

The *in vitro* and *in vivo* anti-oxidative and anti-diabetic effects of some African medicinal plants and the identification of the bioactive compounds

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Submitted in fulfillment of the academic requirements for the degree of Doctor of Philosophy in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville campus), Durban 4000, South Africa

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COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCES

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DECLARATION 2 - PUBLICATIONS AND PRESENTATIONS

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 designed the work, performed all the experiments and wrote all the publications. The co-authors contributed by conducting an editorial work, checking the scientific content of the work and my correct interpretation of the findings.

Already published papers from this thesis:

Publication 1

Ibrahim, M. A., Koorbanally, N. A., Kiplimo, J. J. Islam, M. S. (2012). Anti-oxidative activities of the various extracts of stem bark, root and leaves of *Ziziphus mucronata* (Rhamnaceae) *in vitro*. **Journal of Medicinal Plant Research**. 6(25): 4176-4184.

Publication 2

Ibrahim, M. A., Koorbanally, N. A. and Islam, M. S. (2013). *In vitro* anti-oxidative activities and GC-MS analysis of various solvent extracts of *Cassia singueana* parts. **Acta Poloniae Pharmaceutica**. 70(4): 709-719.

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Ibrahim, M. A., Koorbanally, N. A. and Islam, M. S. (2013) *In vitro* anti-oxidative activities of various parts of *Parkia biglobosa* and GC-MS analysis of extracts with high antioxidant activity. **African Journal of Traditional, Complementary and Alternatives Medicines**. 10(5): 282-291

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Ibrahim M. A., Koorbanally N. A. and Islam M. S. Butanol fraction of *Ziziphus mucronata* (Willd) root ethanolic extract contains anti-oxidative agents and potent inhibitors of α -glucosidase and α -amylase. Submitted to the **Indian Journal of Experimental Biology in April, 2013**.

Publication 8

Ibrahim M. A., Koorbanally N. A. and Islam M. S. Anti-oxidative activity and inhibition of key enzymes linked to type 2 diabetes (α -glucosidase and α -amylase) by *Khaya senegalensis*. Submitted to **Acta Pharmaceutica** in August, 2013

Publication 9

Ibrahim M. A., Koorbanally N. A. and Islam M. S. Acetone fraction of *Cassia singueana* stem bark ethyl acetate extract contains anti-oxidative agents and potent inhibitors of α -glucosidase and α -amylase. (**in preparation**)

Publication 10

Ibrahim M. A., Koorbanally N. A. and Islam M. S. Butanol fraction of *Parkia biglobosa* leaves ethanol extract contains anti-oxidative agents and potent inhibitors of α -glucosidase and α -amylase. (**in preparation**)

Publication 11

Ibrahim M. A. and Islam M. S. Effects of butanol fraction of *Ziziphus mucronata* root on glucose homeostasis, serum insulin, hepatic glycogen metabolism and diabetes-related complications in a type 2 diabetes model of rats. (**in preparation**)

Publication 12

Ibrahim M. A., Koorbanally N. A. and Islam M. S. Butanol fraction of *Parkia biglobosa* leaves modulates β cell functions, stimulates insulin secretion and ameliorates diabetic complications in a type 2 diabetes model of rats. (**in preparation**)

Publication 13

Ibrahim M. A., Koorbanally N. A. and Islam M. S. Anti-diabetic activity of butanol fraction of *Khaya senegalensis* root in a type 2 diabetes model of rats and the isolation of bicyclo [2.2.0]hexane-2,3,5-triol as a possible bioactive compound. (**in preparation**)

Publication 14

Ibrahim M. A. and Islam M. S. Modulation of *in vivo* antioxidant status by the butanol fraction of *Ziziphus mucronata* root in a type 2 diabetes model of rats. (**in preparation**)

Publication 15

Ibrahim M. A. and Islam M. S. Modulation of *in vivo* anti-oxidative status by the acetone fraction of *Cassia singueana* stem bark in a type 2 diabetes model of rats. (**in preparation**)

Publication 16

Ibrahim M. A. and Islam M. S. The anti-diabetic activity of the butanol fraction of *Parkia biglobosa* leaves in a type 2 diabetes model of rats could be mediated via modulation of *in vivo* antioxidant status. (**in preparation**)

Publication 17

Ibrahim M. A. and Islam M. S. Attenuation of *in vivo* antioxidant status could be involved in the anti-diabetic activity of the butanol fraction of *Khaya senegalensis* root in a type 2 diabetes model of rats. (**in preparation**)

PRESENTATIONS

Ibrahim M. A. (2012). *In vitro* and *in vivo* anti-diabetic potentials of *Parkia biglobosa*: possible application in the management of type 2 diabetes. A research paper presented at the **School of Life Sciences Research Day, University of KwaZulu Natal**, Pietermaritzburg campus, South Africa on 20th November, 2012.

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Ibrahim M. A., Habila J. D., Koorbanally N. and Islam M. S. (2013). Anti-diabetic activity of acetone fraction of *Cassia singueana* stem bark in a type 2 diabetes model of rats and the isolation of 3 β -O-acetyl betulinic acid as possible bioactive compound. Presented at the **The Three's Company Pharmacy Conference for Independent Community Pharmacy Association, Academy of Pharmaceutical Sciences of South Africa, South African Association of Pharmacists in Industry and South African Society of Basic and Clinical Pharmacology** at Lagoon Beach Hotel, Cape Town, South Africa from 4th – 6th October, 2013. Book of Abstract. P64.

Ibrahim M. A., Habila J. D., Koorbanally N. and Islam M. S. (2013). Anti-diabetic activity of acetone fraction of *Cassia singueana* stem bark in a type 2 diabetes model of rats and the isolation of 3 β -O-acetyl betulinic acid as possible bioactive compound. Presented at the **Postgraduate Research Day of the College of Agriculture, Engineering and Sciences, University of KwaZulu Natal**, Howard college campus, Durban, South Africa on 1st November, 2013.

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PREFACE

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.



Candidate: Auwal Mohammed Ibrahim

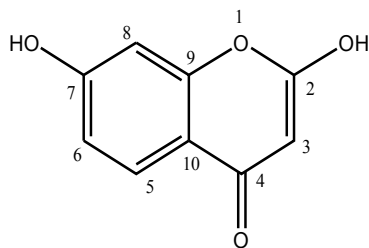
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Supervisor: Dr. M. S. Islam

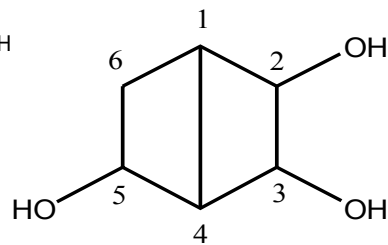
ABSTRACT OF THE THESIS

This thesis examined the *in vitro* and *in vivo* anti-oxidative and anti-diabetic activities of five African medicinal plants which are traditionally used for the treatment of diabetes mellitus viz; *Ziziphus mucronata*, *Cassia singueana*, *Parkia biglobosa*, *Khaya senegalensis* and *Vitex doniana*. Ethanol, ethyl acetate and aqueous crude extracts of the stem bark, root and leaf samples of each of the plants (a total of 45 crude extracts) were investigated for detailed anti-oxidative activity and the most active crude extract from each plant was selected for further fractionation with solvents of increasing polarity. Subsequently, the solvent fractions derived from these crude extracts (a total of 21 fractions) were also subjected to the anti-oxidative assays as well as α -glucosidase and α -amylase inhibitory activities assays. Results from these assays revealed that the butanol fractions from crude extracts of *Z. mucronata*, *P. biglobosa*, *K. senegalensis* and *V. doniana* and the acetone fraction from the crude extract of *C. singueana* were the most bioactive. Kinetic delineation of the types of enzyme inhibitions exerted by these most active fractions as well as measurement of relevant kinetic parameters (K_M , V_{max} and K_i) were done using Lineweaver-Burke's plot. Furthermore, the most active fractions were also subjected to GC-MS analysis and *in vivo* intervention trial in a type 2 diabetes (T2D) model of rats (except fraction from *V. doniana*). The *in vivo* studies revealed that all the fractions possessed potent *in vivo* anti-T2D activity (to varying extent) and the possible mechanisms of actions were proposed. Furthermore, most of the fractions were able to ameliorate the T2D-associated complications. Analysis of *in vivo* oxidative stress markers such as glutathione, thiobarbituric acid reactive substances, superoxide dismutase and catalase in the serum, liver, kidney, heart and pancreas of the animals also gave a clue in to the possible mechanism of action. Bioassay guided isolation was used to track the bioactive anti-diabetic compounds from these fractions via column chromatography. The isolated compounds were characterized by 1H NMR, ^{13}C NMR, 2D NMR (in two cases) and mass spectroscopy (in one case). From this study, 2,7-dihydroxy-4*H*-1-benzopyran-4-one, 3 β -*O*-acetyl betulinic acid, lupeol and bicyclo[2.2.0]hexane-2,3,5-triol were identified as the possible bioactive compounds from *Z. mucronata*, *C. singueana*, *P. biglobosa*, *K. senegalensis* solvent fractions respectively. The findings of this work are important for the relevant government agencies, pharmaceutical industries, scientific community and poor diabetic patients because it might open an avenue for the development of viable and cost effective anti-diabetic herbal products and/or novel plant-derived anti-diabetic drugs.

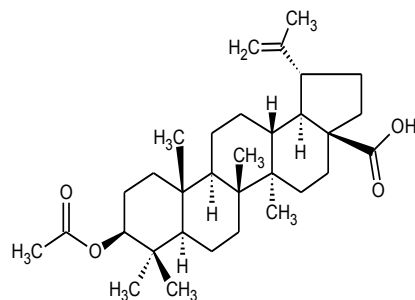
Structure of the isolated anti-diabetic compounds



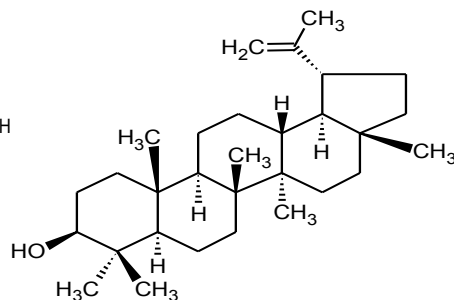
2,7-dihydroxy-4*H*-1-benzopyran-4-one



bicyclo[2.2.0]hexane-2,3,5-triol



3β-*O*-acetyl betulinic acid



Lupeol

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LIST OF ABBREVIATIONS

2D NMR	two dimensional nuclear magnetic resonance
ADP	adenosine diphosphate
AGE	advanced glycation end products
ANG	angiotensin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AO	antioxidants
AR	aldose reductase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BRU	Biomedical Resource Unit
Bw	body weight
¹³ C NMR	carbon 13 nuclear magnetic resonance
CSAF	acetone fraction derived from the ethylacetate crude extract of <i>Cassia singueana</i> stem bark
COSY	correlated spectroscopy
CVD	cardiovascular disease
DAG	diacyl glycerol
DBC	diabetic control
DCL	group of diabetic rats treated with a low dose of a acetone fraction of <i>Cassia singueana</i> stem bark
DCH	group of diabetic rats treated with a high dose of a acetone fraction of <i>Cassia singueana</i> stem bark
dd	double doublet
DEPT	distortionless enhancement by polarization transfer
DETAPAC	diethylenetriaminepentaacetic acid
DFL	group of diabetic rats treated with a low dose of a fraction
DFH	group of diabetic rats treated with a high dose of a fraction
DMF	group of diabetic rats treated with metformin

