistically significant. Treatment with metformin significantly (P<0.05) increased the levels of FRAP, ORAC, GSH levels and GPx activity when compared to their diabetic controls as presented in Table 3.4. Interestingly, the level of GSH in D500 and D1000 groups were 99 % and 93 % higher respectively than in the NC group similar to the effects observed in the liver.

Also, the values of GPx activity in D500, D100 and DME groups were increased compared to that of NC group.

Table 3.4: Effects of CA on renal antioxidant status in type II diabetic rats

Group	End points					
	FRAP	**ORAC**	**TEAC**	**GSH**	**GST**	**GPx**
NC	2.00 ± 0.53	7.90 ± 0.61	2.46 ± 0.16	5.51 ± 0.69	26.22 ± 3.97	5.42 ± 0.77
DC	1.44 ± 0.17 #	5.95 ± 1.47 #	2.45 ± 0.20	4.47 ± 0.53 #	26.62 ± 4.73	4.59 ± 0.39 #
D500	2.14 ± 0.23 *	8.36 ± 1.13 *	2.63 ± 0.24	10.98 ± 1.36 #*	37.28 ± 5.47 *	6.32 ± 1.26 *
D1000	2.06 ± 0.30 *	8.19 ± 0.69 *	2.56 ± 0.17	10.66 ± 1.04 #*	29.15 ± 3.92	6.81 ± 1.42 *
DME	2.35 ± 0.25 *	8.41 ± 0.75 *	2.34 ± 0.21	6.76 ± 0.89 *	26.76 ± 4.78	5.60 ± 0.56 *

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; FRAP - ferric reducing antioxidant power in µmole /g tissue; ORAC - oxygen radical absorbance capacity in µmole trolox equivalents /g tissue; TEAC - trolox equivalent antioxidant capacity in µmol trolox equivalents /g tissue; GSH - reduced glutathione level in µmole /g tissue; GST - glutathione S-transferase activity in µmole GSH-DNB complex formed per minute per mg protein; GPx - glutathione peroxidase activity in µmole GSH utilized per mg protein. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.7 Effects of CA on antioxidant status in brain in type II diabetic rats

The results of the evaluation of the antioxidant status in brain in diabetic rats following treatments with CA and metformin are presented in Table 3.5. DC group rats had significant (P<0.05) decreases in all the antioxidant indices determined in the brain except the ORAC value remained unchanged when compared with those in NC group. Treatment with CA extract (500 and 1000 mg/kg) and metformin significantly (P<0.05) increased FRAP, TEAC levels as well as GST and GPx activities when compared to those of diabetic control rats. In addition, GSH level significantly increased by 175 % (P<0.05) when CA was administered at 500 mg/kg in comparison to the level in diabetic control rats. It was also observed that metformin-treated rats showed a 50 % and 44 % increases (P<0.05) in ORAC values when compared to normal control and diabetic control rats respectively (Table 3.5).

Table 3.5: Effects of CA on antioxidant status in brain in type II diabetic rats

Group	End points					
	FRAP	**ORAC**	**TEAC**	**GSH**	**GST**	**GPx**
NC	0.13 ± 0.04	2.74 ± 0.37	1.46 ± 0.28	4.39 ± 0.40	54.32 ± 7.43	8.02 ± 1.71
DC	0.09 ± 0.02 #	2.84 ± 0.38	0.42 ± 0.08 #	1.98 ± 0.30 #	36.54 ± 10.46 #	5.09 ± 0.79 #
D500	0.15 ± 0.01 *	3.13 ± 0.21	2.02 ± 0.08 *	5.45 ± 0.29 *	64.93 ± 13.29 *	8.69 ± 1.63 *
D1000	0.13 ± 0.02 *	3.40 ± 0.89	1.83 ± 0.10 *	1.56 ± 0.41	64.79 ± 4.67 *	8.63 ± 1.47 *
DME	0.12 ± 0.02 *	4.08 ± 0.40 #*	1.05 ± 0.11*	1.92 ± 0.47	62.42 ± 11.78 *	7.76 ± 1.44 *

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; FRAP - ferric reducing antioxidant power in µmole /g tissue; ORAC - oxygen radical absorbance capacity in µmole trolox equivalents /g tissue; TEAC - trolox equivalent antioxidant capacity in µmol trolox equivalents /g tissue; GSH - reduced glutathione level in µmole /g tissue; GST - glutathione S-transferase activity in µmole GSH-DNB complex formed per minute per mg protein; GPx - glutathione peroxidase activity in µmole GSH utilized per mg protein. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly ($P<0.05$) from NC group and DC group, respectively.

3.8 Effects of CA on hepatic inflammatory cytokines in type II diabetic rats

In the present study, diabetes significantly increased ($P<0.05$) hepatic levels of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and MCP-1 in rats by 73 %, 186 %, 81 % and 90 % respectively (Figure 3.5A-D). The treatment of diabetic rats with CA extract at a dose 500 mg/kg significantly ($P<0.05$) reduced the hepatic concentrations of IL-1β, MCP-1 and TNF-α by 32 %, 23 % and 37 % respectively when compared to diabetic control rats while those of IL-6 were reduced but not significantly. The treatment of diabetic rats at 1000 mg/kg dose of CA only significantly ($P<0.05$) decreased the MCP-1 level. Metformin treatment of diabetic rats also caused a decrease in all the four pro-inflammatory biomarkers these decreases being significant ($P<0.05$) except for IL-1β.

The induction of diabetes in rats significantly increased ($P<0.05$) hepatic levels of anti-inflammatory cytokines IL-4 and IL-10 by 236 % and 52 % respectively (Figure 3.5E-F). However, the treatment of diabetic rats with CA (500 mg/kg) and metformin significantly

(P<0.05) decreased the hepatic concentration of IL-4 while that of IL-10 remained unaffected when compared with diabetic control rats.

3.9 Effects of CA on renal inflammatory cytokines in type II diabetic rats

The results of levels of pro-inflammatory cytokines in kidney in diabetic rats are shown in Figure 3.6A-D. There were significant (P<0.05) increases in levels of IL-1α, IFN-γ, and TNF-α; but not MCP-1 in kidney in diabetic control rats when compared to normal control rats. However, treatment with CA (500 and 1000 mg/kg doses) and metformin significantly (P<0.05) reduced the levels of these pro-inflammatory cytokines to below-normal levels when compared with diabetic control rats. It is worth mentioning that the levels of IL-1α, IFN-γ, and TNF-α levels in D1000 rats were reduced to 75 %, 78 % and 77 % of those in NC rats respectively.

Also, IL-4 levels remain unchanged among groups except in D1000 group where a 39 % significant (P<0.05) reduction was observed when compared to DC group (Figure 3.6E). In addition, level of IL-10 was significantly (P<0.05) reduced (26 %) in DC group in comparison to NC group, but significantly (P<0.05) increased by 18 % and 11 % in D500 and DME groups respectively when compared with DC groups (Figure 3.6F).

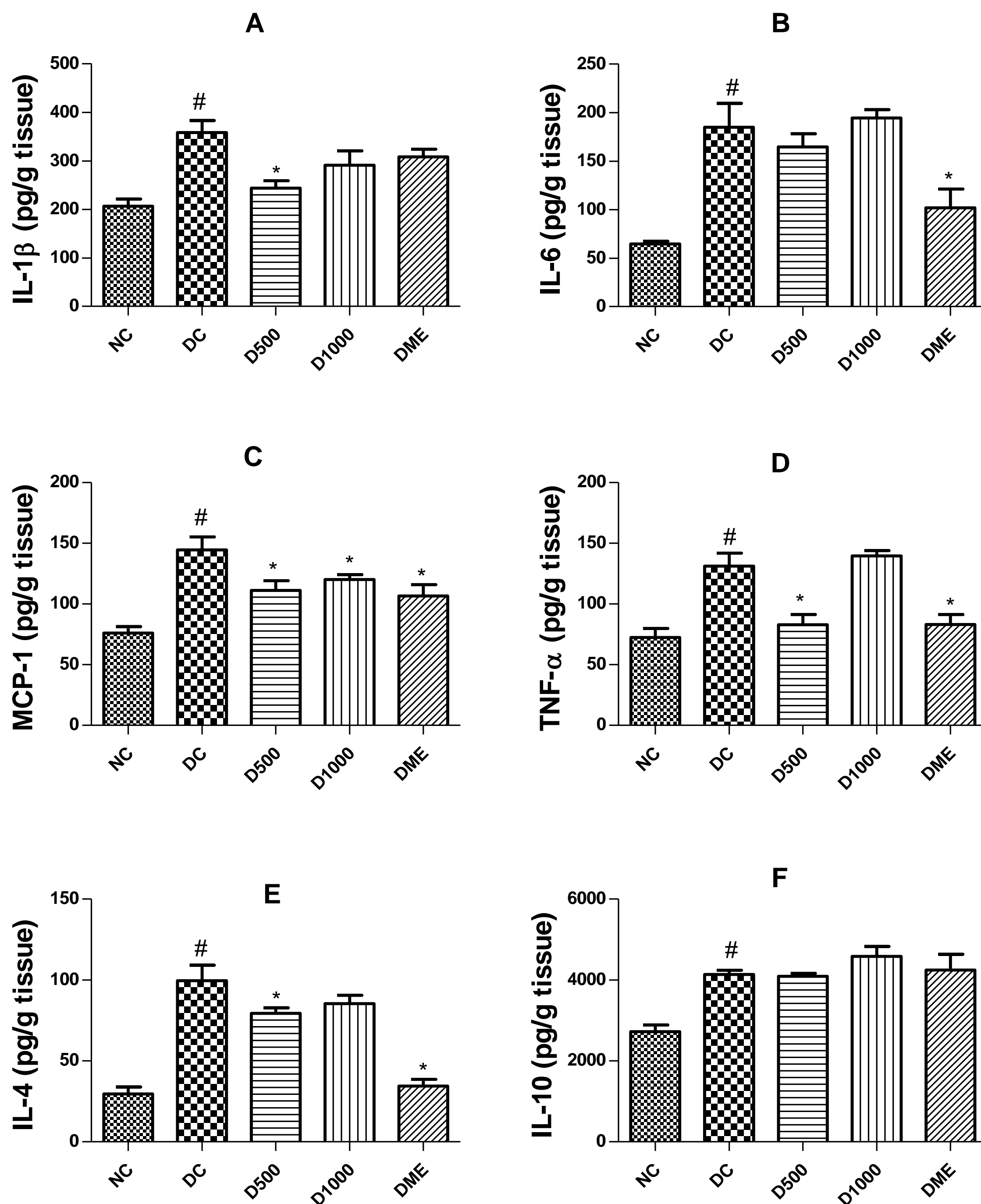


Figure 3.5A-F: Effects of CA on hepatic levels of inflammatory cytokines in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; IL-Interleukins-1β 4, 6, 10; MCP-1 - Monocyte chemoattractant protein-1; TNF-α – Tumor necrosis factor alpha. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly ($P<0.05$) from NC group and DC group, respectively.

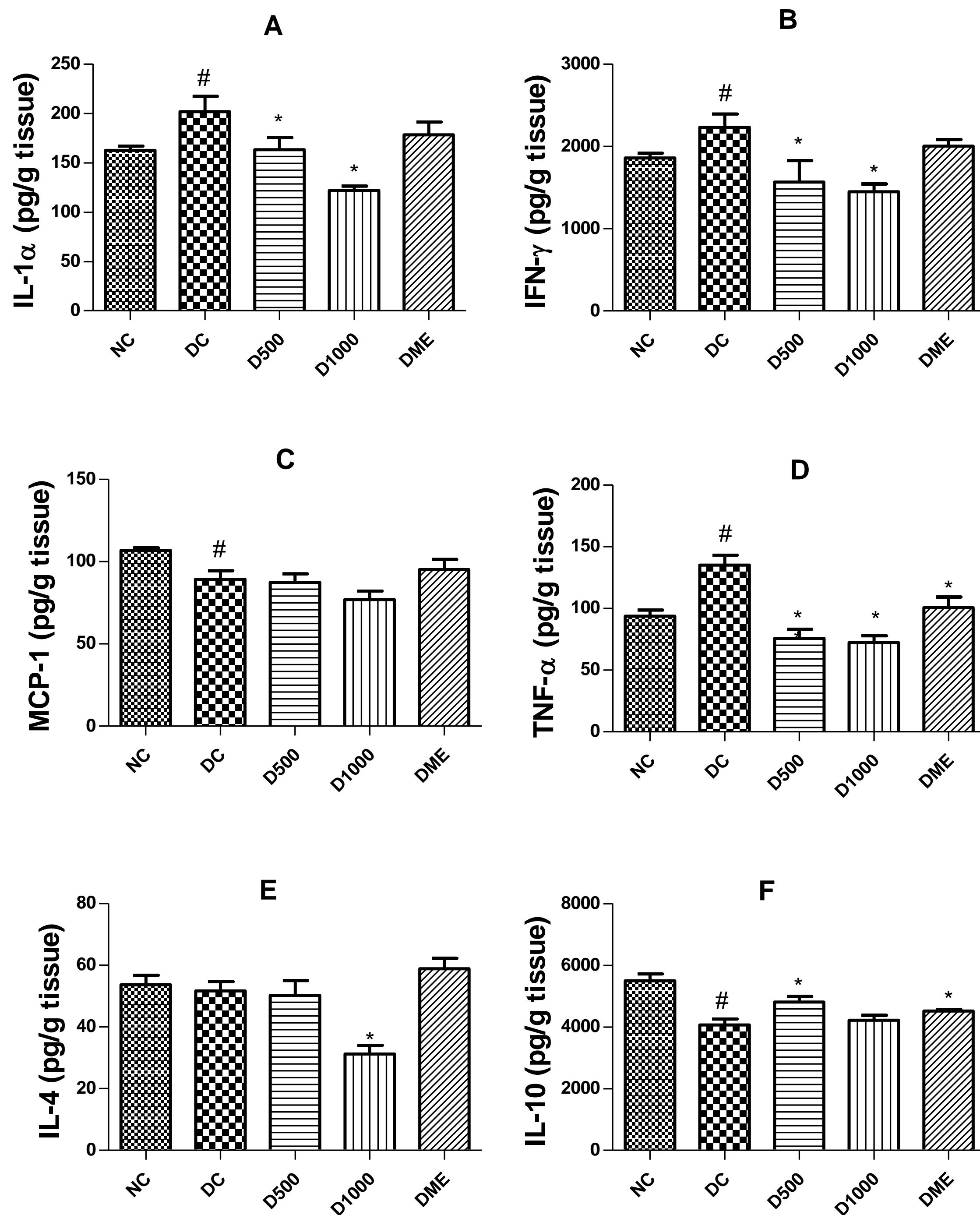


Figure 3.6A-F: Effects of CA on renal levels of inflammatory cytokines in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; IL-Interleukins-1α, 4, 10; IFN-γ – Interferon-γ; MCP-1 - Monocyte chemoattractant protein-1; TNF-α – Tumor necrosis factor-α. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly ($P<0.05$) from NC group and DC group, respectively.

3.10 Effects of CA on inflammatory cytokines in brain in type II diabetic rats

In the brain, diabetic control rats had significant ($P<0.05$) increases of 20 % and 40 % in levels of IFN-γ and TNF-α respectively whereas changes in levels of MCP-1 and MIP-1α were not significant in comparison to normal control rats. However, treatment with CA elicited significant ($P<0.05$) reductions in levels of IFN-γ, MCP-1 and TNF-α (500 and 1000 mg/kg doses) as well as that of MIP-1α (1000 mg/kg dose) when compared to diabetic control rats. Metformin treatment on the other hand, only significantly ($P<0.05$) reduced IFN-γ and TNF-α levels by 14 % and 29 % in comparison with diabetic control rats (Figure 3.7A-D).

Levels of the anti-inflammatory cytokines, IL-4 and IL-10 were significantly ($P<0.05$) decreased by 22 % and 11 % respectively in DC groups in comparison to NC groups. The levels of IL-4 and IL-10 were increased when diabetic control group was compared with the CA and metformin-treated groups except the change in IL-4 level for D1000 group was not significant. Notably, the IL-4 level of D500 was also 50 % greater than that of NC group (Figure 3.7E-F).

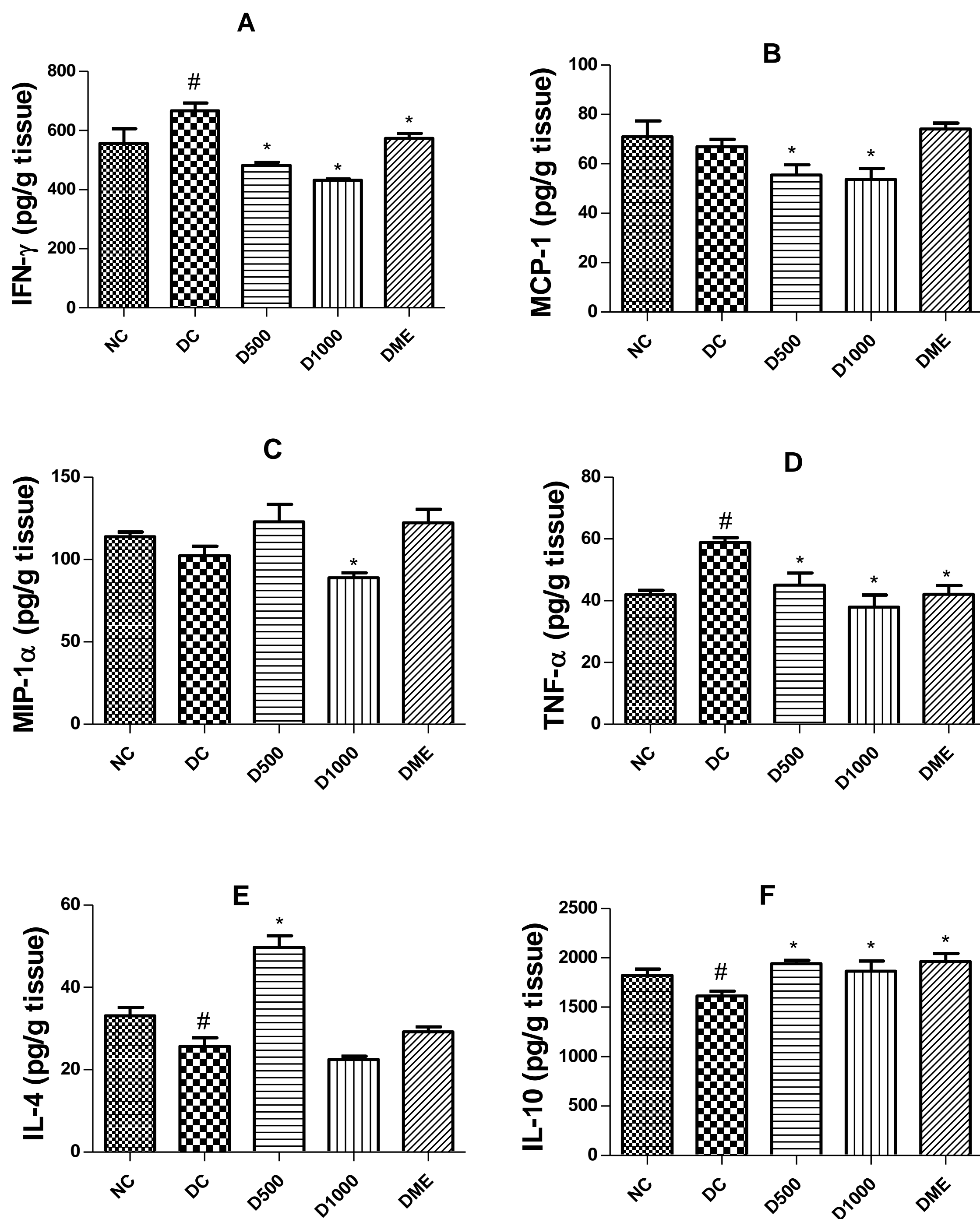


Figure 3.7A-F: Effects of CA on levels of inflammatory cytokines in brain in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; IL-Interleukins-4, 10; IFN-γ – Interferon-γ; MCP-1 – Monocyte chemoattractant protein-1; MIP-1α - Macrophage inflammatory protein-1α; TNF-α – Tumor necrosis factor-α. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly ($P<0.05$) from NC group and DC group, respectively.

3.11 Effects of CA on muscle expression of selected enzymes of insulin signaling pathway in type II diabetic rats

The acute, time-dependent (30, 60 and 90 mins) effects of CA on the activation of p-Akt (Ser 473) and sub-chronic (14 days) effects of CA on the protein expression of GS and GP were investigated. GAPDH was used as a loading control. p-Akt was not detected after 90 mins in muscle samples after several attempts and increasing the concentrations of protein loaded.

3.11.1 Acute effects of CA on muscle p-Akt protein expression

A higher expression of p-Akt was induced in diabetic rats compared to normal rats after 30 minutes of treatment as shown in Figure 3.8. However, the expressions in CA (500 and 1000 mg/kg doses) and metformin-treated groups were considerably lower than that of the diabetic controls. At 60 mins following treatments, NC rats had p-Akt expression almost equal to DC rats. The highest level of p-Akt expression was observed in D500 while DME rats had the lowest p-Akt expression (Figure 3.9). It is important to note that p-Akt expression greatly reduced (up to 10 folds) with respect to time in all groups and became undetectable at 90 mins.

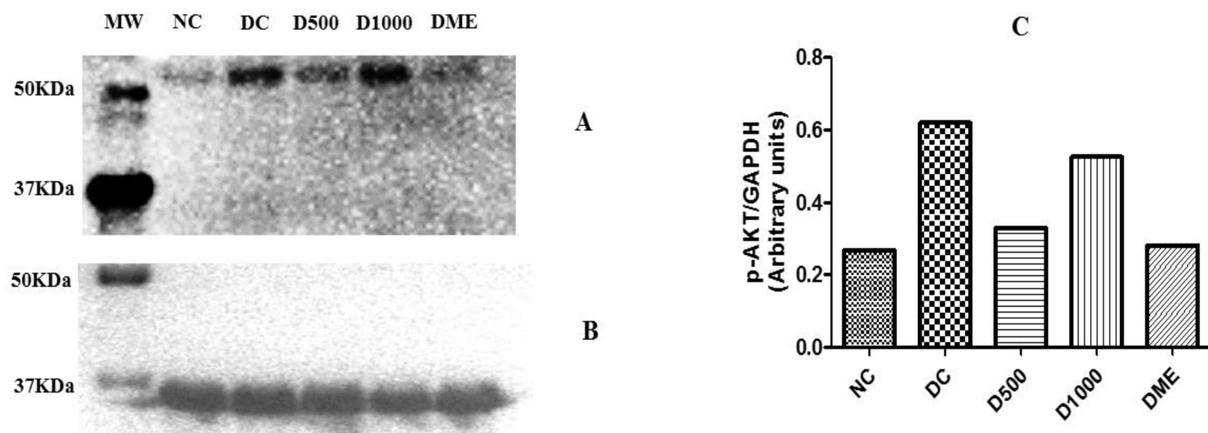


Figure 3.8: Western blots of (A) phospho-Akt (Ser 473) and (B) GAPDH of muscle homogenates 30 mins after treatment. (C) represents the normalized data showing the ratio of phospho-Akt/GAPDH.. 20 µg of protein was loaded for A and 30 µg for B. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight.

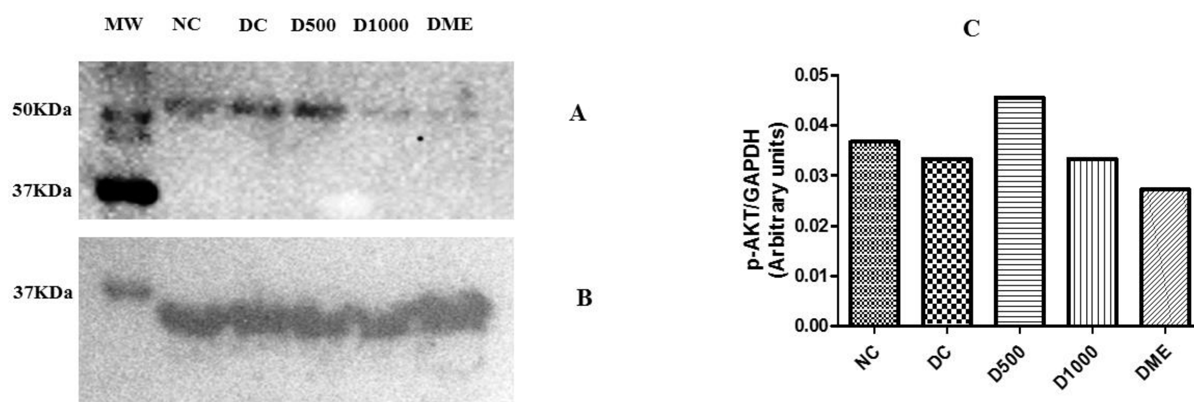


Figure 3.9: Western blots of (A) phospho-Akt (Ser 473) and (B) GAPDH of muscle homogenates 60 mins after treatment. (C) represents the normalized data showing the ratio of phospho-Akt/GAPDH. 20 µg of protein was loaded for A and 30 µg for B. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight.

3.11.2 Sub-chronic effects of CA on muscle GS and GP protein expression

The results of the effects of the oral administration of CA on GS and GP expression in the muscle of type II diabetic rats are shown in Figures 3.10 and 3.11 respectively. Following the induction of diabetes, diabetic rats had lower GS and GP expression when compared to normal control rats. Treatment with CA at both doses and metformin resulted in considerably lower GS expression in comparison with diabetic control rats, with D500 rats having the lowest. On the other hand, GP expression in rats treated with CA at 500 and 1000 mg/kg doses were almost equal with that of diabetic control rats. Metformin-treated rats showed the lowest GP expression.

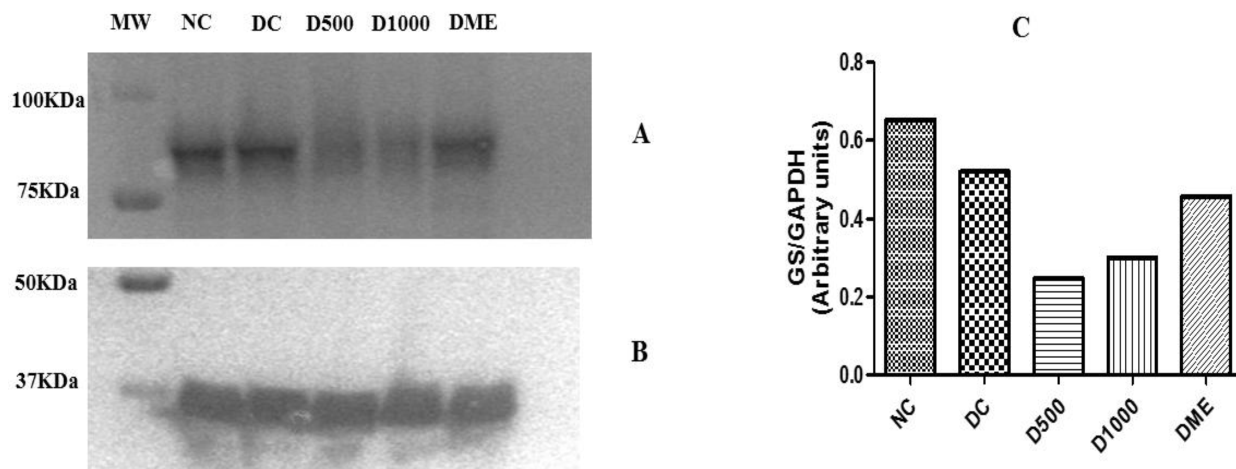


Figure 3.10: Western blots of (A) GS and (B) GAPDH of muscle homogenates 14 days after treatment. (C) represents the normalized data showing the ratio of GP/GAPDH. 60 µg of protein was loaded for A and 30 µg for B. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight.

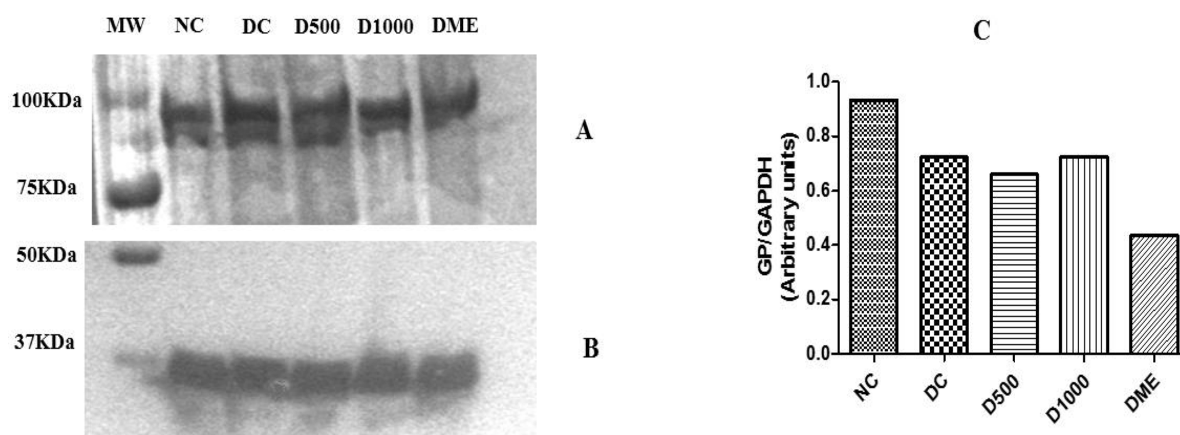


Figure 3.11: Western blots of (A) GP and (B) GAPDH of muscle homogenates 14 days after treatment. (C) represents the normalized data showing the ratio of GS/GAPDH. 40 µg of protein was loaded for A and 30 µg for B. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight.

3.12 Effects of CA on GS and GP activities in muscle in type II diabetic rats

The activity of GS in muscle significantly (P<0.05) reduced by 38 % in diabetic control rats when compared to those of the normal control rats as shown in Figure 3.12. A significant 27 % increase in GS activity in muscle was seen in rats treated with CA at 1000 mg/kg dose and metformin for 14 days in comparison to diabetic control rats whereas that of D500 rats remained unaffected. Diabetes induction had no significant effect on GP activity in muscle (Figure 3.12). However, treatment with CA at 1000 mg/kg dose significantly (P<0.05) increased GP activity by 50 % while that of DME rats led to a significant (P<0.05) decrease of 49 % in GP activity.