DHAP	dihydroxy acetone phosphate
DKL	group of diabetic rats treated with a low dose of a butanol fraction of <i>Khaya senegalensis</i> root
DKH	group of diabetic rats treated with a high dose of a butanol fraction of <i>Khaya senegalensis</i> root
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
DPPIV	dipeptidyl peptidase IV
DPL	group of diabetic rats treated with a low dose of a butanol fraction of <i>Parkia biglobosa</i> leaves
DPH	group of diabetic rats treated with a high dose of a butanol fraction of <i>Parkia biglobosa</i> leaves
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
DTNB	5,5'-dithiobisnitrobenzoic acid
DZL	group of diabetic rats treated with a low dose of a butanol fraction of <i>Ziziphus mucronata</i> root
DZH	group of diabetic rats treated with a high dose of a butanol fraction of <i>Ziziphus mucronata</i> root
ELISA	enzyme-linked immunosorbent assay
EtOAc	ethyl acetate
EtOH	ethanol
FADH ₂	reduced flavin adenine dinucleotide
FBG	fasting blood glucose
FRAP	Ferric cyanide (Fe ³⁺) reducing antioxidant power
G6PDH	glucose-6-phosphate dehydrogenase
GAE	gallic acid equivalent
GC-MS	gas chromatography-mass spectrometry
GFAT	glutamine:fructose-6-phosphate amidotransferase
GIP	glucose dependent insulinotropic polypeptide
GLP-1	glucagon like peptide-1
GLUT	glucose transporter
GSH	reduced glutathione
GSSG	oxidized glutathione

6-HD	6-hydroxydopamine
HDL	high density lipoprotein
HLA	human leukocyte antigen
HMBC	heteronuclear multiple bond coherence
¹ H NMR	proton nuclear magnetic resonance
HOMA-IR	homeostatic model assessment-insulin resistance
HOMA-β	homeostatic model assessment-β cell function
HRS	hydroxyl radical scavenging
HSQC	heteronuclear single quantum coherence
Hz	Hertz
IDDM	insulin-dependent diabetes mellitus
IDF	international diabetes federation
IGF-1	insulin-like growth factor-1
IGT	impaired glucose tolerant
IRS	insulin receptor substrate
<i>K_{cat}</i>	computed index of physiological efficiency of enzyme
<i>K_i</i>	inhibition binding constant
<i>K_M</i>	Michaelis constant
KSBF	butanol fraction derived from the ethanolic crude extract of <i>Khaya senegalensis</i> root
LDL	low density lipoprotein
MDA	malondialdehyde
MS	mass spectrometry
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NC	Normal control
NCT	group of non-diabetic rats treated with a high dose of a acetone fraction of <i>Cassia singueana</i> stem bark
ND	not determined
NEFA	non-esterified fatty acids
NFBG	non-fasting blood glucose

NFT	group of non-diabetic rats treated with a high dose of a fraction
NIDDM	non-insulin dependent diabetes mellitus
NKT	group of non-diabetic rats treated with a high dose of a butanol fraction of <i>Khaya senegalensis</i> root
NMR	nuclear magnetic resonance
NO	nitric oxide
NOESY	nuclear overhauser effect spectroscopy
NPT	group of non-diabetic rats treated with a high dose of a butanol fraction of <i>Parkia biglobosa</i> leaves
NZT	group of non-diabetic rats treated with a high dose of a butanol fraction of <i>Ziziphus mucronata</i> root
OGTT	oral glucose tolerance test
OS	oxidative stress
PBBF	butanol fraction derived from the ethanolic crude extract of <i>Parkia biglobosa</i> leaves
pNPG	p-nitrophenyl- α -D-glucopyranoside
PKC	protein kinase C
PPAR δ	peroxisome-proliferator-activated receptor gamma
ROS	reactive oxygen species
RT	retention time
SD	standard deviation
SDS	sodium dodecyl sulphate
SGLT	sodium-glucose cotransporter
SOD	superoxide dismutase
STZ	streptozotocin
T2D	Type 2 diabetes
T1D	Type 1 Diabetes
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	tricarboxylic acid
TLC	thin layer chromatography
TNF	tumor necrosis factor

Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UDP	Uridine diphosphate
VEGF	vascular endothelial growth factor
V _{max}	maximum velocity
WHO	world health organization
WK	week
ZMBF	butanol fraction derived from the ethanolic crude extract of <i>Ziziphus mucronata</i> root

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 DIABETES MELLITUS

Diabetes mellitus could be defined as a heterogeneous group of a complex metabolic disorder associated with high blood glucose concentrations (hyperglycemia) and alterations in the metabolism of major macromolecules resulting from impairments in the secretion of insulin or its action [1, 2]. Diabetes is commonly accompanied with polydipsia, polyuria, microvascular problems involving eyes, kidney and peripheral nerves as well as cardiovascular problems such as hypertension [3, 4]. These complications affect about 50% of diabetic patients and can lead to their death [5, 6] thereby making diabetes a recognized fatal disease in different parts of the world for centuries.

Insulin is a hormone synthesized by the β -cells at the pancreatic isles of Langerhans and its primary role is to tightly control blood glucose levels which usually rise after dietary intake. Thus, insulin is released from the pancreatic β -cells to normalize the glucose level. Therefore, in diabetic patients, anomalies in the production of insulin and/or its utilization cause hyperglycemia. Insulin is also vital to carbohydrates and lipids metabolism because it lowers blood glucose levels by enhancing the glucose uptake by cells and by stimulating glycogenesis as well as inhibiting glycogenolysis. It also retards the breakdown of fats to free fatty acids and ketone bodies [7]. This hormone also encourages the storage of fat into the adipose tissues and reduces gluconeogenesis in liver and kidneys.

At present, diabetes is the fifth major cause of death in the world affecting 366 million individuals globally and this figure is projected to increase to a staggering 552 million by the year 2030 as reported by the International Diabetes Federation [8]. It is also projected that the greatest number of diabetic patients are between 40-59 years of age and about 50% of diabetic cases are still undiagnosed [8]. In 2011 alone, 4.6 million deaths were attributed to diabetes and about USD 465 billion dollars were spent as healthcare expenditures due to diabetes [8]. In Africa, the prevalence rate of diabetes is 4.3% and the region was reported to have the highest mortality rate due to the disease [9]. More specifically, South African population is greatly affected by diabetes because it is the fourth major cause of death in the country [10] affecting all race groups. However, the Indian origin-South Africans have the highest prevalence because of a gene pool that predisposes them to diabetes [10, 11]. Also the black community is at risk because of rapid lifestyle and cultural changes [12].

1.1 CLASSIFICATION

Diabetes mellitus is broadly classified, based on the clinical manifestations, into two main types such as type 1 diabetes (T1D) or insulin-dependent diabetes mellitus (IDDM) and type 2 diabetes (T2D) or non-

insulin dependent diabetes mellitus (NIDDM). However, a number of authors have also recognized a third category called gestational diabetes or type 3 diabetes [13, 14]. Apart from these common types of diabetes, another type of diabetes is also found rarely called diabetes insipidus where the synthesis, transport or release of anti-diuretic hormone (vasopressin) are impaired resulting in polyuria and polydipsia [15].

1.1.1 Type 1 Diabetes

Type 1 diabetes (T1D) is responsible for approximately 5-10% of the total diabetic patients worldwide. It is caused by destruction of the insulin-secreting pancreatic β -cells by cellular mediated autoimmunity which leads to complete deficiency of insulin in the circulation. A number of factors have been proposed for the autoimmune destruction of the β -cells which includes immunological, genetic and environmental factors. The first proposal on the pathogenesis of T1D was made by George Eisenbarth in 1986 that observed the presence of one or more autoantibodies to islet cells in diabetic patients with polyendocrine deficiencies and consequently proposed a chronic autoimmune process caused by unknown factors as the basis for the destruction of the β -cells by autoreactive lymphocytes [16]. Subsequently, autoantibodies to insulin, glutamic acid decarboxylase and tyrosine phosphatases were also implicated [17] in the pathogenesis of T1D. On the other hand, the most powerful genetic risk factor for the disease pathogenesis is associated with human leukocyte antigen (HLA) locus which is categorized into class I and II. The class II HLA locus is the high-risk which represents the strongest genetic association with T1D [18]. However, apart from HLA loci, over 40 non-HLA polymorphisms that are associated with T1D have also been identified through genome-wide association studies and other novel loci have also been confirmed in prospective population-based studies [18]. The environmental factors involved in the pathogenesis of T1D include parasites, viruses and bacteria which mediate the direct infection of the pancreatic β -cells or shape the immune system to mutually benefit the parasite and the host [19, 20].

Apart from hyperglycemia, diabetic ketoacidosis is another hallmark of T1D which is caused by decreased glucose utilization by body tissues with concomitant increase in lipid catabolism to compensate for the body energy demand [21]. Prolonged lipid catabolism leads to the accumulation of acetyl coA which could trigger ketogenesis with concomitant disturbances in the body's pH homeostatic mechanism (ketoacidosis) and death could result if proper action is not taken. Other important features of T1D are polyuria, polydipsia, polyphagia, unexplained weight loss, lethargy, fatigue and abdominal pain [21]. Insulin therapy is the most predominantly and commonly used method for the treatment of T1D. However, because of the very low prevalence of T1D, most of the diabetes related deaths are caused by the T2D and its associated complications.

1.1.2 Type 2 Diabetes

Type 2 diabetes (T2D) could be defined as a complex metabolic disorder that is associated with a gradual loss in the action of insulin (insulin resistance), followed by the failure of pancreatic β -cells to account for the insulin resistance (β -cell dysfunction) [2].

T2D is a product of a complex interaction between environmental and genetic factors. The genetic factors are insulin resistance and β -cell dysfunction, whereas weight gain, physical inactivity or sedentary life style and oxidative stress exacerbate these metabolic abnormalities. At present, a two-step process has been proposed for the pathogenesis of T2D [22]. In step one, normal individuals progress to impaired glucose tolerant (IGT) individuals with insulin resistance as the primary marker. Blood insulin levels are increased, and β -cell function is usually impaired [23]. In the second step, IGT progresses to T2D due to a decline in β -cell function.

This dissertation intends to mainly focus on T2D and therefore the pathogenesis of the two main features of the disease (insulin resistance and pancreatic β -cell dysfunction) are elaborated below. Furthermore, all subsequent sub-headings (within this chapter) and indeed other chapters will mainly focus on T2D.

1.1.2.1 Insulin resistance

Insulin resistance could be defined as impaired insulin-mediated glucose uptake in skeletal muscle and inhibition of hepatic glucose synthesis. However, in the fasting state, the muscle is also responsible for a small percentage of glucose disposals (less than 20%) while endogenous glucose production accounts for all the glucose that enters the blood circulation. Endogenous glucose production is elevated in T2D patients or impaired fasting glucose [24]. Therefore, the driving force of hyperglycemia in T2D is hepatic insulin resistance. Research findings have indicated that a number of factors act individually or synergistically in the development of insulin resistance [2]. These factors include obesity, molecular defects in insulin signaling and oxidative stress.

❖ Obesity

Sedentary life style, physical inactivity and obesity are important causes of insulin resistance in the developed world. The mechanisms involved appeared to be complex but cytokines and circulating hormones as well as metabolic fuels such as non-esterified fatty acids (NEFA) in the adipocyte are implicated in the modulation of insulin activity. An increase in the mass of stored triglycerides in adipose tissues usually leads to large adipocytes which resist the insulin action towards retarding lipids breakdown. Consequently, an elevation in the levels of glycerol and circulating NEFA occurs and these are known to stimulate insulin resistance in liver and skeletal muscle [25]. Although the mechanism of fatty acid-induced insulin resistance is not completely understood, the most important concept is that fatty acids interfere with insulin signaling events via serine phosphorylation of insulin receptor substrate-1

(IRS-1) by protein kinase C [26]. Another molecular target involved in the inhibition of insulin action is tumor necrosis factor- α (TNF- α) which blocks insulin signaling by altering the interactions between IRS-1 and insulin receptor [27]. Resistin is also another adipocyte-associated factor linked to the obesity-mediated insulin resistance which acts via deactivation of AMP-kinase and consequently impairs the normal effects of this enzyme on transcriptionally regulated gluconeogenic enzymes. This suggests that resistin has an important regulatory role over hepatic glucose production in obese subjects.