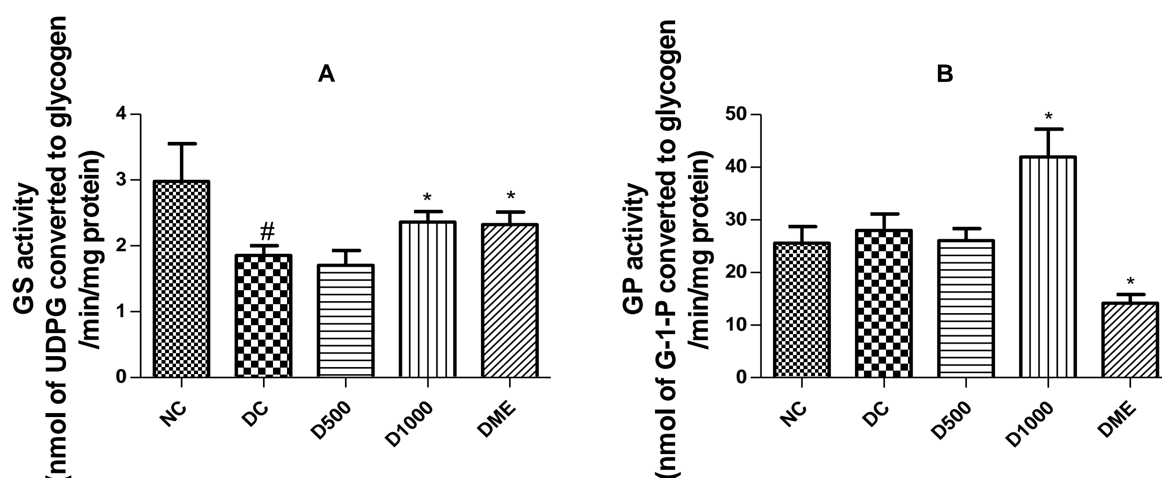


Figure 3.12: Effects of CA on activities of GS (A) and GP (B) in muscle in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; UDPG - uridine diphosphate glucose; G-1-P - glucose-1-phosphate. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.13 Effects of CA on GS and GP activities in liver in type II diabetic rats

The induction of diabetes caused a 49 % (P<0.05) reduction of in the activity of GS in liver in diabetic control rats when compared to that of the normal control rats (Figure 3.13). Treatment with CA at 1000 mg/kg dose and metformin for 14 days significantly (P<0.05) restored the GS activity to normal values while D500 had no effect when compared to diabetic control rats. On

the other hand, GP activity in diabetic control rats was not statistically different from normal control rats as shown in Figure 3.13. Although treatment of diabetic rats with CA at 500 mg/kg dose significantly (P<0.05) increased GP activity by 81 % in liver, those treated with 1000 mg/kg dose and metformin had no significant changes when compared to diabetic control rats.

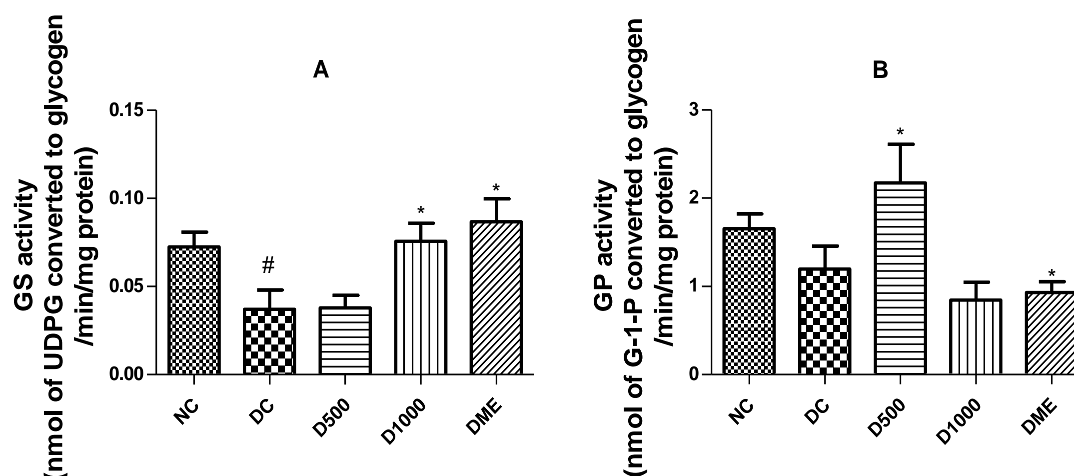


Figure 3.13: Effects of CA on activities of GS (A) and GP (B) in liver in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; UDPG - uridine diphosphate glucose; G-1-P - glucose-1-phosphate. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.14 Effects of CA on glycogen levels in liver and muscle in type II diabetic rats

The induction of diabetes in rats led to significant (P<0.05) decreases in glycogen levels in liver (79 %) and muscle (34 %) as shown in Figure 3.14. Treatment with CA at 1000 mg/kg dose for 14 days significantly (P<0.05) increased glycogen levels by 31 % and 47 % in liver and muscle when compared to diabetic controls. Similarly, metformin treatment increased glycogen levels in both tissues but was only significant (P<0.05) in muscle (53 %) in comparison to diabetic controls.

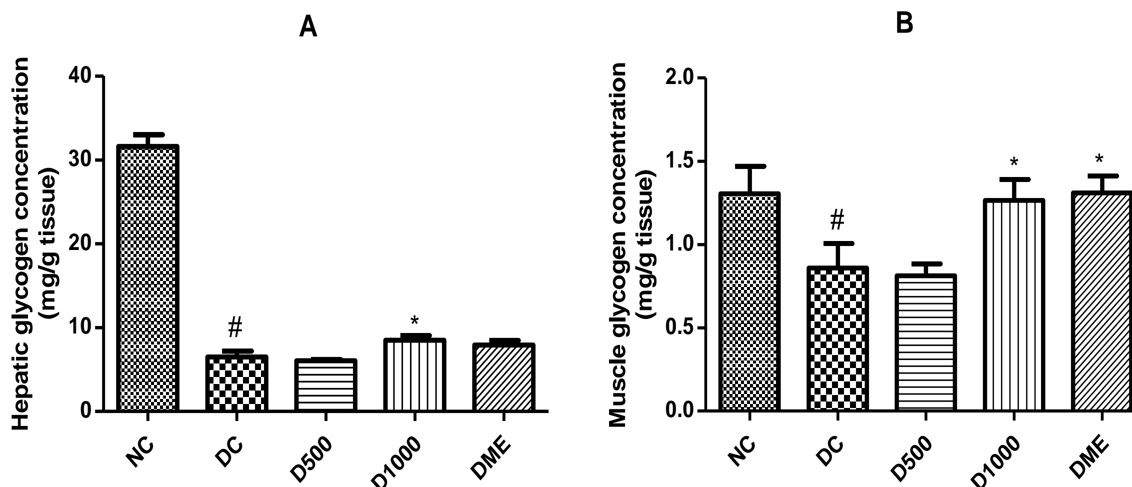


Figure 3.14: Effects of CA on glycogen concentration in liver (A) and muscle (B) in type II diabetic rats. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight. Data are presented as mean ± standard error of mean; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.15 Effects of CA on serum lipids in type II diabetic rats

Alterations in serum lipids observed as a result of diabetes induction and its treatment with CA or metformin are presented in Table 3.6. Diabetes caused a significant (P<0.05) elevation in the serum levels of TG, Tc and HDL-c however the increase in serum levels of LDL-c was not statistically significant (P>0.05). Treatment of diabetic rats with CA significantly (P<0.05) decreased serum TG concentration at 500 mg/kg dose to the value seen in normal control rats while that of D1000 remained unchanged when compared to diabetic control rats. The levels of Tc and LDL-c in D500 rats were increased further beyond DC while D1000 reduced them to 93 % and 78 % of the DC values respectively. HDL-c level among treated rats was not statistically different to that of diabetic control rats. Metformin treatment elicited no significant (P>0.05) change in serum lipids when compared with diabetic control rats.

Table 3.6: Effects of CA on serum lipids in type II diabetic rats

Group	TG (mg/dl)	Tc (mg/dl)	HDL-c (mg/dl)	LDL-c (mg/dl)
NC	60.50 ± 15.02	66.5 ± 8.53	17.17 ± 1.47	33.52 ± 5.44
DC	77.50 ± 7.55 #	76.00 ± 6.69 #	25.00 ± 2.71 #	37.80 ± 6.71
D500	60.00 ± 9.83 *	80.00 ± 4.32	23.50 ± 1.00	42.33 ± 4.83
D1000	76.00 ± 4.58	70.50 ± 8.50	23.80 ± 3.35	29.47 ± 5.19
DME	84.00 ± 6.24	80.17 ± 6.58	26.00 ± 2.65	40.90 ± 7.60

NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight; bw – body weight. Data are presented as mean ± standard deviation; n = 8. Symbols # and * indicate value differs significantly (P<0.05) from NC group and DC group, respectively.

3.16 Histopathological examination of pancreas in type II diabetic rats

Figure 3.15 shows representative sections of histopathological examinations of pancreas from all experimental animal groups. The induction of diabetes in rats elicited abnormal morphological changes in pancreatic islets as observed in DC, D500, D1000 and DME groups when compared to NC group which can lead to reduction in the population of pancreatic β-cells. However, treatment with CA and metformin for 14 days slightly ameliorated these changes when compared with that of untreated diabetic rats.

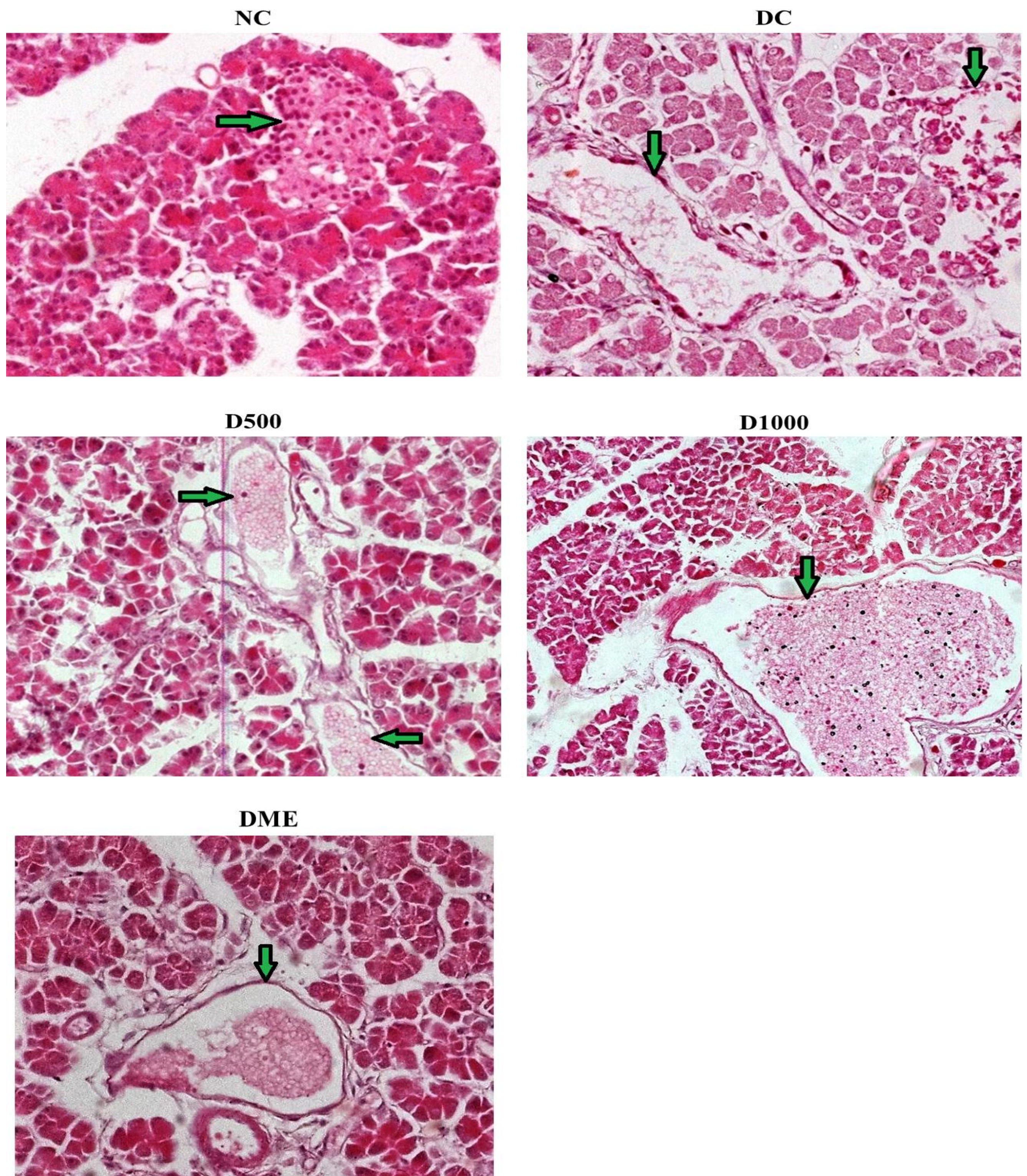


Figure 3.15: Representative sections of histopathological examinations of pancreas from type II diabetic rats showing pancreatic β islets containing β-cells (green arrows). Magnification: 20X - 100µm. NC - Normal Control; DC - Diabetic Control; D500 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 500 mg/kg body weight; D1000 - Diabetic rats treated with *Centella asiatica (L.) Urban* extract at a dose of 1000 mg/kg body weight; DME - Diabetic rats treated with metformin at a dose of 300 mg/kg body weight.

3.17 GC-MS analysis of crude extract of CA

The GC-MS chromatogram of the crude extract of leaves of CA revealed the presence of peaks representing over 50 compounds with matching similarity index (SI) greater than 70% in the National Institute of Standards and Technology (NIST) library as shown in Figure 3.16 below. Some of the peaks are as a result of column bleeding. The fragmentation pattern of mass spectrum of 6 compounds identified in the crude extract of CA were selected (Figures 3.17 – 3.22) based on their identification and pharmacological activities reported previously in literature (Brinkhaus et al., 2000, Mauri and Pietta, 2000, Oyedeji and Afolayan, 2005, Medeiros et al., 2007, Apichartsrangkoon et al., 2009, Wongfhun et al., 2010, Dakhale et al., 2011, James and Dubery, 2011, Wang et al., 2012c, Kumar et al., 2013, Ramachandran and Saravanan, 2013, Ramos et al., 2013, Maulidiani et al., 2014, Pakdeechote et al., 2014, Lee et al., 2016). The molecular formula, molecular weight, and retention times (RT) of these medicinal compounds; ascorbic acid, asiatic acid, oleanolic acid, stevioside, stigmasterol and α-humulene are presented in Table 3.7.

Techniques such as electrospray mass spectrometry (ESMS), high performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS) and thin layer chromatography (TLC) have been used to identify the presence of medicinal compounds in the crude extracts of CA. These compounds include asiatic acid, asiaticoside, centellasaponins, humulene, madecassic acid, madecassoside, stigmasterol, and quercetin among others (Brinkhaus et al., 2000, Mauri and Pietta, 2000, James and Dubery, 2011, Maulidiani et al., 2014). GC-MS analysis has also showed the presence of terpenoids such as humulene in the essential oils (Oyedeji and Afolayan, 2005), water juice (Apichartsrangkoon et al., 2009, Wongfhun et al., 2010), ethanol extract (Orhan, 2012) and acetone extract (Siddique et al., 2014) of CA. This is the first study reporting the presence of ascorbic acid and stevioside in methanol extract of CA leaves identified by GC-MS analysis.