❖ **Molecular mechanisms involved in insulin resistance**

In order to understand how molecular defects in insulin signaling causes insulin resistance, some basic understanding into the molecular mechanisms involved in insulin signaling pathway is paramount.

The molecular events via which insulin promotes glucose uptake by target cells commences with the binding of the hormone to specific cell surface receptors with tyrosine kinase activity that are located on insulin target tissues [28]. Once the insulin binds the receptor, second messengers are generated which eventually initiate a chain of phosphorylation-dephosphorylation reactions that promote glucose metabolism inside the cells. The first event in intracellular glucose metabolism is the activation of glucose transport system (GLUT4) which leads to influx of glucose into the cells. Upon entry, the free glucose is metabolized by a cascade of enzymatic reactions in glycolytic and TCA cycle pathways. Most of these enzymatic reactions are also directly regulated by insulin. Among these, the most important are hexokinase which catalyze the phosphorylation of glucose, glycogen synthase which catalyzed glycogen synthesis, phosphofructokinase which regulate glycolysis.

Thus, insulin resistance also results due to blockage of one or more of the molecular insulin signaling events listed below.

- Insulin receptor tyrosine kinase activity [28]
- Phosphorylation and dephosphorylation of insulin receptor substrate (IRS) proteins [29]
- Translocation of GLUT 4 and glucose transport [2]
- NF κ B activity

❖ **Oxidative stress**

The involvement of oxidative stress in the different aspect of T2D pathology has been the subject of intensive clinical and biomedical investigations. More specifically, the involvement of oxidative stress in the pathogenesis of insulin resistance also appears to be complicated and poorly understood at the moment. *In vitro* studies have provided evidence that H₂O₂, a reactive oxygen species (ROS), can cause a marked decrease in the insulin stimulation of the insulin signaling proteins (such as IRS and Akt) and of

course, glucose transport activity thereby leading to insulin resistance [30] (**Figure 1.1**). Studies have also shown that other stress-activated serine kinases are involved in the pathogenesis of oxidant-induced insulin resistance. For example, serine kinase I κ B kinase β (IKK β) controls the function of the redox-sensitive transcription factor NF- κ B which leads to stimulation of the expression of proinflammatory genes as well as modulate cell survival, immune responses and metabolic regulation. Interestingly, IKK β could also be activated by ROS and inhibition of IKK β is related with diminished insulin resistance [31]. Interestingly, these studies are strongly supported by several other studies which showed that excess mitochondrial H₂O₂ productions play a pivotal role in causing insulin resistance in the skeletal muscle of individuals with excess energy [31]. On the other hand, the renin-angiotensin system is also associated with skeletal muscle insulin resistance and T2D (**Figure 1.1**). Angiotensin II (ANG II) is an important component of the system and was reported to stimulate the activity of NADPH oxidase to produce superoxide ions through a reduction of oxygen and the oxidation of NADPH (**Figure 1.1**). Interestingly, these NADPH-derived superoxides were found to be critical in the ANG-II induced impairment of insulin signaling systems as well as insulin resistance [31].

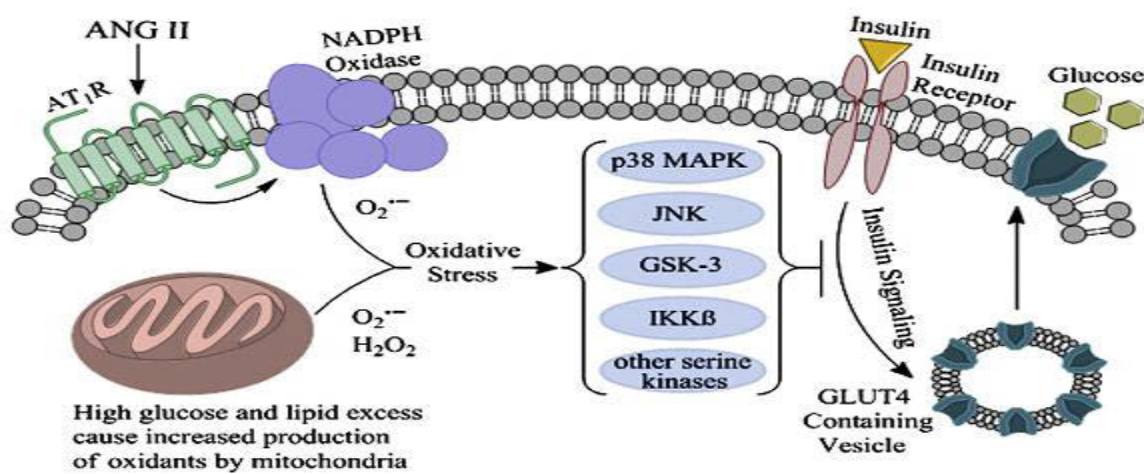


Figure 1.1: The involvement of oxidative stress in the development of insulin resistance. Excess generation of ROS from NADPH oxidase and other mitochondrial sources in muscle leads to the attenuation of stress-activated kinases and consequently diminish insulin- signaling molecular events as well as reduce glucose transport process (Adapted without permission from Henriksen et al. [31])

Apart from *in vitro* experiments, studies with animal models have provided strong evidence for the connection of oxidative stress and insulin resistance. Antioxidant (α -lipoic acid) was found to improve insulin sensitivity in animal models of diabetes and was also reported to have a direct stimulatory effect on GLUT 4 translocation or activation. Furthermore, many clinical trials have revealed that administration of antioxidant vitamins (C and E) or glutathione stimulate insulin sensitivity in human

subjects with insulin resistance [32]. All these findings portray the vital role of oxidative stress-mediated events in causing insulin resistance.

1.1.2.2 Pancreatic β -cell dysfunction

Pancreatic β -cell dysfunction is a gradual progressive decline in insulin secretion and the failure of the β -cells to account for the insulin resistance [33]. Several studies have been carried out to understand the mechanism of β -cell dysfunction in T2D but none of the proposed mechanisms is widely accepted by the experts in the field. However, the hypotheses which have been put forward are briefly discussed below.

❖ Glucose-induced β -cell toxicity

Considerable evidences suggest that hyperglycemia in type 2 diabetic patients contribute to pancreatic β -cell dysfunction although the direct toxic effects of glucose is still an issue of controversy. However, abnormally high glucose level has been reported to impair with the functions of important genes, key enzymes and every major metabolic pathway in the pancreatic β -cells [34]. This suggests that high glucose level is detrimental to the components of insulin producing β -cells leading to decline in insulin secretion.

❖ Beta-cell exhaustion

Several studies have been carried out in animal models and humans that support the β -cell exhaustion. Deterioration of insulin secretion over time has been partly attributed to functional exhaustion in β -cells. Chronic hyperglycemia causes a nonsustainable compensatory increase in insulin secretion which consequently depletes some cellular components for continued insulin secretion [34].

❖ Lipid-induced β -cell toxicity

The effect of lipids on β -cell dysfunction is complex and poorly understood at the moment. However, two mechanisms have been proposed to be involved. The concentrations of free fatty acids are usually increased in type 2 diabetic patients as a result of increased adipocyte lipolysis. The presence of high glucose level inhibits the β -oxidation of these free fatty acids which consequently will lead to the accumulation of long chain fatty acyl coA [35]. Subsequently, the accumulated fatty acyl coA interferes with the normal potassium channel activity and activates uncoupling protein-2 which would uncouple electron transport chain from oxidative phosphorylation leading to diminished ATP production and decreased insulin secretion [36]. Another proposed mechanism for lipid-induced β -cell toxicity involves the biosynthesis of ceramide from free fatty acids. Ceramide was reported to inhibit the expression of insulin gene and can induce β -cell apoptosis via multiple pathways leading to β -cell death [37].

❖ Role of oxidative stress on β -cell dysfunction

Oxidative stress appears to be the common unifying pathogenic factor for the glucose and lipid-induced β -cell dysfunction. The pancreatic β -cells are the most susceptible cells to oxidative stress-induced damages [38] among other cells in the body. This is partly because the β -cells have very low amount of ROS detoxifying enzymes (catalase and superoxide dismutase) as well as redox-regulating enzyme, glutathione peroxidase [39]. It was suggested that hyperglycemia leads to the generation of high amount of ROS via stimulation of mitochondrial electron transport chain resulting in the overproduction of superoxide anions. Also, autooxidation of glucose in the presence of transition metals as well as the generation of advanced glycation end products (AGE) are other various mechanisms of hyperglycemia-induced generation of ROS [40]. On the other hand, *in vitro* and *in vivo* investigations have provided evidences that diminished secretion of insulin and β -cell dysfunction are associated with free fatty acids-induced ROS production [41]. These ROS from the above mentioned sources attack vital cellular components leading to β -cell dysfunction. This is further supported by an *in vitro* study where high blood glucose levels induce mitochondrial ROS and diminished the initial phase of glucose-induced insulin secretion via the suppression of glyceraldehyde-3-phosphate dehydrogenase [42]. An additional mechanism of oxidative stress-induced β -cell dysfunction is via the stimulation of NF κ B which is majorly a pro-apoptotic process in β -cells [38].

❖ Pancreatic islet amyloid

The involvement of amyloidogenic process in the pathophysiology of β -cell dysfunction is not clear at the moment. Islet amyloid is made up of islet amyloid polypeptide deposits called amylin which is simultaneously secreted with insulin and was proposed to act via inhibition of insulin action and/or secretion as well as the inhibition of glucagon. Small aggregates of amyloid polypeptides were found to be cytotoxic to β -cells due to ‘‘channel formation’’ as well as calcium influx into the cells [43]. Thus, it is possible that hyperglycemia promotes amylin aggregation which is in turn cytotoxic to the β cells.

Summarily, although hundreds of studies have been conducted *in vitro* and *in vivo* in order to understand the pathogenesis of β -cell dysfunction, but a number of knowledge-gap still exists in the area. Therefore, more research work is needed to solve some of the puzzles.

1.2 COMPLICATIONS OF TYPE 2 DIABETES

T2D-induced complications affect more than 50% of diabetic patients and accounts for more than 70% of all case-fatalities making these complications the leading mortality cause among type 2 diabetic patients [5, 44]. A number of theories have been put forward to explain the pathophysiology of the vascular complications in T2D which include increase in the metabolic flux of polyol (sorbitol) pathway [45], formation of high amounts of AGE as well as activation of protein kinase C [46]. Interestingly, all these

processes lead to the induction of oxidative stress. Therefore, oxidative stress-mediated events are considered the most important unifying pathogenic factors responsible for the initiation and progression of T2D-associated complications.

1.2.1 Oxidative stress and T2D: focus on ROS generating pathways

Oxidative stress is the most important mechanism that unites the pathogenesis of all diabetic complications and indeed plays a role in all other aspects of diabetes pathology. Therefore, in this sub chapter, oxidative stress will be briefly reviewed in the context of T2D with emphasis on the multiple pathways responsible for the induction of this stress.

Oxidative stress refers to the presence of free radicals and ROS which are produced in normal body processes and are usually neutralized by a chain of endogenous antioxidants systems. However, when there is an excess generation of ROS or the antioxidants are inactivated, the ROS/antioxidants equilibrium usually shift in favor of stress, thereby making the ROS become harmful to the body [40]. The most important ROS are superoxides, hydroxyl radicals and peroxides while the endogenous antioxidants are divided into enzymatic (examples are superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (examples are glutathione, vitamins A, C and E). In T2D, hyperglycemia induces oxidative stress via multiple pathways which include glucose autooxidation, increased metabolic flux of the polyol (sorbitol) pathway, increased production of AGE, activation of protein kinase C as well as increased flux of the hexosamine pathway [3, 47].

1.2.1.1 Glucose autooxidation

This is the single hyperglycemia-induced unifying process that links other pathogenic mechanisms of diabetic complications [48]. In T2D, hyperglycemia leads to the overdrive of the TCA cycle with concomitant increase in the number of electron donors (NADH and FADH₂) that enter the electron transport chain. Consequently, the differences in voltage across the membranes of the mitochondria shoot up until a critical threshold is attained when the transfer of electrons within complex III is blocked making the electrons to revert to coenzyme Q. Subsequently, the coenzyme Q donates the electrons, one after the other, to molecular oxygen, thereby producing high amounts of superoxide [48] that cannot be completely be neutralized by the mitochondrial SOD (**Figure 1.2**). It is generally accepted that this mitochondrial-derived overproduction of superoxides is the unifying hypothesis that account for the activation of the four major pathways involved in the induction of oxidative stress in T2D [3].

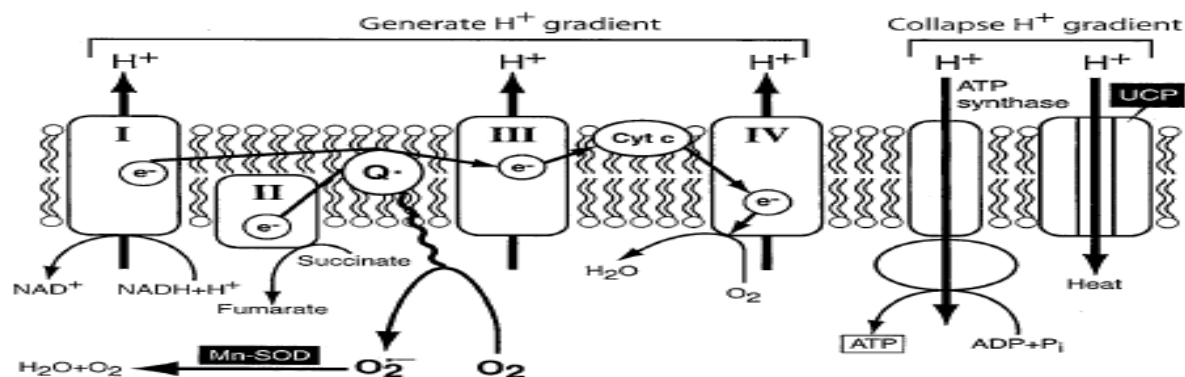


Figure 1.2: Hyperglycemia induced excess generation of superoxides in the mitochondrial electron transport chain (Adapted from Brownlee [48])

1.2.1.2 Increased metabolic flux of polyol pathway

The role of this pathway as a major player in the T2D-associated oxidative stress was first described by Gabbay et al. [49]. Under normal condition, toxic aldehydes are converted to inactive alcohols by NADPH-dependent aldose reductase (AR) but upon elevation of glucose concentration, the glucose first undergoes reduction to produce its sugar alcohol sorbitol catalyzed by AR. Subsequently, NAD-dependent sorbitol dehydrogenase oxidized the sorbitol to fructose (**Figure 1.3**). Hyperactivity of the polyol pathway under diabetic situation leads to high levels of intracellular sorbitol that results in osmotic stress and production of fructose, which is 10 times more powerful as an agent of glycation than glucose [45]. Another biochemical consequence of increased polyol pathway is the consumption of NADPH by AR which consequently affects the regeneration of the most important endogenous non-enzymatic antioxidant, reduced glutathione (**Figure 1.3**). The overall effect of these events is increased susceptibility to intracellular oxidative stress [48].

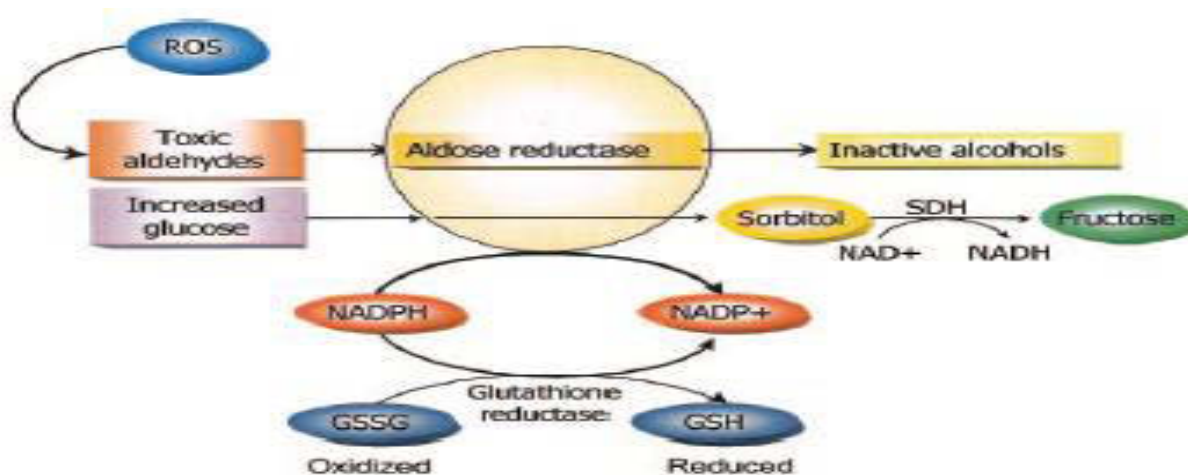


Figure 1.3: Relationship between hyperglycemia, increased polyol pathway and oxidative stress in T2D (Adapted from Brownlee [3]).

1.2.1.3 Increased generation of advanced glycation end products (AGE)

The concept of AGE as biological drivers to T2D-associated oxidative stress started manifesting in 1970s. AGE are a complex group of compounds formed from a non-enzymatic covalent bonding of aldehyde or ketone groups of reducing sugars to the free amine groups on proteins, lipids, or nucleic acids. Research findings have revealed that the major AGE produced *in vivo* is formed from oxoaldehydes or dicarbonyls such as glyoxal, 3-deoxyglucosone and methylglyoxal which are established highly reactive intermediate carbonyl groups. Common examples of AGE in humans are N-carboxymethyl-lysine and pentosidine [50]. The generation of AGEs which is associated by ROS-generating reactions at different steps [51] and under diabetic condition, high glucose stimulates AGEs generation and consequently leads to the overproduction of ROS which cannot be completely neutralized by endogenous antioxidants resulting in oxidative stress. Another ROS-generating step associated with AGEs occurs during binding of the AGEs to various receptors called RAGE [52]. AGEs play a cardinal role in causing biological damages to cells by modifying intracellular proteins involved in gene transcription or modifying extracellular matrix molecules as well as blood proteins like albumin [48].

1.2.1.4 Activation of protein kinase C (PKC)

PKC is a threonine/serine kinase that performs a vital role in signal transduction processes and respond to specific neuronal, hormonal and growth factor stimuli. This enzyme catalyzes the transfer of phosphate moieties from ATP to various substrate proteins, while the enzyme itself undergoes a series of complex phosphorylation steps before it is activated and translocated from the cytosol to the cell membrane [53]. Under normal physiological state, the activation of PKC is achieved in cells through pathways that produce diacyl glycerol (DAG). In hyperglycemic or diabetic situation, there is usually an increase in the metabolic flux of glycolysis which increases the levels of the glycolytic intermediate, dihydroxyacetone phosphate (DHAP). The DHAP is reduced to glycerol-3-phosphate, which subsequently promotes the de novo synthesis of DAG [54] leading to profound activation of PKC (**Figure 1.4**). Apart from this major PKC activation pathway, this enzyme is activated by oxidants such as H_2O_2 in a manner unrelated to lipid second messengers and by mitochondrial superoxide induced by elevated glucose levels [55].

The activation of PKC has been linked to the induction of oxidative stress and a number of metabolic and functional aberrations in T2D. Upon activation of PKC under diabetic condition, NADPH oxidase which is the most important source of ROS production in blood vessels is consequently activated by PKC-dependent mechanisms (**Figure 1.4**) and this is thought to be the most essential path responsible for increased ROS production in blood vessels [56]. Furthermore, the pathway has been implicated as a vital process in the increased vascular permeability and neovascularization as well as decreased Na^+-K^+ ATPase activity observed in diabetic animals [57]. Indeed, most of the diabetes-induced vascular complications are associated, at least in part, with the PKC-dependent increase in ROS [53, 55]. A

schematic representation of the biological effects of PKC activation under diabetic condition is provided below.

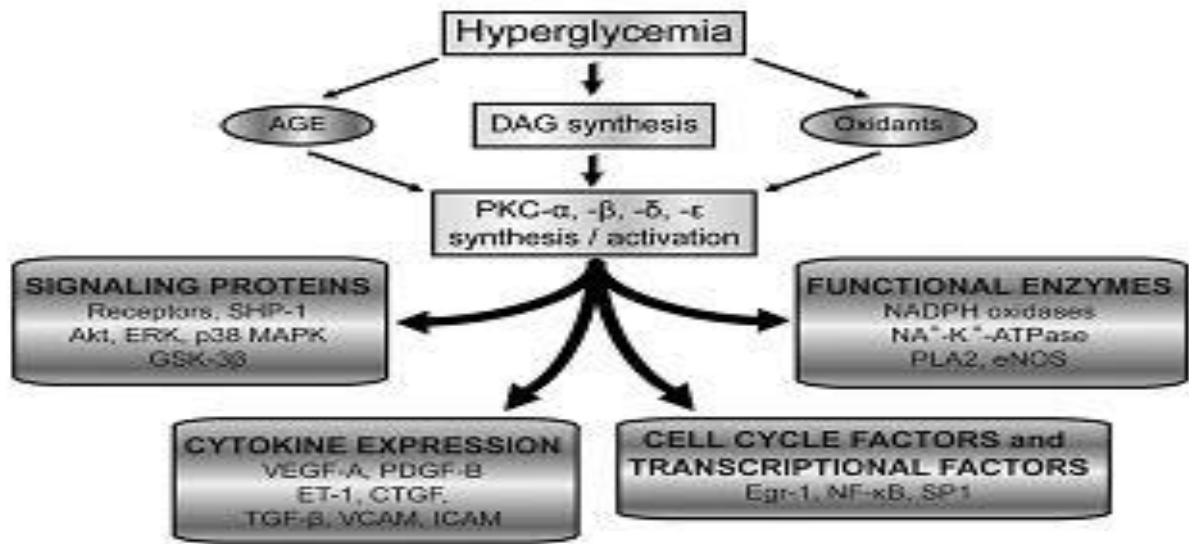


Figure 1.4: Multiple effects of PKC activation in diabetic animals. (Adapted from Geraldine and King [55]).

1.2.1.5 Hexosamine pathway

The role of increased metabolic flux of the hexosamine pathway in the pathophysiology of diabetic complications started coming out in the 1990s. Under hyperglycemic condition, glucose is metabolized through glycolysis while generating fructose-6-phosphate as an intermediate [48]. Some of the fructose-6-phosphate becomes channelled from the glycolytic pathway into a signaling pathway, the hexosamine pathway. The first step of the hexosamine pathway involves the conversion of fructose-6-phosphate to glucosamine-6-phosphate in the presence of glutamine:fructose-6-phosphate amidotransferase (GFAT) which is the rate-limiting enzyme for the pathway [58]. Subsequently, the glucosamine-phosphate is converted to UDP N acetyl-glucosamine (UDP GlcNAc) which happens to be a good substrate for O- and N-glycosylation of protein [59]. Thus, increased hexosamine flux leads to high O-linked glycosylation of proteins and/or inhibition of the hexose monophosphate shunt and this is responsible for the detrimental effects of hyperglycemia in various situations. On the other hand, the glucosamine phosphate could competitively inhibit glucose-6-phosphate dehydrogenase (G6PDH), the rate limiting enzyme of the hexose monophosphate shunt. G6PDH activity is linked to the reduction of NADP^+ to NADPH which is most important redox-regulator because it is a cofactor for glutathione reductase. This enzyme catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) as well as enhances the activity of catalase which decomposes H_2O_2 to H_2O . The foregoing could explain, at least in part, the influence of the increased hexosamine pathway in the induction of oxidative stress under diabetic state.

Indeed, it was reported that high activity of hexosamine pathway induces oxidative stress during chronic exposure to hyperglycemia and leads to β -cell deterioration [60].