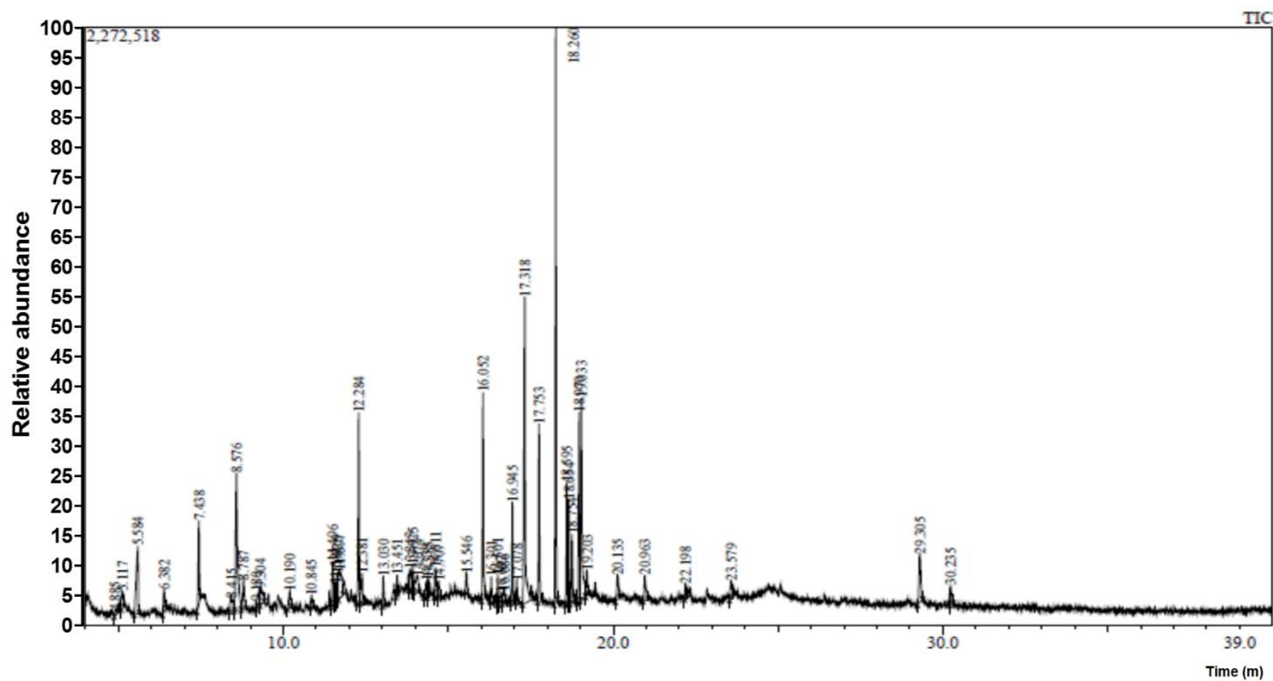


Figure 3.16: The GC-MS chromatogram of crude extract of CA

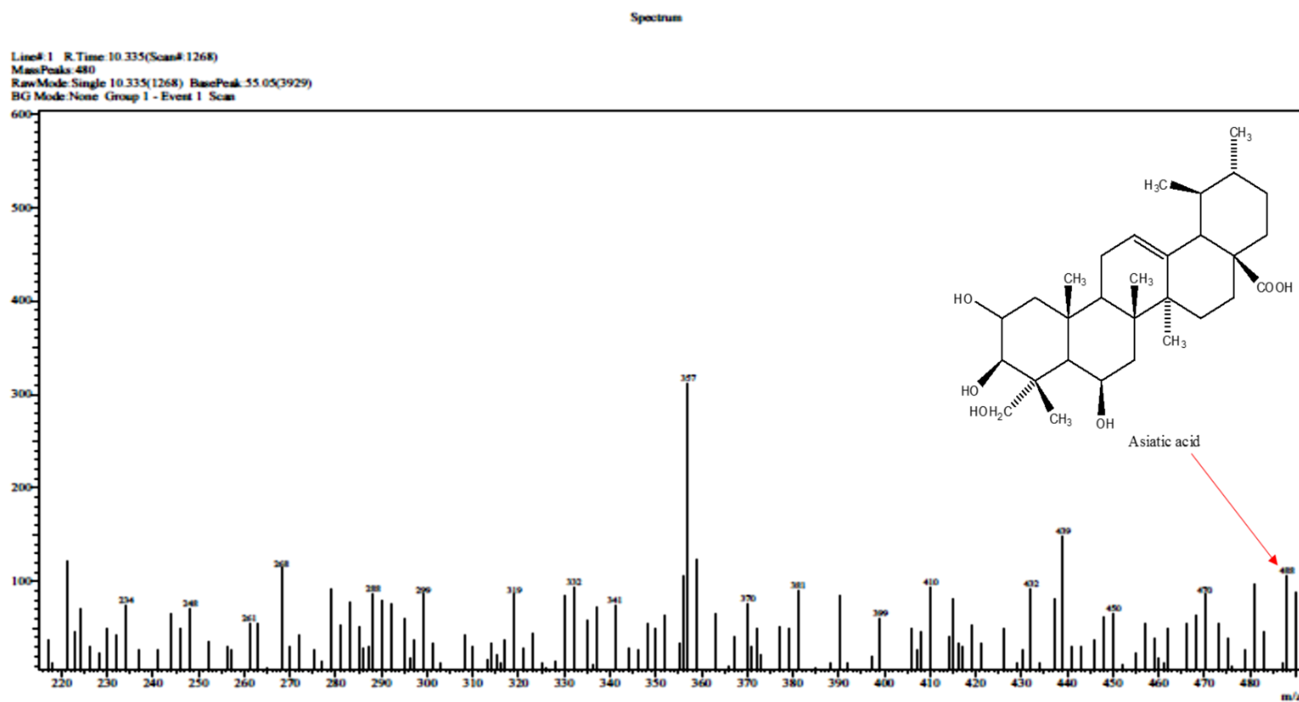


Figure 3.17: Mass spectrum of asiatic acid identified in crude extract of CA

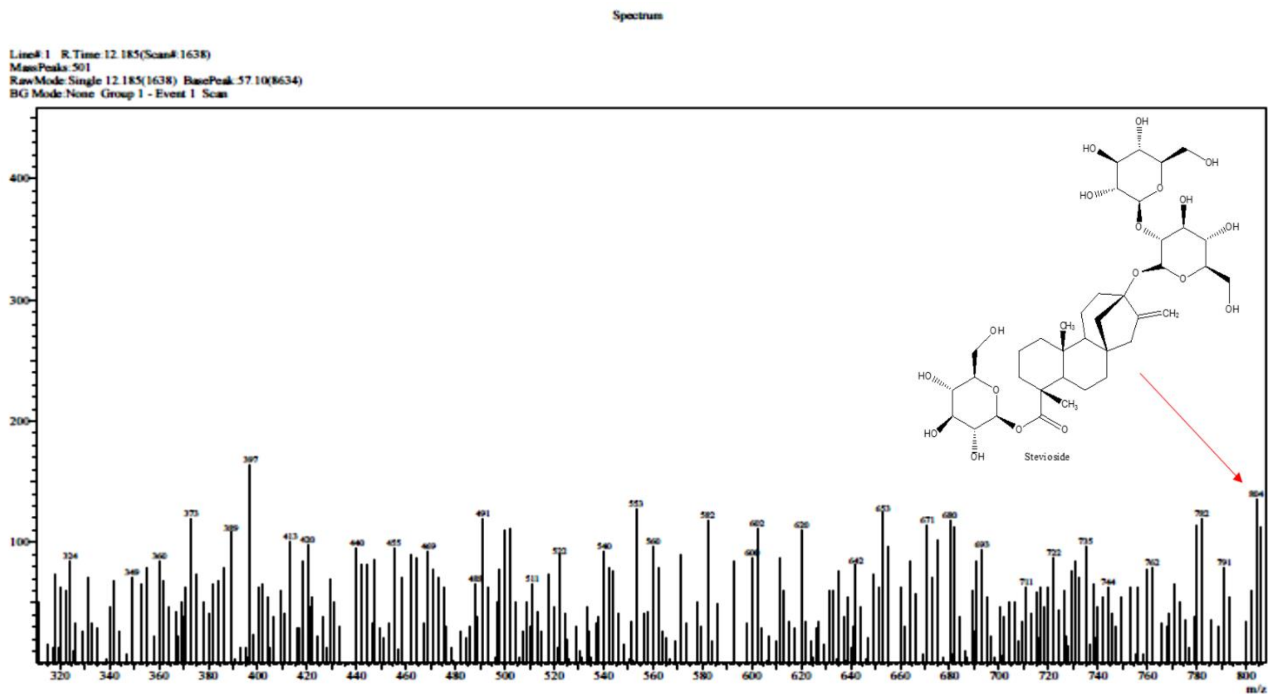


Figure 3.18: Mass spectrum of stevioside identified in crude extract of CA

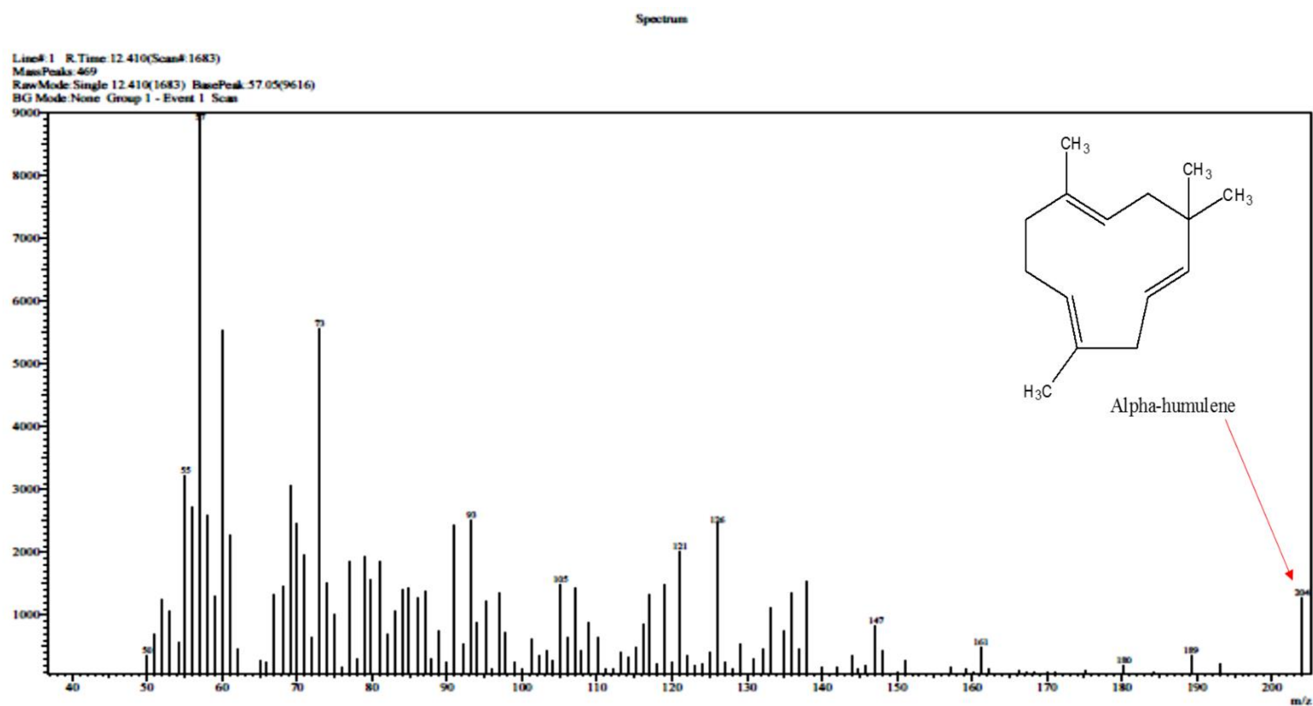


Figure 3.19: Mass spectrum of alpha-humulene identified in crude extract of CA

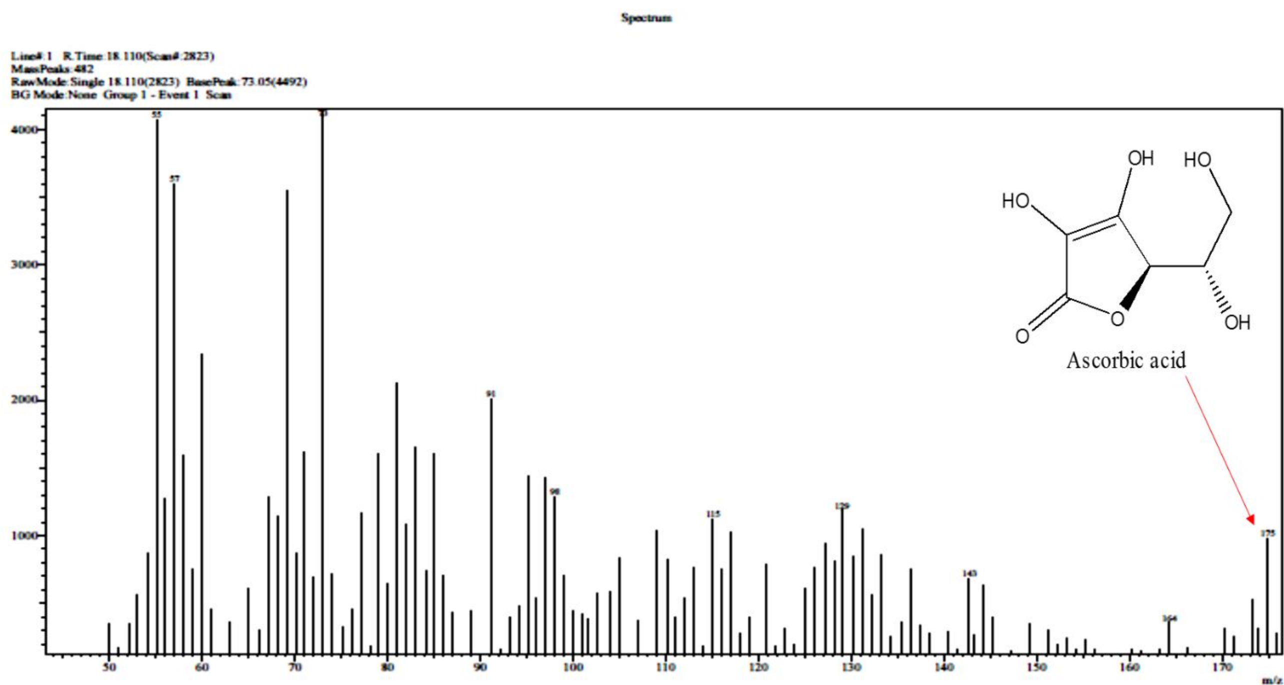


Figure 3.20: Mass spectrum of ascorbic acid identified in crude extract of CA

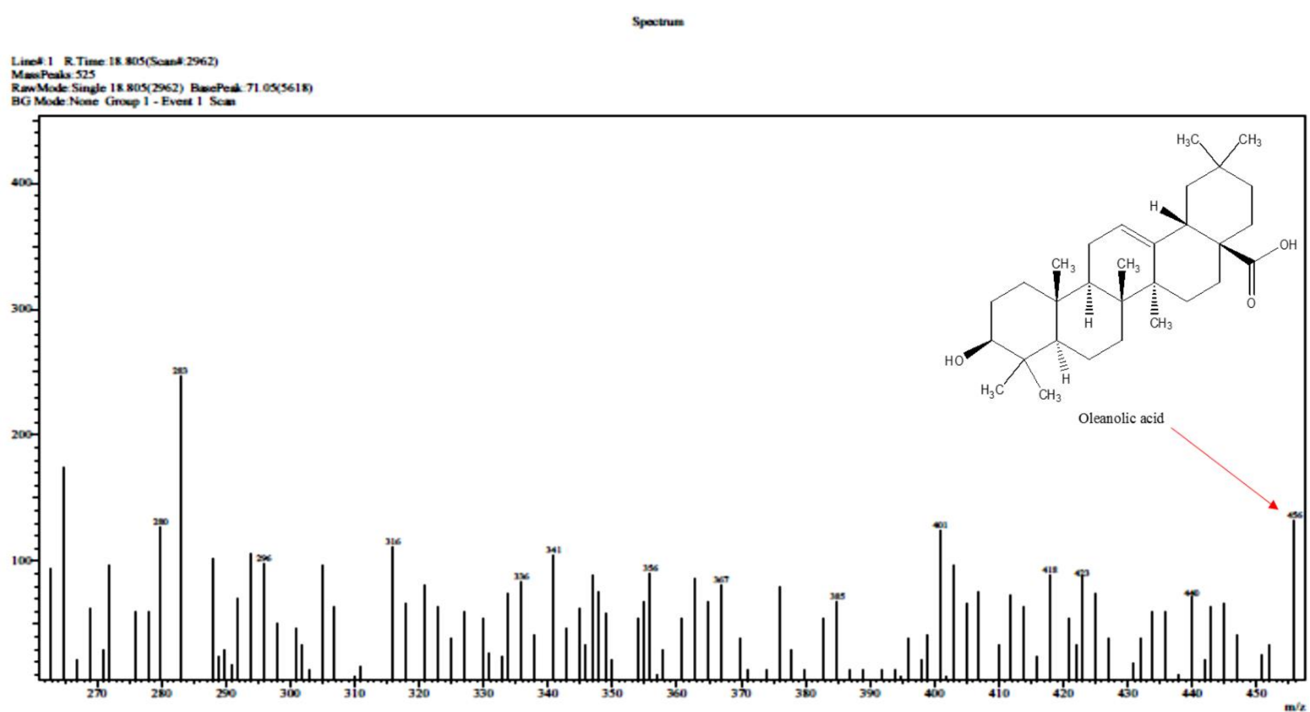


Figure 3.21: Mass spectrum of oleanolic acid identified in crude extract of CA

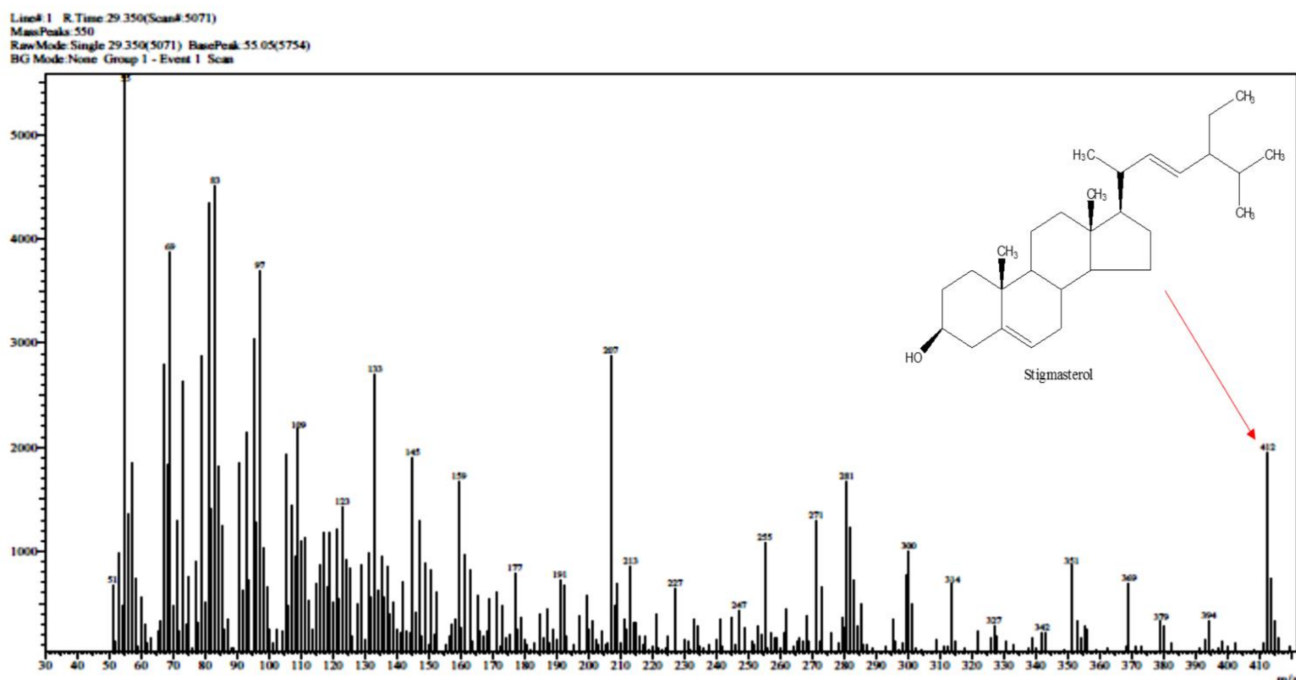


Figure 3.22: Mass spectrum of stigmasterol identified in crude extract of CA

Table 3.7: Chemical properties of selected compounds identified by GC-MS in crude extract of CA

Name of compound	Molecular formula	Molecular weight (g/mol)	Retention time
Asiatic acid	$C_{30}H_{48}O_5$	488.70	10.33
Stevioside	$C_{38}H_{60}O_{18}$	804.87	12.18
Alpha-humulene	$C_{15}H_{24}$	204.36	12.41
Ascorbic acid	$C_6H_8O_6$	176.12	18.11
Oleanolic acid	$C_{30}H_{48}O_3$	456.71	18.80
Stigmasterol	$C_{29}H_{48}O$	412.69	29.35

CHAPTER FOUR: DISCUSSION

This study was designed specifically to investigate the potential effects of crude extract of CA leaves on hyperglycaemia, oxidative stress, inflammatory cytokines, insulin signaling and downstream metabolic effects, based on the importance of these mechanisms in the pathophysiology of type II DM using a rat model that exhibits both insulin resistance and insufficiency. The choice of the plant was due to its extensive use in traditional medicines of Africa and Asia to manage diseases including diabetes (Brinkhaus et al., 2000, Gohil et al., 2010, Orhan, 2012).

An ideal animal model of type II DM is expected to resemble, as much as possible, the diabetic conditions seen in humans. The administration of fructose and low-dose streptozotocin to rats have been recently demonstrated to induce insulin resistance, while also partially destroying pancreatic β-cells leading to insufficient insulin secretion and closely mimicking the symptoms observed in human diabetic patients (Wilson and Islam, 2012, Mohammed et al., 2016). In this study, common symptoms of type II DM such as excessive thirst, urination and hunger were observed among diabetic rats. These effects have been attributed to impaired glucose metabolism due to insufficient insulin secretion and insulin resistance in several animal models (Soumya and Srilatha, 2012). Therefore, the amelioration of polyphagia, polydipsia and polyuria as seen in rats treated with CA and metformin (Figures 3.1 and 3.2) suggests an improvement of glucose metabolism by the drugs.

Although the diabetic control rats consumed more food and water in the present study, they had a lower percentage body weight gain than the normal control rats (Table 3.1). Treatments with CA at the dose of 500 mg/kg and metformin increased the body weight gain of diabetic rats. Again, this implies a restoration of glucose homeostasis leading to the proper utilization of this important fuel for body growth and development. Despite the reduction in the mean body weight of diabetic control rats observed in the present study, the weights of kidney and brain were elevated compared to normal control rats (Table 3.2). Generally, abnormal changes in organ weights are common indicators of ill-health in several animal models of diseases. The increase in the weights of kidney and brain are probably due to hypertrophy of the glucose-sensitive organs in response to persistent hyperglycaemic states (Moroz et al., 2008, Zafar and Naqvi, 2010). In contrast, the decrease in liver weights of diabetic rats may result from the hyperglycaemia-induced apoptosis as excess glucose accumulates in the liver (Frances et al.,

2010). The administration of CA however ameliorated the weight changes in the brain of diabetic rats thereby restoring normal growth in the brain.

Hyperglycaemia is the most obvious consequence of type II DM and results from the inability of the body to regulate glucose metabolism. Excess concentration of glucose in the bloodstream causes tissue damage and promotes the development of diabetic complications (King and Loeken, 2004, Giacco and Brownlee, 2010). Restoring normal glucose concentration in type II DM remains the major target of most therapeutic interventions. Although, the anti-hyperglycaemic property of CA had been previously reported with probable mechanisms of actions such as scavenging free radicals and glycated end-products (Rahman et al., 2012) and inhibition of intestinal glucose absorption (Kabir et al., 2014), this is the first study showing the glucose-lowering effects of CA at 500 and 1000 mg/kg doses (Figure 3.3) in a type II diabetic rat model that combines both insulin resistance and insufficiency.

A summary of the alterations in MDA level and antioxidant indices in liver, kidney and brain following the induction of diabetes in rats is presented in Table 4.1. The effects of CA and metformin on diabetes-induced alterations in MDA level and antioxidant indices in liver, kidney and brain in type II diabetic rats are also summarized in Table 4.2. Under diabetic conditions, hyperglycaemia favours the excess production of ROS and RNS leading to oxidative stress that subsequently overwhelms body's antioxidant capacity to protect (Oyenihi et al., 2015). Oxidative stress has been reported to induce dysregulated insulin signaling and destroy pancreatic β-cells leading to the development of type II DM (Rains and Jain, 2011). Lipid peroxidation resulting from oxidation chain reactions induced by ROS and RNS results in the formation of end products such as malondialdehyde (MDA) which has been documented as a primary marker of oxidative stress in diabetic tissues (Kumar et al., 2013). Reports have shown that MDA levels are significantly higher in diabetic rats compared to non-diabetic controls (Aizzat et al., 2010, Hamadi et al., 2012). In type II diabetic subjects, increase in serum levels of MDA and lipid peroxides correlates with the severity of the disease (Peerapatdit and Sriratanasathavorn, 2010). Excess formation of lipid peroxidation end products impair protein functions by forming deleterious cross-links that ultimately damage tissues and promotes the development of diabetic complications (Negre-Salvayre et al., 2008). In the present study, elevated levels of MDA were observed in the liver, kidney and brain of diabetic control rats (Figure 3.4) suggesting an increased state of cellular oxidative injury. Our results also indicate that treatment of diabetic rats with CA at 500 and 1000 mg/kg doses may boost antioxidant

status in rats by inhibiting the formation of lipid peroxidation end products in these tissues. This effect most likely limited oxidative damage not only in the liver, kidney and brain but also in the pancreas another tissue critical in glucose homeostasis.

Table 4.1: Summary of the alterations in MDA level and antioxidant indices in tissues following the induction of diabetes in rats

	MDA	FRAP	ORAC	TEAC	GSH	GST	GPx
Liver	↑	↓	ns	↓	↓	ns	↓
Kidney	↑	↓	↓	ns	↓	ns	↓
Brain	↑	↓	ns	↓	↓	↓	↓

Down arrow [↓] denotes significant ($P<0.05$) decrease while the up arrow [↑] shows significant ($P<0.05$) increase in level or activity of biomarker in diabetic control rats when compared to normal control rats; ns indicates no significant difference was observed in level or activity of biomarkers between DC and NC rats.

Table 4.2: Summary of the effects of CA and metformin on diabetes-induced alterations in MDA level and antioxidant indices in tissues in type II diabetic rats

	MDA	FRAP	ORAC	TEAC	GSH	GST	GPx
Liver	↓ ↓ ↓	↑ ↑ ↑	ns	↑	↑ ↑ ↑	↑ ↑	↑
Kidney	↓	↑ ↑ ↑	↑ ↑ ↑	ns	↑ ↑ ↑	↑	↑ ↑ ↑
Brain	↓ ↓ ↓	↑ ↑ ↑	↑	↑ ↑ ↑	↑	↑ ↑ ↑	↑ ↑ ↑

Down arrows [↓], [↓] and [↓] denote significant ($P<0.05$) decrease in level or activity of biomarker in D500, D1000 and DME rats respectively when compared to DC rats. Up arrows [↑], [↑] and [↑] show significant ($P<0.05$) increase in level or activity of biomarkers in D500, D1000 and DME rats respectively when compared to DC rats; ns indicate no significant difference was observed in level or activity of biomarkers between diabetic control and treated rats.

The capability of a tissue to take up peroxyl radicals, scavenge free radicals and reduce ferric ions to ferrous ions as determined by oxygen radical absorbance capacity (ORAC), trolox equivalent absorbance capacity (TEAC) and ferric reducing antioxidant power (FRAP) values respectively, can be used to assess the ability to withstand the onslaught of excess ROS and RNS observed in diabetes (Katalinic et al., 2005, Ayepola et al., 2014). Decrease in the values of these indices have been observed in diabetic subjects (Mancino et al., 2011) and rats (Ayeleso et al., 2014) when compared to their non-diabetic counterparts. In this study, induction of diabetes in rats resulted in reduction in levels of ORAC (kidney) and TEAC (liver and brain) in addition to a general decrease in FRAP level in all tissues studied. These effects indicated that the antioxidant capacity of tissues to withstand cellular damage was depleted probably as a result of an elevation in hyperglycaemia-mediated oxidative stress that was propagated by increased formation of lipid peroxidation end products. In further support of the antioxidant action of CA, an improvement in antioxidant capacity was observed in diabetic rats given CA. This was evidenced by an increase in the levels of ORAC and TEAC in kidney and brain respectively in addition to the elevated FRAP values in all tissues studied in CA-treated (500 and 1000 mg/kg doses) rats.

Oxidative stress was also assessed by the activities of antioxidant enzymes such as glutathione S-transferase (GST) and glutathione peroxidase (GPx) and non-enzymatic antioxidant level of reduced glutathione (GSH). Similar to other studies (Kasznicki et al., 2012, Gawlik et al., 2016), the data obtained in the present investigation also revealed that diabetes induced alterations in the functions and amount of these antioxidants (Tables 3.3 -3.5). The tripeptide GSH, a prominent non-enzymatic antioxidant, plays a major role in restoring other antioxidants such as vitamins C and E to reduced states, itself becoming oxidized in the process and requiring NADPH to re-convert it back to its reduced form (Valko et al., 2007). GSH has also been shown to directly inactivate H_2O_2 in the presence of GPx and detoxify numerous reactive species like lipid peroxides by the catalytic actions of GST (Farombi et al., 2013). Therefore, the maintenance of GSH level and actions of the GSH-dependent enzymes are very critical to the antioxidant defense mechanism of a cell (Ghosh et al., 2015). In the present study, depletions in GSH level and GPx activity were observed in liver, kidney and brain tissues of diabetic rats. These effects suggest the increased utilization of GSH and GPx against excess RONS such as lipid peroxides that was greater than their synthesis or activation. However, treatment of diabetic rats with CA restored both antioxidants to near-normalcy indicating that oxidative stress in tissues was reduced. Interestingly, the level of GSH increase in CA-treated

rats was significantly higher than the normal rats which suggests that CA has the capacity to activate GSH synthesis to the point of overcompensating its depletion that resulted from diabetes-induced oxidative stress. Although the activity of GST did not differ between the diabetic control and normal control rats in liver and kidney; it was reduced in the brain. The increase in the activity of GST by CA in all tissues studied implies an increased rate of conversion of excess highly reactive metabolites to less toxic, more hydrophilic conjugates that were easily excreted from the body thereby reducing oxidative tissue damage. In addition, the increase in the activity of GPx seen in CA-treated rats may help tissues convert ROS such as H_2O_2 to excretable products that can effectively halt the propagation of oxidative reactions. The effects of CA on GSH level, GPx and GST activities in diabetic tissues are comparable to those observed in metformin-treated rats except the GSH level in brain and GST activity in kidney where no significant difference was observed in comparison to those of diabetic control rats.