Based on the above discussions on the oxidative stress-induction pathways under diabetic condition, the following figure (**Figure 1.5**) is provided to give an interconnection between the different pathways.

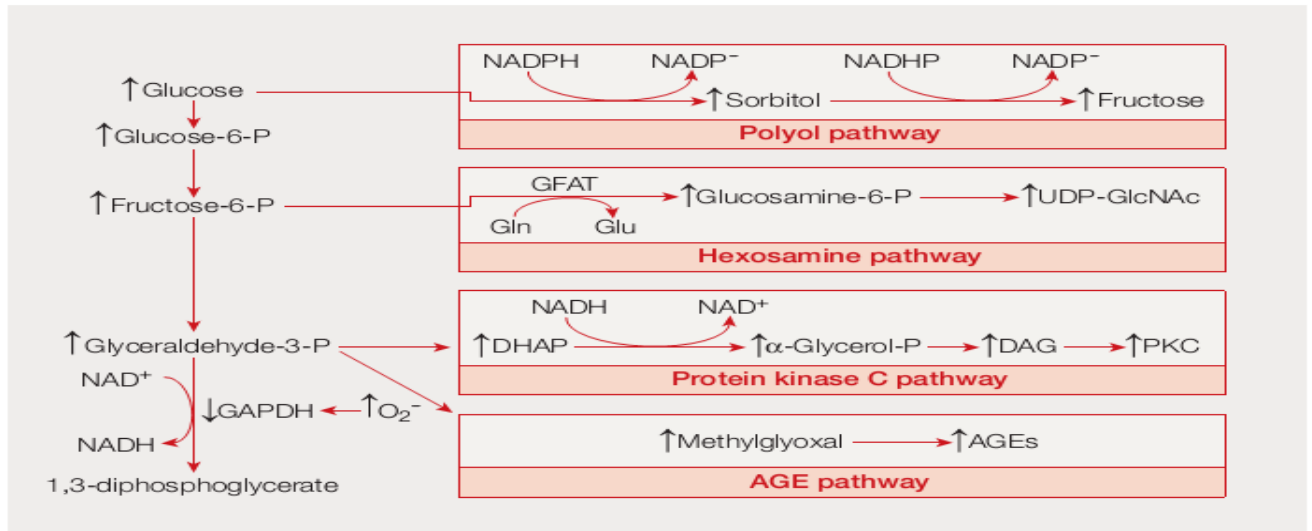


Figure 1.5: Interrelationship between hyperglycemia and oxidative stress-induced pathways in type 2 Diabetes (Adapted from Ceriello [61]).

We now have an insight into the multiple oxidative stress generating pathways, thus, we can now turn the focus to the complications of T2D themselves.

Broadly speaking, all complications associated with T2D are categorized into two broad classes; microvascular and macrovascular complications.

1.2.2 Microvascular complications

These involve small vessels such as capillaries and the most common, and perhaps most important, forms of these complications are called diabetic nephropathy, diabetic retinopathy and diabetic neuropathy.

❖ Diabetic nephropathy

This is also referred to as nodular diabetic glomerulosclerosis or Kimmelstiel-Wilson syndrome. It is a serious and progressive complication of T2D that affect approximately 25% of people with T2D [62]. The primary marker for this pathological change is microalbuminuria which usually progresses to full albuminuria and eventually to renal failure. Several mechanisms contribute to diabetic nephropathy which includes hemodynamic pathways such as the renin-angiotensin-aldosterone systems, non-enzymatic protein glycosylation, increased PKC activity as well as impaired polyol pathway [63]. However,

oxidative stress is the common theme to all these pathways. The oxidative stress elicits multiple negative biological effects including modulation of nephron functionality, membrane lipids peroxidation, proteins oxidation, DNA damage and renal vasoconstriction [63]. All these effects of oxidative stress eventually lead to the pathological features associated with T2D-induced nephropathy. This is supported by a study where histological analysis of type 2 diabetic patients' kidney showed deposits of glycation and lipid oxidation products in the mesangial matrix and glomeruli which were not detected in non-diabetic individuals [63].

❖ **Diabetic neuropathy**

Diabetic neuropathy is a complex disorder that impairs both peripheral and autonomic nervous system. Several possible pathogenic mechanisms have been proposed for diabetic neuropathy but the pathogenesis still remains unclear suggesting that the overall mechanism could be complex and perhaps multifactorial [64]. A number of vascular and metabolic factors have been implicated in the development of T2D-related neuropathy. These include:

- Increase in the metabolic flux of polyol pathway which leads to high levels of sorbitol and fructose, as well as diminished $\text{Na}^+\text{-K}^+$ ATPase activity.
- Distortions in the membrane structure of the nerves as well as hemorrheologic and microvascular anomalies due to impairments in the metabolism of essential fatty acid and prostaglandin
- Activation of the NF- κ B, and increased activity of PKC, endoneural microvascular deficits with ischemia and hypoxia.
- Reduced levels of neurotrophic agents, including neurotrophin-3, nerve- and insulin like growth factors, which causes a decline in neurotrophism and distortions in axonal transport
- High levels of non-enzymatic AGE on nerve and vessel proteins
- Generation of auto-antibodies against sympathetic ganglia, vagal nerve and adrenal medulla as well as inflammatory changes [65].

❖ **Diabetic retinopathy**

This is a microvascular complication that impairs normal function of the peripheral retina and/or the macula. It causes visual disability as well as partial or complete blindness in patients with T2D. The primary marker for this complication is the loss of pericytes which affect capillary constriction, new capillary generation and indeed other continuous exposure to noxious molecules [66]. Consequently, these would lead to partial or total loss of vision via a retinal detachment or vitreous hemorrhage [67].

Currently, the pathogenesis of diabetic retinopathy is unclear. However, multiple biochemical events appear to interplay in causing this complication. These include activation of PKC, high activity of the polyol pathway, high amount of AGE, high expression of growth factors such as insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), activation of the renin-angiotensin-

aldosterone system, haemodynamic changes and sub clinical inflammation and capillary occlusion [68]. However, a close analysis of the possible contributory role of each of the mechanisms suggests that oxidative stress is the most important biological phenomenon to the pathogenesis of T2D-related retinopathy [69]. Evidences from animal studies suggest that there is a diminished activity of ROS-detoxifying enzymes in the retina and the prevention of experimental diabetic retinopathy by the use of antioxidants [70]. It was proposed that the ROS induces DNA damage which could activate poly-(ADP-ribose)-polymerase (PARP) and reduce the activity of glyceraldehyde phosphate dehydrogenase leading to the accumulation of glycolysis metabolites. These metabolites could activate the polyol pathway leading to impaired function of the lens [45]. Furthermore, the ROS were found to activate metalloproteinase-2 in the development of diabetic retinopathy and this is believed to elicit death of the retinal cells [68]. Although hundreds of animal and human-based studies have unequivocally show oxidative stress as the most vital pathogenic mechanism for the development of diabetic retinopathy, but more specific details on the ROS-mediated molecular events that actually leads to visual impairments are still unknown.

1.2.3. Macrovascular complications

This involves large vessels such as arteries and veins and is sometimes referred to as cardiovascular complications because cardiovascular events are the most predominant forms of the complications.

1.2.3.1 Cardiovascular complications

Cardiovascular disease (CVD) accounts for >70% of death among people with T2D [44] and therefore CVD is regarded as the leading cause of death in type 2 diabetic patients. Research investigations have provided evidence that type 2 diabetic patients have a four-fold higher risk for developing CVD compared to non-diabetic human subjects after the traditional risk factors for CVD such as tobacco use, age, dyslipidemia, obesity, and hypertension are controlled. This indicates that T2D is an independent risk factor for CVD [71]. At present, the mechanism(s) of pathogenesis of T2D-associated CVD is poorly understood despite extensive research activities in the area. However, a number of plausible biological mechanisms have been proposed as possible explanations to the development of atherosclerosis in T2D.

❖ Endothelial cells dysfunction

Endothelial cells refer to a mono layer of cells that line the inner portion of blood vessels and play a crucial role in vessel homeostasis. The endothelial cells are known to perform crucial functions in the regulation of blood flow, nutrient delivery, leukocyte trafficking and vascular smooth muscle cell proliferation and migration [44]. Therefore, dysfunction of the endothelial cells is a highly detrimental event and plays a vital role in atherogenesis.

A number of experiments have been carried out to determine the cause(s) of endothelial dysfunction in T2D but it is generally agreed based on evidences from such experiments that oxidative stress is the major cause of endothelial dysfunction in T2D [44]. High blood glucose levels lead to the generation of AGE with concomitant stimulation of PKC which in turn stimulate endothelial NADPH oxidase activity [56]. Subsequently, the increased NADPH oxidase activity uncouples eNOS and directs electron transfer to oxygen rather than L-arginine to generate superoxide (**Figure 1.6**) instead of nitric oxide radicals [47]. Thus, the bioavailability of nitric oxide is decreased and this is associated with impairment of endothelial-directed vasodilation. Another mechanism of oxidative stress-mediated endothelial dysfunction is through stimulation of platelet-derived growth factor and plasminogen activator-1 activation of NFkB as well as vascular permeability [47].

❖ Vascular smooth muscle

Atherosclerosis is associated with vascular smooth muscle proliferation and migration. In T2D, hyperglycemia increase superoxide generation in vascular smooth muscle cells which reacts with nitric oxide from endothelial cells and consequently impair with smooth muscle relaxation (**Figure 1.6**), vasodilation and enhance the formation of atherosclerotic lesions [47].

❖ Inflammatory cells

Monocytes and macrophages penetrate into damaged endothelial cells and move to the inner vessels to ingest oxidized LDL and generate foam cells (**Figure 1.6**). These foam cells become the most important component of an early marker of macrovascular disease, atherosclerotic fatty streaks. The levels of adhesion molecules are also elevated type 2 diabetic patients, thereby aggravating the phenomenon of foam cell formation [72].

❖ Oxidized LDL

Oxidized low density lipoproteins play a pivotal role in vascular cell injury and atherosclerosis [47]. This is because the influx and maintainance of lipoproteins in the vascular wall is an important step in atherogenesis. LDL in diabetic patients easily undergoes oxidative modification through oxidative stress-dependent processes which consequently increased the rate of LDL transvascular transport (**Figure 1.6**) and affect vascular tone [47].

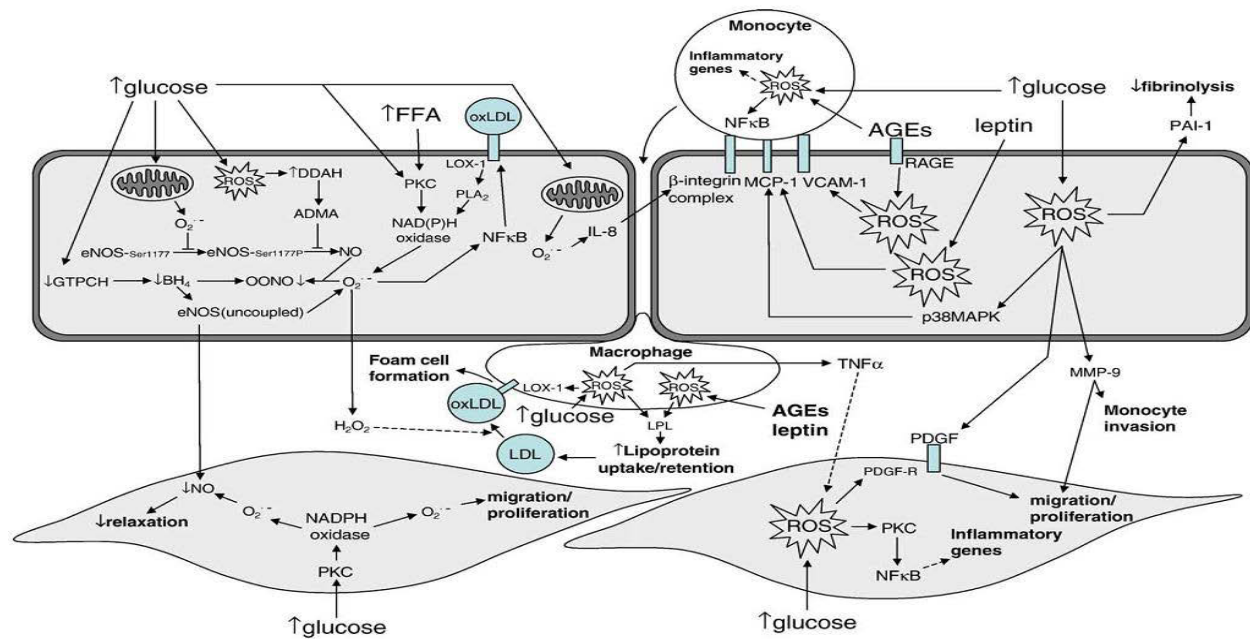


Figure 1.6: The role of oxidative stress in T2D-associated cardiovascular complications (Adapted from Jay et al. [47]).