The summary of the changes in levels of pro-inflammatory and anti-inflammatory cytokines in liver, kidney and brain that result from induction of diabetes in rats is given in Table 4.3. The effects of CA and metformin treatment of type II diabetic rats in levels of pro-inflammatory and anti-inflammatory cytokines in liver, kidney and brain are summarized in Table 4.4. There is compelling evidence that inflammation, a constant companion of oxidative stress participates centrally in the pathogenesis/pathophysiology of diabetes and its complications (Vinagre et al., 2014). Hyperglycaemia-induced over-production of RONS in type II DM is a ‘perfect’ condition for inflammatory processes to thrive. Under this environment, macrophages, monocytes and leukocytes are massively recruited into cells leading to the activation of pro-inflammatory mediators that further contributes to the progression of the disease (Vinagre et al., 2014). Increased levels of pro-inflammatory cytokines have been reported in type II DM (Akash et al., 2013, Cruz et al., 2013). Activities of these mediators lead to destruction of pancreatic β-cells by inducing apoptosis as well as initiating insulin resistance by inhibiting proteins of the insulin signaling pathways (Esser et al., 2014). In this study, the induction of diabetes increased the production of IL-1β, IL-6, MCP-1 and TNF-α in liver; IL-1α, INF-γ and TNF-α in kidney; INF-γ and TNF-α in brain in rats. TNF-*α* has been implicated to play a key role in insulin resistance *via* reduction of insulin receptor activity, the depression of the insulin receptor substrate (IRS)-1 and glucose transporter (GLUT)-4 (Evans et al., 2005). TNF-α also produces ROS, increases endothelial cell permeability, alters intraglomerular haemodynamics

and induces apoptosis that contribute to tissue damage (Navarro-Gonzalez et al., 2011). Therefore, a reduction in TNF-α levels in all tissues studied upon treatment with CA may help prevent TNF-α-mediated tissue damage in diabetic rats. Increased production of IL-1 pro-inflammatory cytokines lead to multiple protein phosphorylations and activation of phosphatases such as enzymes in the JNK pathway in pancreatic β-cells that eventually result in insulin dysfunction (Major and Wolf, 2001, Banerjee and Saxena, 2012). IL-1α and IL-1β have also been shown to 'partner' with TNF-α in the induction of NO that mediates cell death (Banerjee and Saxena, 2012). The significant reductions of IL-1α level in kidney and IL-1β level in liver in diabetic rats treated with CA protect these tissues from damage and may help increase insulin secretion in the pancreas of diabetic rats. INF-γ has been reported to cause harmful effects on pancreatic β-cell viability through the activation of molecular signaling pathways like signal transducer and activator of transcription-1 (STAT-1) and has been shown to upregulate iNOS expression *via* IL-1β activation in insulin-producing cells (Kacheva et al., 2011). A decrease in the production of INF-γ in kidney and brain in diabetic rats upon treatment with CA as revealed in this study, may improve pancreatic β-cell functions leading to more insulin secretion. Similarly, the reduction in MCP-1 level in liver and brain of diabetic rats following treatment with CA suggests a reduction in the recruitment of monocytes into the tissues that can terminate the activation of pro-inflammatory processes.

Furthermore, IL-4 and IL-10 are generally recognized as anti-inflammatory cytokines due to their capacity to regulate the release and activities of pro-inflammatory cytokines although this classification may have been over-simplification as inflammatory responses are more complicated involving several other influencing factors such as site of actions, type of stimulus etc (Cavaillon, 2001). In this study, the significant increases observed in the hepatic levels of IL-4 and IL-10 following induction of diabetes in rats may have been triggered as a direct response to the increasing concentrations of IL-1β, IL-6, MCP-1 and TNF-α. The administration of CA and metformin only ameliorated the changes in hepatic IL-4 levels but not those involving IL-10. On the other hand, lower level of IL-10 observed in the brain and kidney of diabetic rats was significantly increased after CA-treatment. The renal level of IL-4 did not differ significantly in CA-treated diabetic rats compared to diabetic control rats, while elevated level of IL-4 was observed in the brain. Differences in the concentrations of these inflammatory cytokines in different tissues and their varying responses to treatments suggest their complexity in regulating inflammatory and immunological processes in diabetes (Galley

and Webster, 1996, Opal and DePalo, 2000). It is noteworthy to mention that the different doses of CA used to treat diabetic rats in this study may also elicit different cytokine responses. For example, the dose of 500 mg/kg significantly decreased TNF-α concentration in the liver of diabetic rats, while the 1000 mg/kg dose increased TNF-α though statistically insignificant when compared to that of diabetic controls. In the brain, a 500 mg/kg dose considerably increased IL-4 levels to 150 % and 194 % of those in NC and DC group respectively, while that of 1000 mg/kg dose reduced to 68 % and 88 % of NC and DC group respectively. These differences in cytokine levels suggest that the effects of CA on some inflammatory cytokine responses in diabetes may be dose-dependent.

Table 4.3: Summary of the changes in levels of pro-inflammatory and anti-inflammatory cytokines in tissues following the induction of diabetes in rats

	TNF-α	MCP-1	IL-4	IL-10	IFN-γ	IL- 1α	IL-1β	IL-6	MIP-1α
Liver	↑	↑	↑	↑	—	—	↑	↑	—
Kidney	↑	↓	ns	↓	↑	↑	—	—	—
Brain	↑	ns	↓	↓	↑	—	—	—	ns

Down arrow [↓] denotes significant (P<0.05) decrease while the up arrow [↑] shows significant (P<0.05) increase in level or activity of biomarker in diabetic control rats when compared to normal control rats; ns indicates no significant difference was observed in level or activity of biomarkers between DC and NC rats.

Table 4.4: Summary of the effects of CA and metformin on levels of pro-inflammatory and anti-inflammatory cytokines in tissues in type II diabetic rats

	TNF-α	MCP-1	IL-4	IL-10	IFN-γ	IL- 1α	IL-1β	IL-6	MIP-1α
Liver	↓ ↓	↓ ↓ ↓	↓ ↓	ns	—	—	↓	↓	—
Kidney	↓ ↓ ↓	ns	↓	↑ ↑	↓ ↓	↓ ↓	—	—	—
Brain	↓ ↓ ↓	↓ ↓	↑	↑ ↑ ↑	↓ ↓ ↓	—	—	—	↓

Down arrows [↓], [↓] and [↓] denote significant ($P<0.05$) decrease in level or activity of biomarkers in D500, D1000 and DME rats respectively when compared to DC rats. Up arrows [↑], [↑] and [↑] show significant ($P<0.05$) increase in level or activity of biomarker in D500, D1000 and DME rats respectively when compared to DC rats; ns indicate no significant difference was observed in level or activity of biomarkers between diabetic control and treated rats.

Considering the role of inflammatory processes in the promotion of hepatic, renal and neuronal injury in diabetes, the anti-inflammatory activities of CA as observed in the present investigation may be a mechanism to protect tissues from cytokine-mediated toxicity that can be of therapeutic relevance in halting the progression of complications of type II DM.

The secretion of insulin by pancreatic β-cells activate insulin receptor substrates (IRS) (especially IRS 1 & 2) and the subsequent binding of phosphatidylinositol-3-kinase (PI3K) leads to the recruitment of protein kinase B (PKB/Akt). The activation of Akt is thought to be responsible for most of insulin's metabolic actions (Frojdo et al., 2009, Rains and Jain, 2011). Besides its role in insulin-mediated glucose metabolism and transport, Akt activation has been shown to regulate key proteins in other cellular processes such as apoptosis, cell proliferation and NO production (Zdychova and Komers, 2005). For example, activated Akt has been shown to phosphorylate inhibitor of κB (IκB) kinase allowing the degradation of IκB that results in the translocation and activation of NF-κB. In the nucleus, NF-κB induces the transcription of pro-inflammatory mediatiors responsible for cell survival (Ozes et al., 1999, Romashkova and Makarov, 1999). In type II DM, hyperglycaemia has been shown to cause dysregulation of

normal insulin signaling by activating PKC which in turn increases serine/threonine phosphorylation of insulin receptor. The phosphorylation decreases insulin-stimulated tyrosine phosphorylation of IRS, PI3K activation and ultimately reduces Akt activation contributing to insulin resistance (Zierath and Kawano, 2003). Several experimental studies have confirmed decreased Akt phosphorylation in diabetic animal models (Shao et al., 2000, Bruss et al., 2005, Choi and Kim, 2010, Khorami et al., 2015). However, most of these reports were based on effects observed in the presence of above-normal insulin concentrations. In the present study, serine phosphorylation of Akt was increased in muscle of diabetic rats compared to normal rats but was reduced in CA and metformin-treated rats after 30 mins (Figure 3.8). It seems probable that hyperglycaemia, in the presence of normal or reduced insulin levels, independently increases p-Akt concentration in muscle indicating that glucose may also directly influence IRS-PI3K-Akt signaling in type II DM. Other studies have also reported elevated Akt activation in brain (Clodfelder-Miller et al., 2005), glomeruli, podocytes (Mavroeidi et al., 2013), and platelets (Stolla et al., 2013) of diabetic animals. The elevated phosphorylation of Akt in diabetic rats may also activate the NF-κB-dependent transcription of pro-inflammatory genes. The muscle Akt-phosphorylating effect was however reduced in CA and metformin-treated rats after 30 mins probably due to a decrease in excess blood glucose level in treated rats. At 60 mins, the p-Akt levels in all groups considerably diminished when compared with their levels at 30 mins (Figure 3.8 and 3.9) and the p-Akt level in DC rats at 60 mins was almost equal to that of NC rats (Figure 3.9). Treatment of diabetic rats with CA at 500 mg/kg dose seemingly retained the phosphorylation of Akt while that of D1000 rats was about the same as NC rats at this time point.

In the present study, the induction of diabetes slightly reduced GS and GP protein expression as well as decreased GS activity in muscle of rats. The glycogen level in muscle of diabetic rats was also depleted in comparison to normal rats. These effects suggest a dysregulation in insulin signaling and downstream effects in the muscle of diabetic rats. The activity of GS is regulated by an allosteric phosphorylation/dephosphorylation mechanism. The phosphorylation of GS by glycogen synthase kinase-3 (GSK-3) results in its inhibition while dephosphorylation by protein phosphatase type 1 (PP1) leads to its activation. The activity of GSK-3 is also tightly regulated *via* phosphorylation by Akt (Cohen et al., 1997, Taylor et al., 2006). Alterations in gene expression and activities of Akt, GSK-3 and PP1 leading to GS inhibition in skeletal muscle have been reported in diabetes (Nikoulina et al., 2000, Munro et al., 2005, Kaidanovich-Beilin and Eldar-Finkelman, 2006, Khorami et al., 2015). Decreased

protein expression of GS and GP has also been reported in skeletal muscle of diabetic rats (Reynet et al., 1996, Mukundwa et al., 2016). The reduction in muscle glycogen levels in diabetic rats is probably due to the decrease in GS protein expression and activity suggesting an inhibition of glycogenesis in the rats. Treatment of diabetic rats with CA at 500 and 1000 mg/kg doses reduced GS protein expression in muscle (Figure 3.10). Also, the dose of 1000 mg/kg significantly increased activities of GS and GP; and increased glycogen concentration in muscle when compared to diabetic control rats. These effects imply that CA may regulate glycogen metabolism in diabetes *via* alterations in protein expression and phosphorylation/dephosphorylation states of GS and GP. The dual activating effects of CA on GS and GP activities result in the elevation of muscle glycogen concentration, likely due to increased activation of GS which seems to overcome the transient activation of GP thus, favoring glycogen synthesis. Metformin treatment of diabetic rats however, increased muscle glycogenesis by decreasing GP protein expression and activity while also inducing GS activity.

In the liver, there was a drastic reduction in glycogen concentration in diabetic rats with a concomitant decrease in GS activity while GP activity remain unchanged when compared to normal rats. Diabetes has been reported to cause changes in the insulin signaling pathway that ultimately increases the phosphorylation of GS thereby deactivating it, and leading to decreased glycogen synthesis in the liver (Dimitriadis et al., 2011). When glycogenesis is suppressed, excess glucose is not stored but rather accumulates in the tissues and blood, worsening diabetic conditions (Inzucchi et al., 2012). In the present study, treatment of diabetic rats with CA at a dose of 1000 mg/kg increased hepatic GS activity (Figure 3.13) which may partly explain the resulting increase in hepatic glycogen level as excess glucose is being stored. Surprisingly, the treatment of diabetic rats with CA at 500 mg/kg increased hepatic GP activity but a higher dose of 1000 mg/kg caused a decrease in GP activity. This effect observed in the D1000-treated rats may have counteracted the effect of the lower dose as the net effect is increased glucose storage in the form of glycogen. Metformin treatment of diabetic rats increased the GS activity but did not lead to elevated glycogen level in the liver.

Insulin resistance, a prominent feature in type II DM has been associated with dyslipidaemia which further exacerbates the disease conditions (Gadi and Samaha, 2007). Diabetes-induced hyperlipidaemia or dyslipidaemia is a major risk factor for atherosclerosis and cardiovascular complications. Hyperlipidaemia can be easily described as increased blood levels of total cholesterol (Tc), triglycerides (TG), low-density lipoproteins cholesterol (LDL-c) and

decreased high-density lipoproteins cholesterol (HDL-c) (Mahamuni et al., 2012, Ma et al., 2014b). Abnormal levels of lipids have been observed in type II diabetic patients (de Vries et al., 2015) and diabetic rat model (Li et al., 2011). An elevation of serum TG and Tc levels following the induction of diabetes observed in the present study may be due to the presence of excess blood glucose level which stimulate hepatic *de novo* lipogenesis rather than glycogenesis (Jornayvaz et al., 2010, Taskinen and Boren, 2015). Increased serum TG and Tc concentrations have been reported to further promote the over-production of LDL-c that may accumulate within the vascular arterial walls forming plaques implicated in the development of atherosclerosis and coronary heart diseases (Gadi and Samaha, 2007, Mahamuni et al., 2012). Treatment of diabetic rats with CA at 500 mg/kg dose however, significantly lowered the high serum TG concentration (Table 3.6) indicating that it has anti-hyperlipidaemic effects that could be beneficial in the management of type II DM. HDL has been reported to play cardio-protective roles by transporting excess cholesterol from peripheral tissues into the liver for subsequent excretion (Van Linthout et al., 2010). Therefore, increase in serum HDL-c levels of diabetic control rats in the present study may indicate its accumulation and hence increased release into the serum from the liver. Elevated serum HDL-c have also been reported in type II diabetic patients (Maduka et al., 2007). In addition, the increase in LDL-c values observed in diabetic rats in this study (though not statistically significant) is probably due to increase in TG and Tc. However, treatment with CA at a dose of 1000 mg/kg reduced LDL-c value to below normal (though not statistically significant) suggesting the plant may help delay the development of plaques in the vascular arterial walls. Furthermore, the oxidation of excess circulating free fatty acids synthesized as a result of increased hyperglycaemia-induced hepatic *de novo* lipogenesis may trigger the formation of advanced lipid end-products (ALEs). These ALEs cause pancreatic β-cell apoptosis and dysfunction *via* induction of iNOS, IL-1 and TNF-α dependent cellular stress pathways (Boada and Martinez-Moreno, 2013). The ability of CA to restore blood glucose concentration to near-normal levels as observed in this study, may also contribute to the prevention of ALE-mediated pancreatic β-cell destruction.

Pancreatic β-cells are highly responsive to glucose concentration in the blood. This property is important in regulating glucose homeostasis and metabolism through stimulating insulin secretion/actions (Cernea and Dobreanu, 2013). Under hyperglycaemic environment such as that seen in type II DM, pancreatic β-cells become highly susceptible to damage leading to reduction in insulin secretions. High blood glucose concentration has been shown to

downregulate insulin gene expression (Poitout and Robertson, 2008, Kim and Yoon, 2011); contribute to endoplasmic reticulum stress (Prentki and Nolan, 2006) and increase β-cell apoptosis (Butler et al., 2003, Donath et al., 2005). In addition, hyperglycaemia contributes to oxidative stress through the over-activation of hexosamine pathway that causes pancreatic β-cell dysfunction (Kaneto et al., 2006). Impairment in the activities of antioxidant enzymes under hyperglycaemic conditions also make the β-cells highly vulnerable to oxidative damage (Leibowitz et al., 2011). In the present study, diabetes caused alterations in the morphology of pancreatic β islets (Figure 3.15) that may cause a reduction in pancreatic β-cell population. However, the daily administration of CA for 14 days at both 500 and 1000 mg/kg doses ameliorated diabetes-induced pancreatic β islets damage probably by decreasing hyperglycaemia, inhibiting the formation of MDA as well as enhancing cellular antioxidant capacity. These effects can lead to increased insulin secretion/actions in type II DM.