1.3 CURRENT TREATMENTS OF TYPE 2 DIABETES

Several clinical and experimental studies have provided strong evidences that T2D and its related complications can be controlled, prevented or delayed by achieving tight glycemic control. Therefore much effort has been devoted to the search and development of optimal therapeutic agents for the management of T2D. However, the most commonly used approach is via oral therapies and at present, several classifications exist for the currently available oral anti-T2D drugs (depending on the approach) but for the sake of simplicity and ease of understanding, these drugs will be classified into six classes in this dissertation based on their mechanism of actions. These are the sulfonylureas, biguanides, thiazolidinediones, α -glucosidase inhibitors, dipeptidyl peptidase IV (DPP IV) inhibitors and SGLT2 inhibitors.

1.3.1 Sulfonylureas

This class of anti-T2D drugs became available since 1954 and act to lower blood glucose mainly by stimulating insulin release from functioning pancreatic β -cells [73]. Sulfonylureas bind, with high affinity, to their specific receptors on the pancreatic β -cells surface which are associated with ATP-sensitive potassium channel that inhibits the efflux of potassium ions through the channel resulting in depolarization of the cell membrane [73]. Subsequently, depolarization causes the opening of a voltage-gated calcium channel that leads to calcium entry and insulin release from secretory granules within the cell. Insulin sensitivity is not directly influenced by sulfonylureas and the potentiation in insulin

sensitivity usually observed after using these drugs is only as a result of better control of metabolic processes [74].

Sulfonylureas are generally divided into first and second generation sulfonylureas. The first generation includes chlorpropamide, tolbutamide, acetohexamide, and tolazamide while the second generation includes glyburide, glipizide, glimepiride and glibenclamide. The second generation drugs are more powerful and safer compared to the first generation drugs [75].

Despite interesting anti-T2D activity, the sulfonylureas are not without side effects. Hypoglycemia and weight gain are the major adverse effects of these drugs. The sulfonylureas-associated hypoglycemia results from the direct stimulation of insulin secretion from the pancreatic β -cells regardless of blood glucose concentrations [74]. On the other hand, the weight gain could also be detrimental, especially to obesity prone patients. Other side effects of the sulfonylureas are skin rash, hyponatremia and alcohol-induced flushing.

1.3.2 Biguanides

So far, three types of biguanides have been introduced into the market. The first two, phenformin and buformin, were introduced into the markets since 1970s but they were withdrawn from circulation because of their association with lactic acidosis [76]. However, the third biguanide, metformin was later introduced into the market in 1995 after safety concerns were cleared. Metformin was originally derived from the French lilac, *Galega officinalis* L., a perennial herb used for centuries to reduce the diabetic symptoms. The active compound is galegine, a guanidine derivative.

At present, the mechanism of action of metformin is not completely known. However, the predominant mechanism of lowering blood glucose levels is via decreasing hepatic glucose production in the presence of insulin, primarily by decreasing gluconeogenesis [77]. Metformin also decreases the peripheral insulin resistance to a lesser extent and this is achieved by increasing insulin-mediated glucose uptake [73]. It is therefore considered as an insulin sensitizer. Another proposed mechanism of action of metformin is delaying glucose absorption in the gastrointestinal tract. Metformin has no effect on insulin secretion and therefore it does not cause hypoglycemia when applied in monotherapy.

Metformin therapy has side effects which include gastrointestinal discomfort, such as nausea, abdominal pain, bloating, anorexia and diarrhea in approximately 50% of patients [78] and the reasons for these effects is not completely known. In some patients, metformin also causes weight loss [79] and the risk of lactic acidosis observed with earlier biguanides is also reported, though rarely in some patients. Approximately, the risk of the lactic acidosis is 100 times less than that with phenformin therapy [80].

1.3.3 Thiazolidinediones

Thiazolidinediones is a recently introduced class of oral antidiabetic drug that enhances insulin sensitivity. In 1997, the first thiazolidinediones was introduced in the United Kingdom, United States and Japan but was later withdrawn from the circulation due to high risk of liver failure. At present, the two commercially available thiazolidinediones are rosiglitazone and pioglitazone. These agents improve the sensitivity of muscle and adipose tissue to insulin via a unique mechanism which is not completely understood.

Thiazolidinediones are pharmacological ligands for a nuclear receptor called peroxisome-proliferator-activated receptor gamma (PPAR γ). These nuclear receptors bind with response elements on DNA to alter the expression of vital genes responsible for the control of carbohydrate and lipid metabolism. Indeed, insulin-stimulated uptake of glucose in the skeletal muscle cells is profoundly increased by thiazolidinediones. Thus, thiazolidinediones improve insulin sensitivity in peripheral tissues. Glucose production in the liver is also reduced but only occurs when high doses are administered. The PPAR γ activation also facilitates adipocyte differentiation and decreases lipid breakdown. It is also noteworthy that the adipocytes produced the highest amount of the nuclear receptors compared to other tissues. Therefore, stimulation of muscle glucose uptake is an indirect effect elicited as a result of the drugs interaction with adipocytes. Other biomolecules affected by thiazolidinediones are tumor necrosis factor, free fatty acids, leptin, resistin and adiponectin [75]. Apart from the above-mentioned mode of action, some authors have postulated that thiazolidinediones improve β -cell function by reducing free fatty acids. Furthermore, thiazolidinediones were reported to decrease the proinsulin-insulin ratio in T2D indicating an improvement in β -cell function [81].

Thiazolidinediones are highly costly and usually regarded as the most expensive anti-T2D drugs. In addition, they have multiple side effects which include anemia, edema, weight gain and congestive heart failure, thereby making it highly unsuitable for patients with hepatic impairments [75].

1.3.4 α -Glucosidase inhibitors

The commercially available α -glucosidase inhibitors were first introduced in 1996. These drugs do not target any specific mechanism of diabetic pathogenesis. Rather, they act by decreasing the rate of absorption of products of carbohydrates digestion (monosaccharides) in the small intestine. The α -glucosidase inhibitors inhibits the activity of α -glucosidases located at the brush border of the small intestine as well as α -amylase found in the pancreas in a competitive manner. The α -Amylase breakdown starch and other polysaccharides to oligosaccharides whereas α -glucosidases hydrolyze these oligosaccharides into absorbable glucose. Therefore, these agents interact with the carbohydrate-binding region of these enzymes and inhibit the digestion of the ingested carbohydrates [73]. Consequently, the

absorption of monosaccharides (mainly glucose) derived from dietary carbohydrates is delayed. This impedes the entry of glucose into the bloodstream and reduces postprandial blood glucose levels [74]. A number of α -glucosidase isoenzymes exist but the crucial ones are sucrase, glucoamylase, maltase, and isomaltase.

The side effects of α -glucosidase inhibitors are mainly gastrointestinal problems which include flatulence, abdominal discomfort, diarrhea and bloating [74].

1.3.5 Dipeptidyl peptidase (DPP) IV inhibitors

This class of anti-T2D drugs was first introduced into the market in 2006. Sitagliptin was the first drug to be introduced followed by vildagliptin, saxagliptin, linagliptin, and alogliptin.

Glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are incretin hormones that cause glucose-dependent insulin secretion, increase β - cells mass and decrease glucagon secretion. They also retard gastric emptying and induce satiety [82]. However, these incretin hormones are rapidly inactivated by DPP IV thereby making the half-life of the active forms of these hormones to be approximately 1-2 minutes [82]. Therefore, the therapeutic basis of DPP-IV inhibitors depends on their ability to bind and inhibit DPP-IV and consequently increase the half-life of the hormones thereby elevating the levels of the biologically active form of the incretin hormones in circulation. These would obviously have multiple advantages on metabolic processes [83] such as a more sustained potentiation of insulin secretion and suppression of glucagon release.

The most frequently encountered adverse effects of DPP IV inhibitors are headache, nausea, hypersensitivity and skin reactions as well as nasopharyngitis [84].

1.3.6 SGLT 2 inhibitors

These are most likely the next generation of anti-T2D drugs that may flood the diabetic market because several of them are currently at advance stages of clinical trials. They use an insulin-independent approach and based on the inhibition of renal glucose reabsorption. Canagliflozin has been approved in the USA while Dapagliflozin was rejected by FDA but marketed in Europe. Ipragliflozin, Tofogliflozin and Empagliflozin are at phase III clinical trials.

Glucose transport into the cells is mediated via two main families of glucose transporters, SGLT and GLUT. The SGLT family has 12 members which are usually situated at the renal tubular cells and also at the brush border membrane of the gut epithelium. In enterocytes, the main glucose transporter is SGLT 1 which simultaneously transports glucose and sodium with a stoichiometry of 1:2. However, this transporter is thought to possess another vital function in the kidney which is to reabsorb about 10% of glucose in the straight S3 segment of the proximal renal tubule. Apart from SGLT 1, scientific findings

have revealed that a complementary role of SGLT 2 is greatly required for the filtered glucose to be reabsorbed in the kidney. By contrast, the expression of SGLT 2 occurs mainly at the S1 segment of the proximal renal tubule, where it simultaneously transports glucose and sodium using a stoichiometry of 1:1 and with 50% efficiency in humans. Interestingly, apart from the kidney, SGLT 2 is also synthesized in other tissues [85]. Therefore, SGLT 2 inhibitors act via modulating the function of SGLT 2 in proximal convoluted tubules of the kidney and consequently block the glucose reabsorption. This leads to excretion of the glucose in urine (glycosuria). Thus, SGLT 2 inhibitors induce glycosuria which leads to a decrease in blood glucose concentrations. They also cause a net decrease of body calories and maintain an overall negative energy balance [86].

Interestingly, these drugs present other multiple advantages such as prevention of blood pressure elevation in hypertensive prone patients because of reduced sodium reabsorption thereby causing a decrease in the activity of the renin-angiotensin-aldosterone system.

Despite interesting reports on several potential beneficial effects of these drugs, they are still not without adverse effects. The most recognized side effect of SGLT 2 inhibitors is increased risk of genitourinary infections which are more prevalent in females than males [85]. Furthermore, these drugs do not seem to optimally act in patients with some renal impairment because of their dependence of glomerular filtration and other kidney dependent processes. Thus, they find more application in early diagnosed T2D cases where complications have not been fully developed. Clinical trials have provided evidence of the risk of developing mild form of secondary hyperparathyroidism with SGLT 2 inhibitors.

Based on the above discussion on the currently available anti-diabetic drugs, it is evident that all the drugs have detrimental side effects and therefore the search for alternative therapies is appealing.

1.4 THE ROLE OF TRADITIONAL HERBAL MEDICINES IN THE TREATMENT OF T2D

1.4.1 Medicinal plants as sources of therapeutics

Throughout the history of mankind, nature has been the primary source of all kinds of medicines for the treatment of wide spectrum of diseases. Among all the natural sources of medicines, plants have particularly been the most important source of complex traditional medicine systems. The earliest records of traditional use of plants for treating diseases dates back to 2600 BCE where approximately 1000 plant materials were documented in the traditional medicine system of Mesopotamia [87]. Also, the Egyptian traditional medicine dates back to 2900 B.C with over 700 plant-derived drugs used in their traditional medicine. Furthermore, Chinese system of traditional medicine dates back to 1100 B.C. with over 850 plant-derived materials in use since then [87]. On the other hand, Africa has a rich diversity of plants harbouring about 25% of the total numbers of higher plants in the world where more than 5400 medicinal plants have been found to have over 16,300 medicinal uses [88]. Unfortunately however, African

traditional system, though as old as others, is verbal and therefore some of the precious information about the plants and their medicinal values along with dates was not recorded leading to the loss of the information over the course of time. Based on the above, it appears therefore that the use of herbal products in the treatment of wide array of diseases is as old as human existence itself.

Interestingly, in this modern era, the use of these herbal products also continues to play a crucial role in the healthcare delivery systems of many parts of the world [89]. Indeed, according to World Health Organization [90], 60% of the total population of the world relies on traditional medical systems directly or indirectly, while 80% of the population in developing countries depends almost exclusively on traditional medical practices, more specifically, plant medicines for their primary health care needs [91]. Reports from developed countries also indicated that a number of patients of some chronic disease are gradually turning to herbal remedies as alternatives to modern synthetic drugs [92] because of the toxic side effects and high cost of the modern drugs as well as the more perceived effectiveness of the plant-based remedies.

An investigation of plant-originated pure compounds used as drugs in countries with WHO-Traditional Medicine Centers indicated that, out of the 122 compounds so far identified, 80% were applied for the same or similar ethno-medical purposes and were obtained from 94 plant species only. Relevant examples of currently used drugs which are originally derived from medicinal plants are quinine and artemisinin for treating malaria, reserpine for treating hypertension, khellin as bronchodilator, salbutamol and salmetrol for treating asthma and more relevant to this dissertation, the widely used anti-T2D drug metformin was originally developed from medicinal plant [87]. In fact, 75% of the anti-infectious agents and 60% of the anticancer drugs approved from 1981-2002, are originally derived from natural sources [93] whereas 61% of all new chemical compounds marketed as drugs worldwide during the same period are also derived from natural products [94]. In another report by Newman and Cragg [95], from 1981 to 2010, a total of 1130 new drugs were approved for the treatment of 61 diseases and an impressive number of 543 of those drugs were directly derived from medicinal plants. Therefore, in view of the impressive role of medicinal plants in drug discovery, biochemists, pharmacologists, medicinal chemists, pharmacists, microbiologists and botanists from all over the world have intensify efforts on investigating medicinal plants for novel phytochemicals and lead compounds that could be developed for the treatment of various diseases.

1.4.2 African medicinal plants in the treatment of T2D

At present, medicinal plants are extensively used in the treatment of diabetes mellitus in all the sub-regions of Africa and perhaps by all African cultures as well. This is evident by the number of publications on ethnobotanical surveys for medicinal plants used in local diabetes therapy from different

geographical regions of the continent. These reports could further denote that most diabetic patients in the region perhaps depend on medicinal plants for the treatment of the disease.

Starting from West Africa, Gbolade [96] reported that fifty medicinal plants from different families are exploited in the local treatment of diabetes in Lagos state, Nigeria. These herbal recipes are often orally taken without serious adverse effects. Among the fifty plants prescribed, the most highly cited medicinal plants by the diabetes trade-specialists are *Vernonia amygdalina*, *Ocimum gratissimum*, *Carica papaya*, *Citrus aurantifolia* and *Bidens pilosa*. Another study from the South Western part of Nigeria where one hundred traditional medicine practitioners were interviewed revealed that thirty one medicinal plants are the most commonly used for the treatment of diabetes mellitus [97]. Among these plants, *Vernonia amygdalina*, *Cassia alata* and *Momordica charantia* top the list. In another ethnobotanical survey from the North Western part of Nigeria, one hundred and twenty eight herbalists were interviewed [98]. These herbalists have no formal education and inherited these indigenous knowledge systems from their ancestors. From the study, thirty four medicinal plants were cited by the traditional practitioners and based on informant consensus, *Vernonia amygdalina*, *Mangifera indica*, *Calotropis procera*, *Khaya senegalensis* and *Cassia singueana* were ranked highest among other plants. Available ethnobotanical study from another West African country comes from Guinea [99]. A total of one hundred and twelve people were interviewed where one hundred and forty six plant species belonging to fifty five families were recommended for the treatment of diabetes mellitus. The most cited plant species were *Anacardium occidentale*, *Aframomum melegueta*, *Ficus glumosa* and *Ficus capensis*.

From Southern Africa, Oyedemi et al. [100] conducted a survey on anti-diabetic plants in the Eastern Cape Province of South Africa. Results from the study indicated that fifteen different plant species belonging to thirteen families are used by the traditional healers while *Strychnos henningsii* and *Leonotis leonorus* were the most cited plants in the study. In a more recent report from South Africa, though from the Limpopo Province, fifty two traditional healers were interviewed where they recommended twenty four plant species belonging to twenty families for the treatment of diabetes mellitus [101]. The most repeatedly mentioned plants by the healers were *Mimusops zeyheri*, *Helichrysum caespititium*, *Plumeria obtuse*, *Aloe marlothii*, *Hypoxis iridifolia* and *Moringa oleifera*. Another ethnobotanical study for anti-diabetic plants from the Southern sub-region of Africa was from DR Congo [102]. Thirty one plant species were cited by the traditional healers of Kisangani city of the country and among them, *Catharanthus roseus*, *Aloe vera*, *Morinda lucida*, *Morinda morindoides* and *Cassia occidentalis* were the most highly cited.

Available data from Eastern Africa emanates from the Lower eastern Province of Kenya [103]. In this study, thirty-nine plant species belonging to thirty three genera and twenty six families were reported to

be used in the local therapy of diabetes mellitus. The most frequently cited plants were *Cassia abbreviate*, *Zanthoxylum chalybeum*, *Azadirachta indica* and *Cactus species*.

Reports from Morocco (North Africa) indicated that about ninety four medicinal plants belonging to thirty eight families are used in the traditional management of diabetes mellitus and hypoglycemic effects of some of the plants have been confirmed by Moroccan researchers [104]. Members of Compositae, Lamiaceae, Leguminosae and Liliaceae families are generally the most commonly used anti-diabetic plants in Morocco. In an ethnopharmacological survey from the South Eastern Morocco [105], a total of four hundred people were interviewed and forty five medicinal plant species were mentioned as potent anti-diabetic plants used for diabetes treatment in the area. From the results of the study, the most frequently cited plants to treating diabetes include *Nigella sativa*, *Allium cepa*, *Rosmarinus officinalis*, *Artemisia herba-alba*, *Carum carvi*, *Peganum harmala*, *Ajuga iva*, *Lepidium sativum*, *Olea europaea*, *Zygophyllum gaetulum* and *Phoenix dactylifera*.

Based on the above discussions, it is obvious that medicinal plants play a fundamental role in the treatment of diabetes in Africa. However, most of the above-mentioned plants are used by traditional healers as simple concoction and not standardized by relevant authorities. In order to further portray the crucial role of phytomedicines in diabetes therapy, some standardized herbal products are investigated and approved for sales in the South African market. A brief run-down of these standardized herbal products present in South African market is given below.

The most commonly used standardized herbal products in South Africa are probetix, Diavite™, Diabecinn and Cinnachrome.

Probetix is a herbal supplement developed from the leaf extract of South African *Sutherlandia frutescens*. Scientific findings have confirmed that this herbal product has anti-diabetic activity which is mediated through decreasing intestinal glucose uptake as well as maintaining normoinsulineamic state in diabetic subjects [106]. Furthermore, the extract was found to normalize some physico-metabolic abnormalities associated with diabetes. In another study, this extract was found to have a profound effect on the development of insulin resistance by reducing the levels of free fatty acid [107]. In a more recent study [108], this herbal product was also reported to directly affect the biosynthesis of lipid and mitochondrial activity and consequently restore insulin sensitivity via modulation of fatty acid biosynthesis. Interestingly, findings from a clinical trial revealed that no indication of toxicological effects is associated with treatment of 800 mg/day of this herbal product for a period of three months [109]. The possible bioactive agents identified are pinitol, asparagines, L-arginine, γ -aminobutyric acid, L-canavanine, γ -sitosterol, saponin, parabens and α -lipoic acid [110].

Diavite™ is an anti-diabetic food supplement developed from the dried and ground pods of *Prosopis glandulosa*, commonly known as honey mosquito. Scientific findings have indicated that this herbal supplement reduces blood glucose levels as well as the glycemic index value of foods. Furthermore, Diavite™ was found to stimulate insulin secretion, improves insulin sensitivity of cardiomyocytes as well as confers some protection to β -cells [111].

Another anti-diabetic herbal product in the South African market is Diabecinn. This product is water based cinnamon bark extract that may reduce blood sugar levels, triglycerides and cholesterol in type 2 diabetic patients. Scientific investigation done with the cinnamon extract showed that the extract prevents the development of insulin resistance and lowers plasma glucose concentrations by enhancing insulin signaling process [112]. In a more recent study by Couturier et al. [113], polyphenols were found to be main bioactive agents that decrease blood glucose levels, improves insulin sensitivity and alters body composition.

Cinnachrome is another cinnamon-derived herbal product that is used by diabetic patients in South Africa. Apart from the cinnamon extract, this herbal preparation also contains chromium polynicotinate which is a natural form of chromium that provides free chromium and niacin. [114]. The bioactive agent was proposed to be methyl-hydroxy chalcone polymer.

Based on the above discussions coupled with ethnobotanical revelations (described in earlier part of this sub-section), it is obvious that Africa is naturally blessed with huge medicinal plant resources that could be exploited either directly as standardized anti-T2D herbal products or indirectly by providing novel chemical leads for the development of newer alternative therapies for the treatment of T2D. Unfortunately, these abundant African medicinal plant natural resources remain largely untapped in both respects.

Therefore, there is a crucial need for thorough studies on the anti-diabetic potential of a number of African medicinal plants with a view to harness their potentials as anti-diabetic herbal products or as possible providers of chemical leads for the development of novel and more effective chemotherapeutic agents against T2D.

1.5 AIM OF THE STUDY

The aim of this study is to conduct a thorough investigation on the anti-oxidative, anti-diabetic potentials and possible toxicological effects of five African medicinal plants namely; *Ziziphus mucronata*, *Parkia biglobosa*, *Khaya senegalensis*, *Cassia singueana* and *Vitex doniana* using a number of *in vitro* and *in vivo* models and to also isolate the possible bioactive anti-T2D agents as well as their mechanism of action.

1.5.1 Objectives

The research objectives are:

1. To evaluate the *in vitro* anti-oxidative activities of aqueous, ethanol and ethyl acetate crude extracts of various parts (stem barks, roots and leaves) of the *Ziziphus mucronata*, *Parkia biglobosa*, *Khaya senegalensis*, *Cassia singueana* and *Vitex doniana*
2. Based on the results of the *in vitro* anti-oxidative studies above, the best crude extracts from each plant will further be partitioned and the fractions will be investigated for the *in vitro* anti-oxidative activities as well as α -glucosidase and α -amylase inhibitory effects, which are crucial carbohydrates-hydrolyzing enzymes.
3. Based on the results of the *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory effects of the fractions, the best fraction from each plant will be subjected to a comprehensive study on the *in vivo* anti-diabetic activity of the fractions in a T2D model of rats as well as their possible mechanism of actions.
4. To identify the possible bioactive agents in the extracts and fractions from each plant using GC-MS.
5. To isolate the possible bioactive anti-T2D compounds in their pure forms, characterize them using NMR and MS and subject them to the α -glucosidase and α -amylase inhibitory activities assays.

1.5.2 STRUCTURE OF THE DISSERTATION

Chapter 1: This chapter gives detailed background introduction and literature review on diabetes mellitus. Epidemiology, classification, pathogenesis, complications, role of oxidative stress, currently available oral anti-diabetic drugs and their limitations as well as the role of African medicinal plants in the treatment of diabetes mellitus are discussed.