Plant-derived compounds possessing therapeutic potentials have been leading the way in the search for novel antidiabetic drugs. Recently, thin layer chromatography (TLC) and liquid chromatography-mass spectroscopy (LC-MS) techniques have revealed the presence of triterpenes (asiatic acid, asiaticoside, madecassic acid, madecassoside, centellasaponins) and flavonoids (quercetin, kaempferol) in the crude ethanol extract of CA. The pharmacological activities of these extracts were attributed to the presence of these compounds (James and Dubery, 2011, Maulidiani et al., 2014). GC-MS analysis of the crude methanol extract of CA leaves used in this study identified ascorbic acid, asiatic acid, oleanolic acid, stevioside, stigmasterol and α-humulene among several other compounds. Notably, asiatic acid has been reported to reduce blood glucose by modulating key enzymes of carbohydrate metabolism in diabetic rats (Ramachandran and Saravanan, 2013) and ameliorates metabolic alterations in metabolic syndrome rats (Pakdeechote et al., 2014). Also, stevioside and stigmasterol have been previously reported to exhibit potent antidiabetic and antioxidant properties in animal models of DM (Wang et al., 2012c, Kumar et al., 2013). Ascorbic acid and oleanolic acid are potent antioxidants that have been utilized as a supplementary or alternative medicine in the management of type II DM (Dakhale et al., 2011, Lee et al., 2016). Oleanolic acid was recently shown to induce insulin-mediated changes in Akt signaling and downstream effects in skeletal muscle of diabetic rats (Mukundwa et al., 2016). In addition, α-humulene has shown exemplary anti-inflammatory activities in a variety of animal models of diseases (Medeiros et al., 2007, Ramos et al., 2013). Therefore, the presence of a mixture of these medicinal compounds in the

methanol extract of the leaves of CA may be responsible for the effects observed in the CA-treated type II diabetic rats in this study.

CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

This study demonstrates for the first time the *in vivo* effects of *Centella asiatica (L.) Urban* on insulin signaling and downstream processes, inflammatory cytokines and oxidative stress in a type II diabetic animal model that exhibits both insulin insufficiency and resistance. In addition, this may be the first study reporting the presence of ascorbic acid and stevioside in methanol extract of CA leaves that is identified by GC-MS analysis.

The overall data obtained from this research revealed the promising potentials of leaves of *Centella asiatica (L.) Urban* in the management of type II *diabetes mellitus* primarily due to its anti-hyperglycaemic and anti-hyperlipidaemic properties. Its modulatory effect on inflammatory processes through reduction in the levels of crucial pro-inflammatory cytokines and elevation of anti-inflammatory cytokines may be an important mechanism by which the plant help limit diabetic damage to the liver, kidney and brain in diabetic rats thereby preventing the progression to diabetic complications. The insulin-independent effects of the plant on insulin signaling and glycogen metabolism that result in increased glycogen level in the muscle and liver of diabetic rats may also contribute meaningfully to the overall antidiabetic qualities. The protective effects of *Centella asiatica (L.) Urban* against oxidative stress proven by the elevation in the synthesis and activities of antioxidants in addition to inhibiting lipid peroxidation in the tissues of diabetic rats is also notable. The ability of *Centella asiatica (L.) Urban* to ameliorate diabetes-induced abnormalities in pancreatic β islets may be important in increasing the secretion of insulin needed to regulate hyperglycaemia in type II *diabetes mellitus*. The pharmacological actions of the plant are probably attributed to the presence and combinations of a plethora of phyto-compounds within its leaves that act with different but synergistic cellular mechanisms.

Limitations of study and recommendations

Although this research suggested that crude methanol extract of leaves of *Centella asiatica (L.) Urban* had effects on hyperglycaemia, hyperlipidaemia, oxidative stress, inflammatory cytokine levels, insulin signaling and glycogen metabolism in type II *diabetes mellitus*, further studies that focus on specific molecular targets such as NF-κB, Nrf2, ARE, JNK, etc that are relevant to the pathophysiology of type II *diabetes mellitus* will be helpful. This will assist in proper understanding of the molecular basis of the antidiabetic properties of the plant. In addition, the effects of *Centella asiatica (L.) Urban* on p-Akt signaling should be investigated

in detail in view of the results obtained at 60 and 90 mins after treatment in this study. Other proteins and enzymes in the insulin signaling pathway can be studied to be able to conclude on the probable mechanisms of *Centella asiatica (L.) Urban* in type II *diabetes mellitus.*

Also, a more comprehensive isolation and identification of phyto-compounds present within the leaves of *Centella asiatica (L.) Urban* are required that may lead to the discovery of an antidiabetic drug or analogues in the near future.

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APPENDIX

15 December 2014

Reference: 024/15/Animal

Mr A Oyenihi
Biochemistry
School of Life Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Oyenihi

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2015** on the following project:

"Antidiabetic properties of *Catharanthus roseus* and *Centella asiatica* in Type II diabetic rats."

Yours sincerely

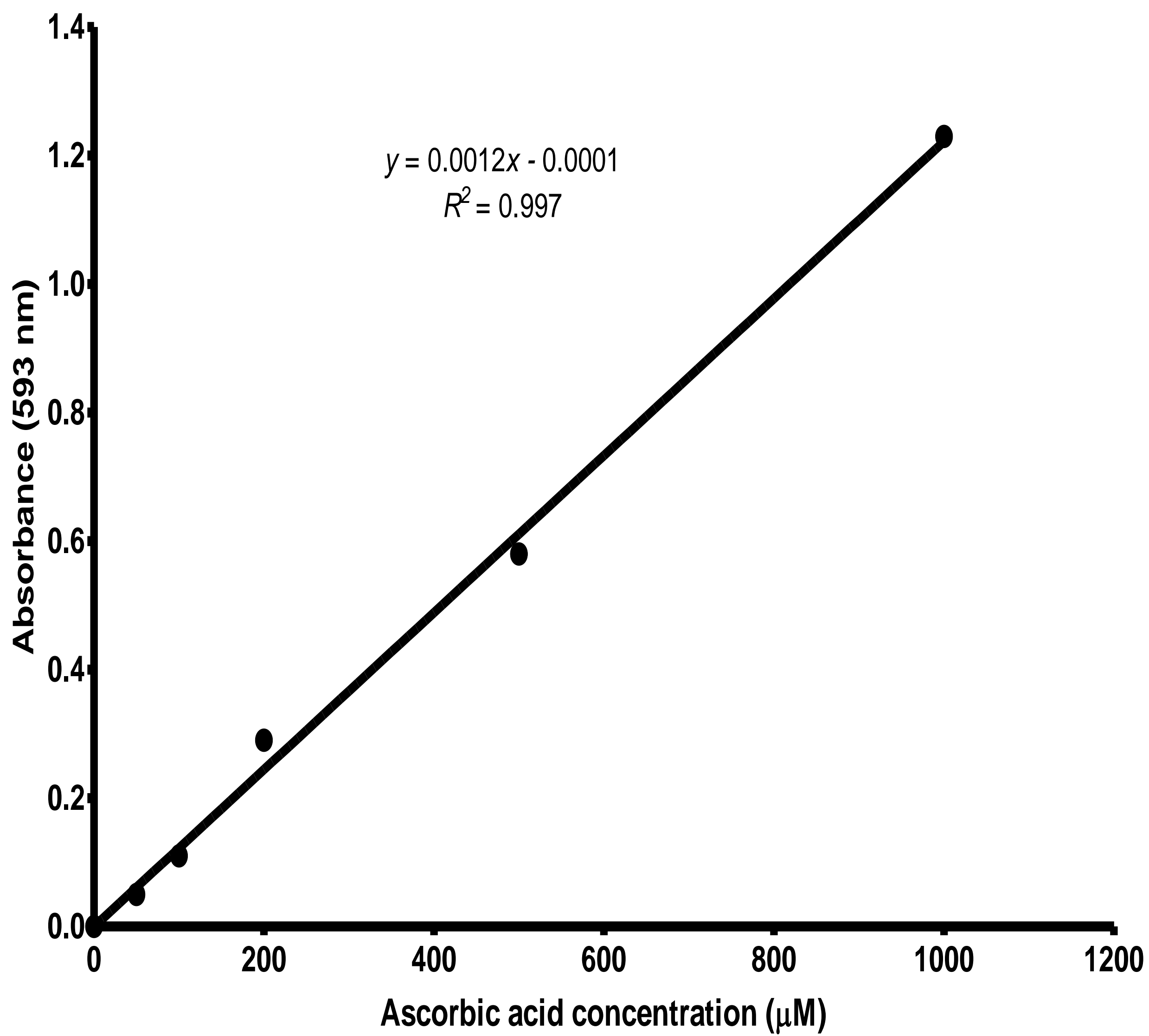
Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar
Research Office – Dr N Singh
Supervisor – Dr B Masola
Head of School – Prof. S Mukaratirwa
BRU – Dr S Singh

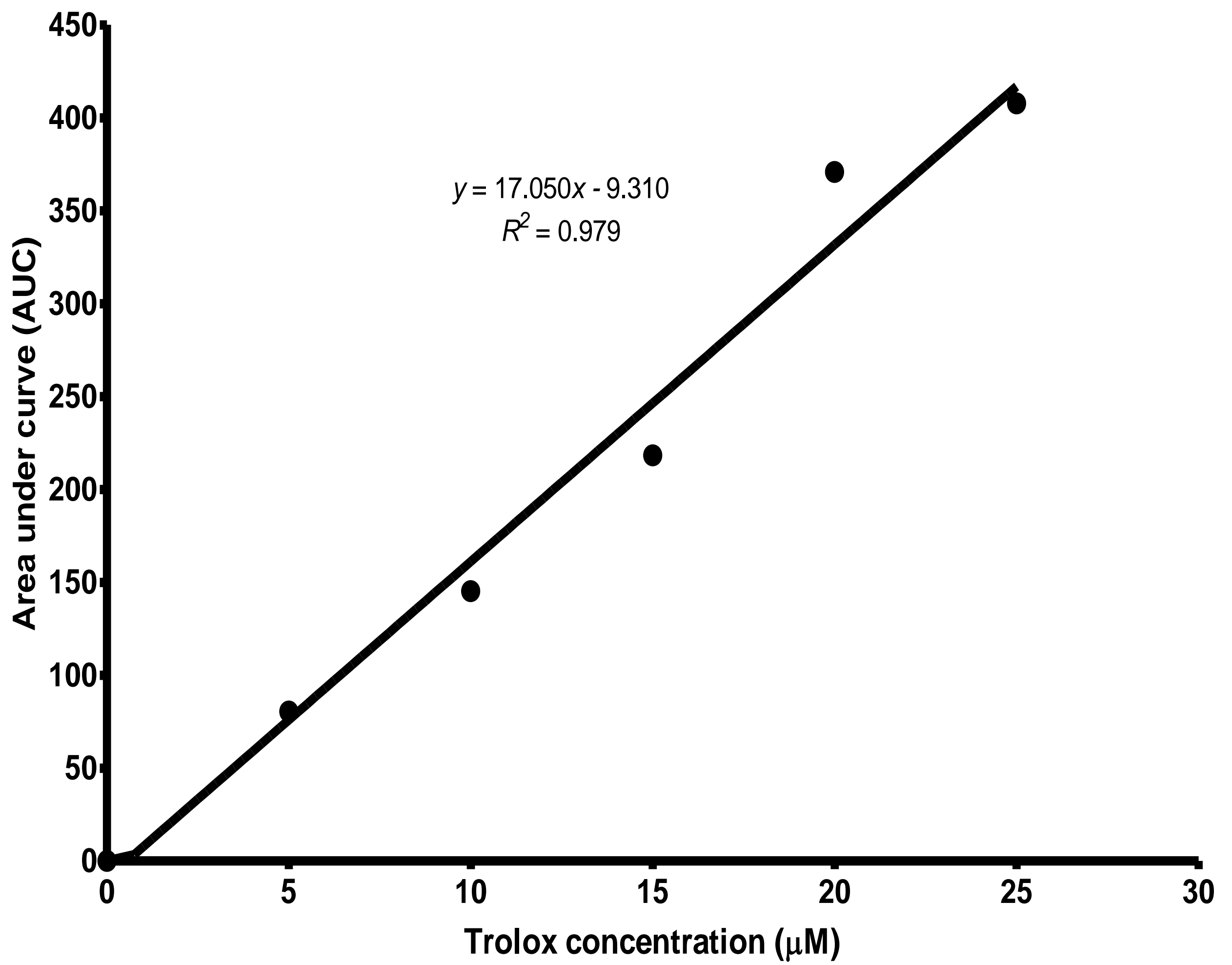
Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
phone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
unding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

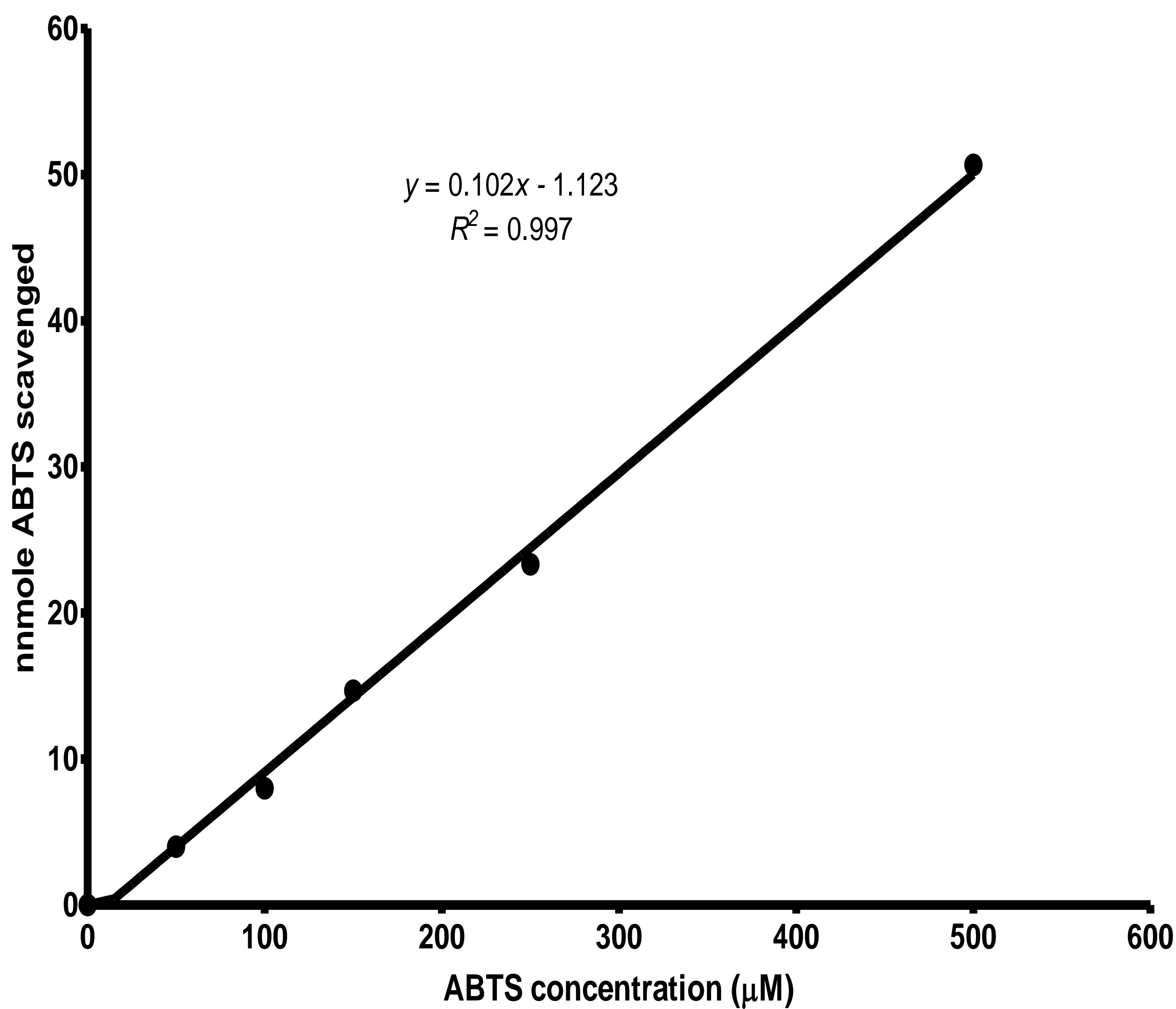
Appendix 1: The clearance letter issued by the University Animal Research Ethics Committee to conduct *in vivo* study using Sprague-Dawley rats



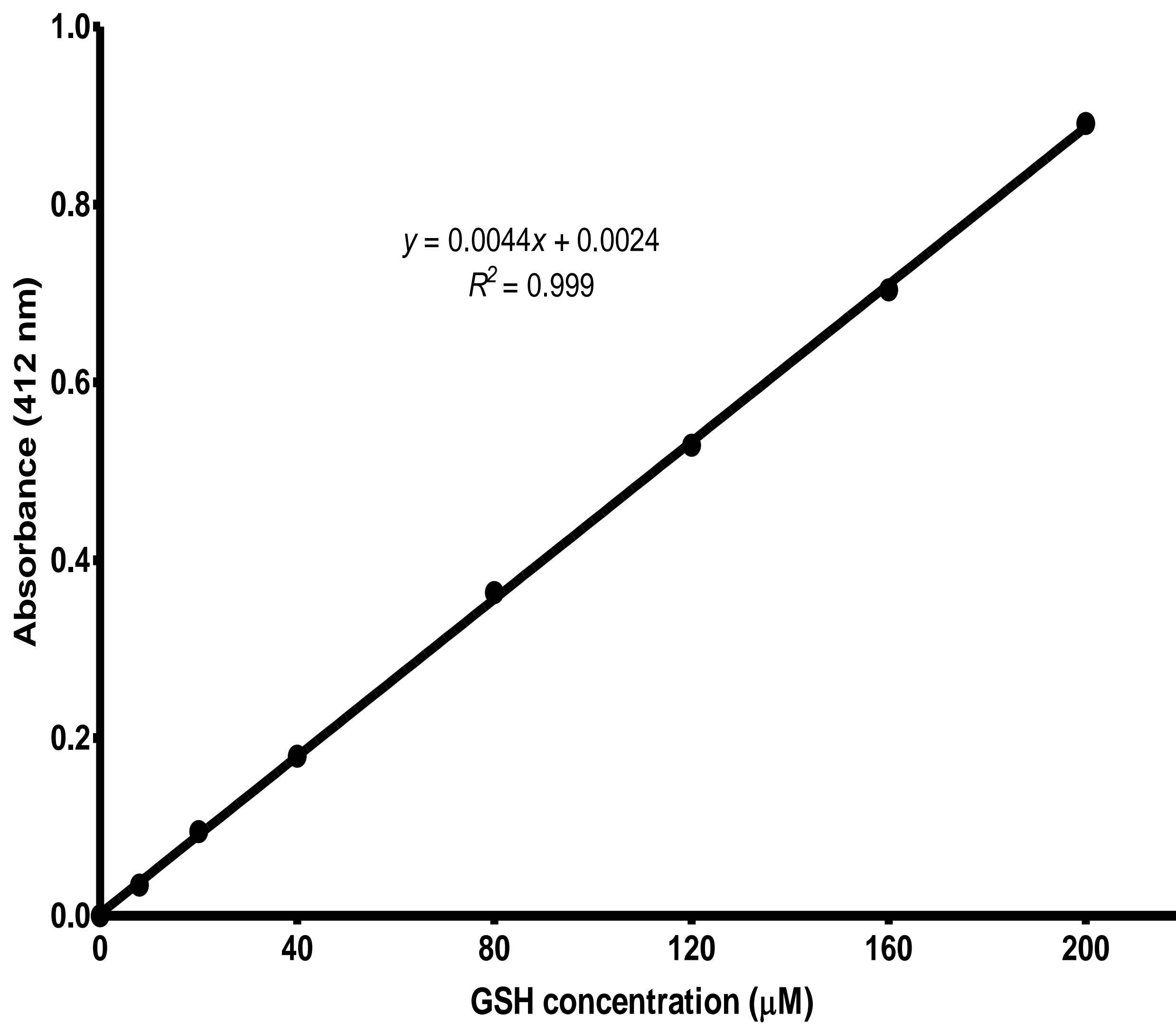
Appendix 2: Standard ascorbic acid curve used in the determination of FRAP



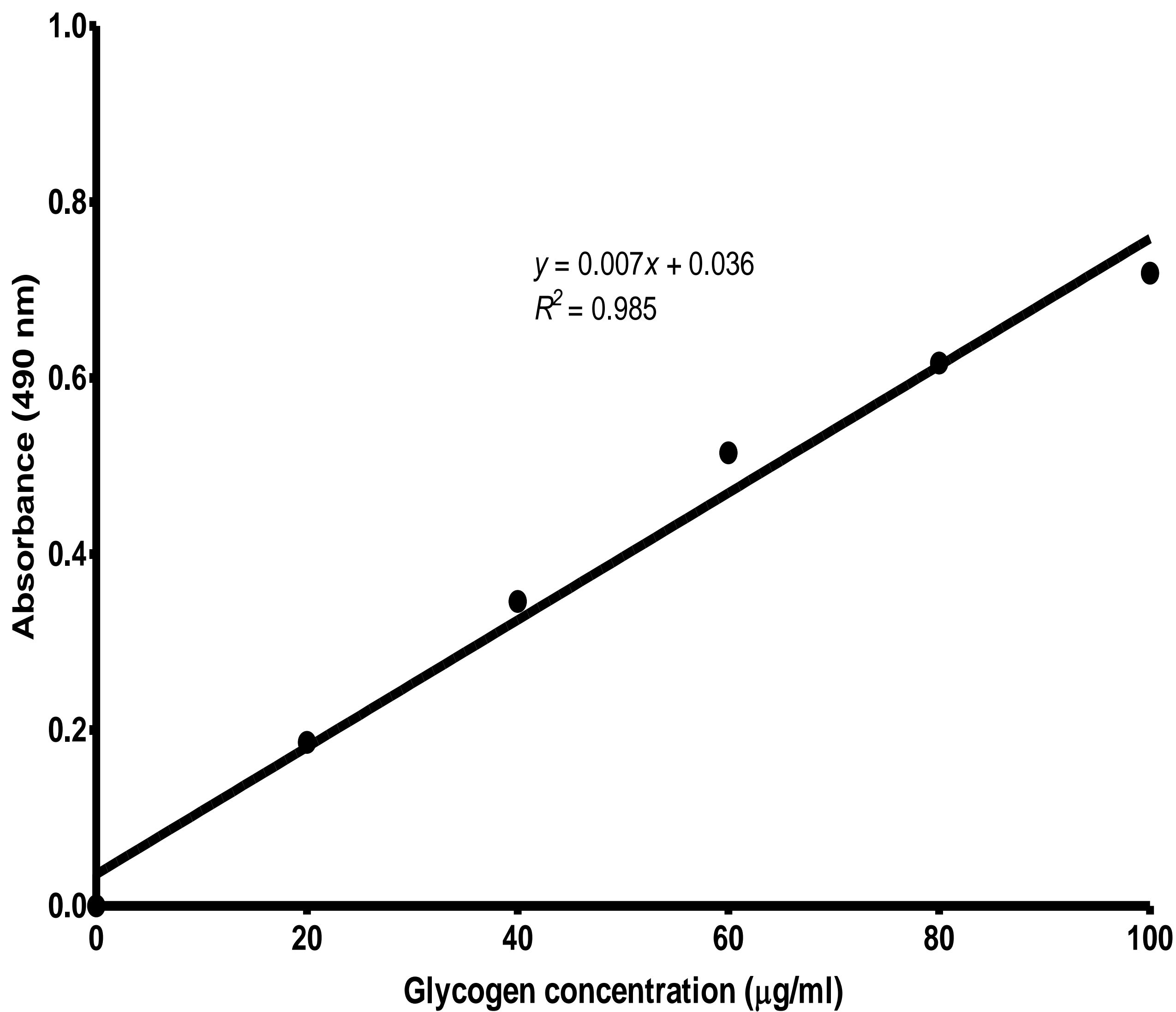
Appendix 3: Standard Trolox curve used in the determination of ORAC



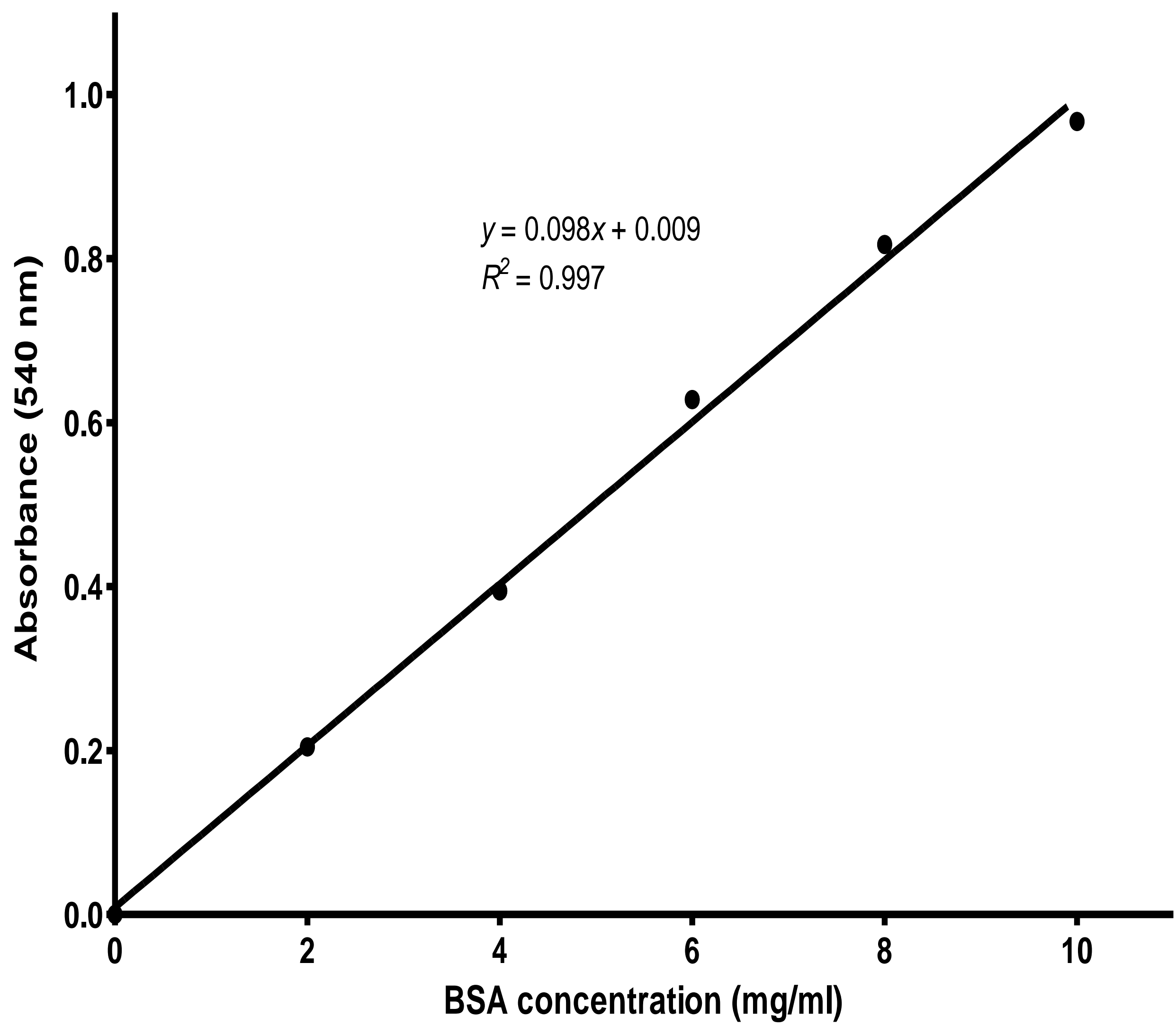
Appendix 4: Standard ABTS curve used in the determination of TEAC



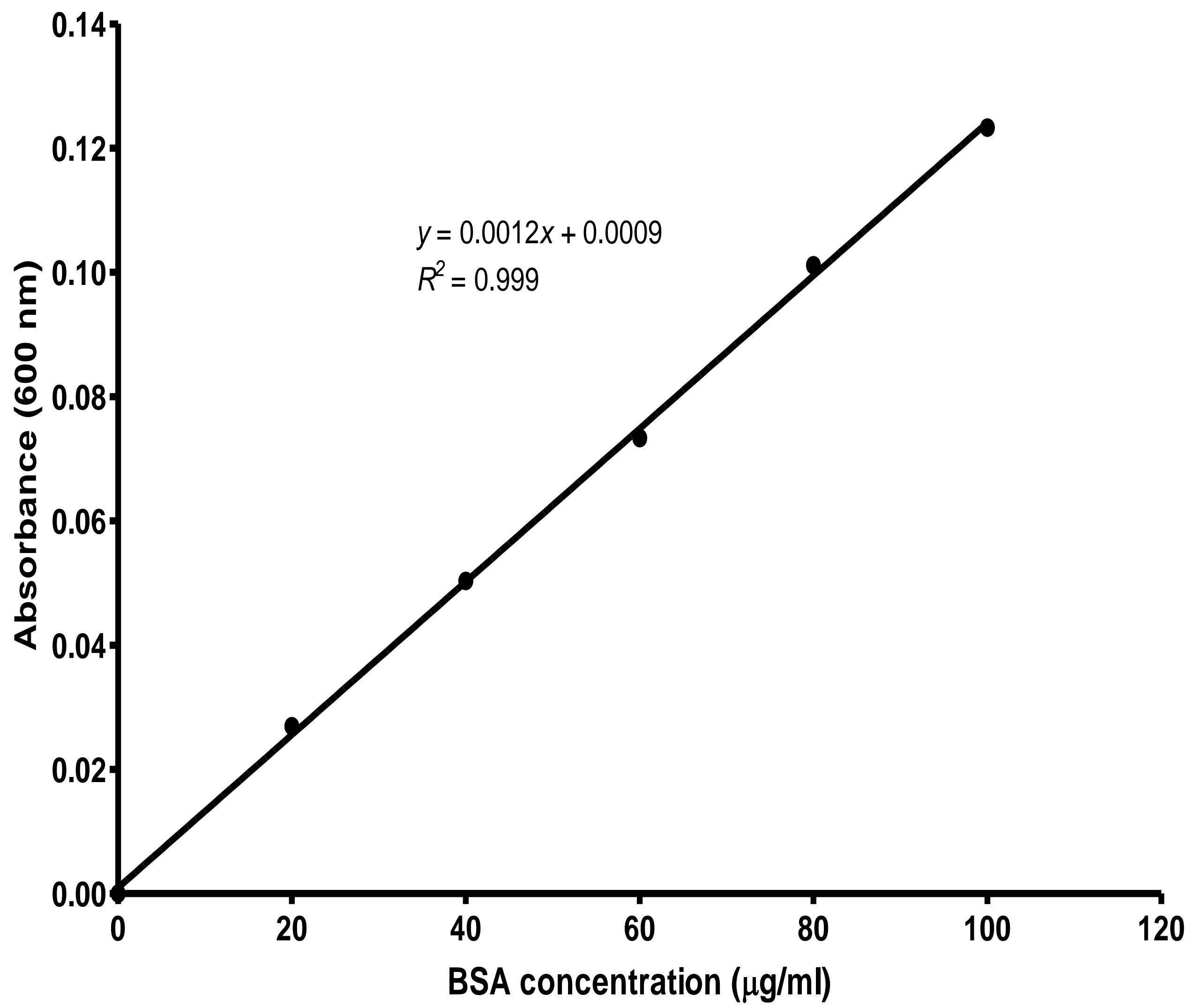
Appendix 5: Standard GSH curve used in the determination of GSH concentration in samples



Appendix 6: Standard glycogen curve used in the determination of glycogen level in samples



Appendix 7: Standard BSA curve used in the Biuret method of protein determination



Appendix 7: Standard BSA curve used in the Lowry method of protein determination