Chapter 2: This chapter describes the materials and methods used in the study

Chapter 3: The *in vitro* and *in vivo* anti-diabetic activities of *Ziziphus mucronata* have been described in this chapter. Identification, isolation and characterization of the possible bioactive compounds are also discussed.

Chapter 4: The *in vitro* and *in vivo* anti-diabetic activities of *Cassia singueana* have been described in this chapter. Identification, isolation and characterization of the possible bioactive compound are also discussed.

Chapter 5: The *in vitro* and *in vivo* anti-diabetic activities of *Parkia biglobosa* have been described in this chapter. Identification, isolation and characterization of the possible bioactive compound are also discussed.

Chapter 6: The *in vitro* and *in vivo* anti-diabetic activities of *Khaya senegalensis* have been described in this chapter. Identification, isolation and characterization of the possible bioactive compound are also discussed.

Chapter 7: The *in vitro* anti-oxidative and anti-diabetic activities of *Vitex doniana* have been described in this chapter.

Chapter 8: This chapter gives a general discussion of all the findings of the study and the conclusions drawn from the study. Recommendations are also suggested in this chapter.

Chapter 9: Appendices

References: References for each chapter are presented at the end of the chapter.

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CHAPTER 2

2.0 MATERIALS AND METHODS

Experimental design

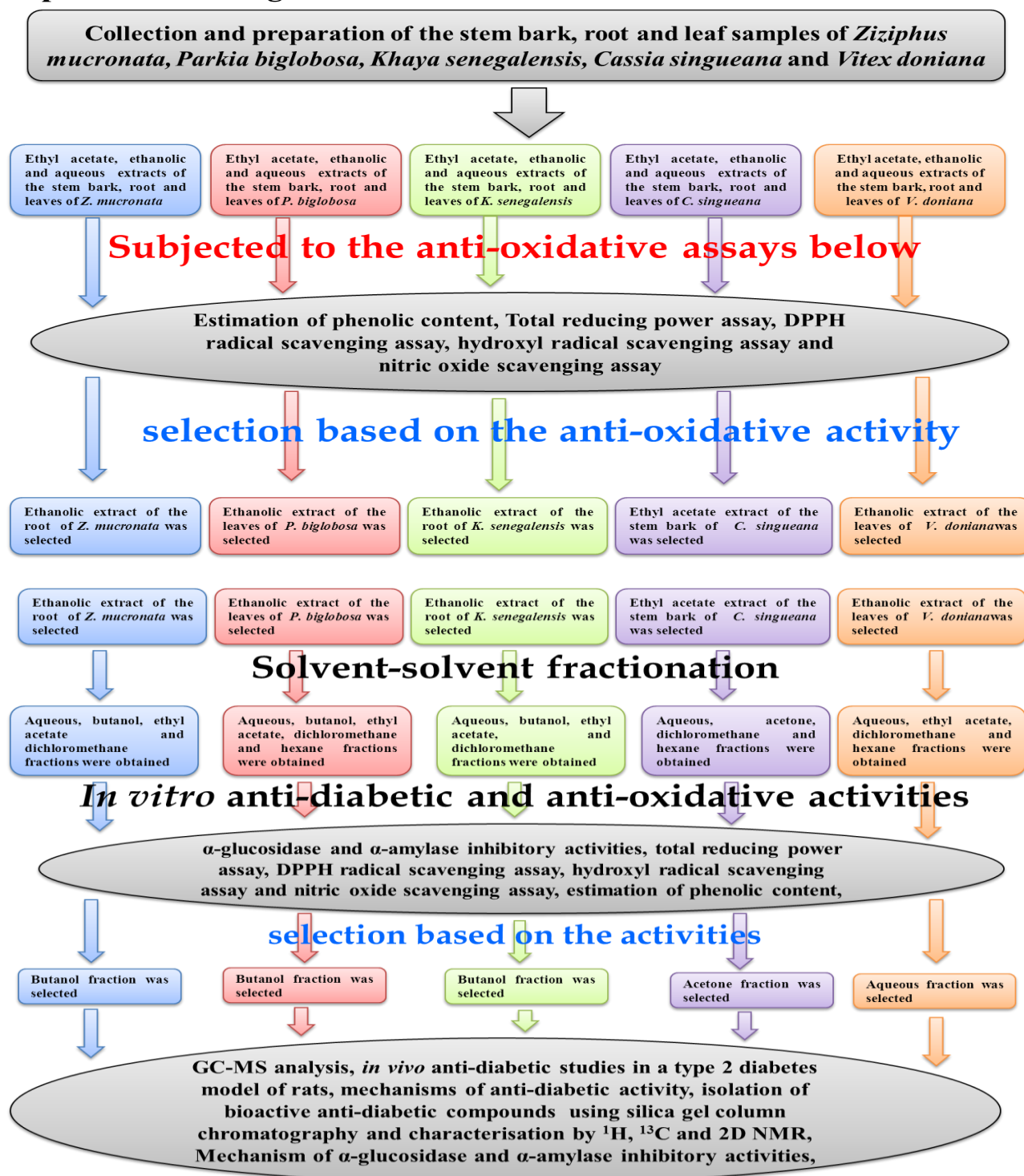


Figure 2.1: Flow charts to describe how the experiment was systematically arranged and executed

2.1 Chemicals and reagents

Streptozotocin, yeast α -glucosidase, porcine pancreatic amylase, p-nitrophenyl- α -D-glucopyranoside (pNPG), p-nitrophenol, acarbose, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 6-hydroxydopamine, 5,5'-dithiobisnitrobenzoic acid (DTNB), 2 deoxy-D-ribose, diethylenetriaminepentaacetic acid (DETAPAC), ascorbic acid, gallic acid and potassium ferricyanide were obtained from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Starch, dinitrosalicylic acid (DNS), maltose, absolute ethanol, ethyl acetate, trichloroacetic acid, hydrogen peroxide, ferric chloride, Griess reagent, sodium nitroprusside, thiobarbituric acid, reduced glutathione, Folin ciocalteau reagent, deuterated chloroform and deuterated methanol were purchased from Merck Chemical Company, Durban, South Africa. An ultra sensitive rat insulin ELISA kit was purchased from Mercodia AB, Uppsala, Sweden. Reagents to analyze the other blood parameters were from Labtest Diagnostics (Costa Brava, Lagoa Santa, Brazil) purchased via Replamed Company Ltd., Centurion, South Africa.

2.2 Equipment

Buchi Rotavapor II, Buchi, Germany, Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), microcentrifuge, Bruker AvanceIII 400 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), GC-MS 6890 series (Agilent Technologies, USA), Glucoplus glucometer (Glucoplus Inc., Saint-Laurent, Que., Canada), Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil), multi-plate ELISA reader (Biorad-680, BIORAD Ltd., Japan), Leica slide Scanner SCN 4000, (Leicabiosystems Germany) and portable tissue homogenizer.

2.3 Collection and preparation of plant materials

The stem bark, root and leaf samples of *Ziziphus mucronata* Willd, *Parkia biglobosa* A. Jacq, *Khaya senegalensis* A. Juss, *Cassia singueana* Delile and *Vitex doniana* Sweet were collected in January 2011 from Zaria, Kaduna state, Nigeria. The plant samples were authenticated at the herbarium unit of the Department of Biological Science, Ahmadu Bello University, Zaria, Nigeria. The herbarium voucher specimens were deposited and assigned the repository numbers 154 (*Z.mucronata*), 3017 (*P. biglobosa*), 900081 (*K. senegalensis*), 6863 (*C. singueana*) and 1162 (*V. doniana*). The stem bark, root and leaves were immediately washed and shade-dried for about 2-4 weeks (depending on the plant material) to attain constant weights. The dried samples were pounded to fine powder using a kitchen blender, and then stored individually in air-tight containers for transport to the University of KwaZulu-Natal, Westville campus, South Africa for subsequent analysis.

2.4 Preparation of the crude extracts

Forty (40) grams of the fine powdered sample from each part of the plant were separately defatted with hexane. The defatted materials were sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 hours in 200 ml of the respective solvent followed by shaking for 2 hours at 200 rpm. After filtration through Whatmann filter paper (No 1), respective solvents were evaporated in vacuum using a rotary evaporator at 40 °C under reduced pressure to obtain the solvent crude extracts. Aqueous extracts were however dried on a water bath at 50 °C (**Figure 2.1**). The extracts in each case were weighed, transferred to micro tubes and refrigerated at 4 °C until needed.

2.5 *In vitro* anti-oxidative activities of crude extracts from the various parts of the selected plants

2.5.1 Estimation of total phenolic content

The total phenolic content of each extract was estimated (as gallic acid equivalent) using the method described by McDonald et al. [1] with slight modifications. Briefly, 200 µL of the extract (240 µg/mL) was incubated with 1 mL of 10 times diluted Folin ciocalteau reagent and 800 µL of 0.7 M Na₂CO₃ for 30 minutes at room temperature. Then, the Absorbance values were measured at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

2.5.2 Ferric cyanide (Fe³⁺) reducing antioxidant power (FRAP) assay

The total reducing power of the extracts were measured using the FRAP method of Oyaizu [2] with slight modifications. To perform this assay, 1 mL of each extract (15–240 µg/mL) was incubated with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide at 50 °C for 30 minutes. Thereafter, 1 mL of 10% trichloroacetic acid was used to acidify the reaction mixtures. After the acidification, 1 mL of the sample was mixed with 1 mL of distilled water and 200 µL of 0.1% FeCl₃. The absorbance of the resulting solution was read at 700 nm in a spectrophotometer. The absorbance of the samples is proportional to the reduction capability of the extracts [3]. The results were expressed as a percentage of the absorbance of the sample to the absorbance of gallic acid.

$$\text{Ferric reducing antioxidant power (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of gallic acid}} \times 100$$

2.5.3 Free radical scavenging activity

The free radical scavenging activity of the extracts or standards (ascorbic and gallic acids as well as trolox) was determined using a slightly modified method described by Tuba and Gulcin [4]. A 0.3 mM solution of DPPH was prepared in methanol and 500 µL of the DPPH solution was added to 1 mL of the extracts at various concentrations (15–240 µg/mL). These solutions were mixed and incubated in the dark

for 30 minutes at room temperature. The absorbance was read at 517 nm against blank samples lacking scavenger.

2.5.4 Nonsite-specific hydroxyl radical mediated 2-deoxy-D-ribose degradation assay

Ascorbate–EDTA– H₂O₂ system (Fenton reaction) generate hydroxyl radicals which could degrade deoxyribose and measured as products degradation reaction. Thus, the hydroxyl radical scavenging activity was determined by studying the competition between the extracts and deoxyribose for the generated hydroxyl radicals as described by Hinnerburg et al. [5]. The assay was performed by adding 200 µL of premixed 100 µM FeCl₃ and 100 µM EDTA (1:1 v/v) solution, 100 µL of 10 mM H₂O₂, 360 µL of 10 mM 2-deoxy-D-ribose, 1 mL of different extract concentrations (15–240 µg/mL), 400 µL of phosphate buffer (50 mM, pH 7.4) and 100 µL of 1 mM ascorbic acid in sequence. This followed by the incubation of the mixture at 50 °C for 2 hours. Thereafter, 1 mL of TCA (2.8%) and 1 mL of 1.0% thiobarbituric acid (in 25 mM NaOH) were transferred to the reaction tubes. The samples were further incubated in a water bath at 50 °C for 30 minutes to develop the pink chromogen. The extent of oxidation was determined from the absorbance of the samples at 532 nm and the hydroxyl radical scavenging activity of the extract was reported as percentage inhibition of deoxyribose degradation.

2.5.5 Nitric oxide (NO) radical scavenging assay

This assay is based on the ability of aqueous solution of sodium nitroprusside at physiological pH to spontaneously produce nitric oxide (NO), which could interact with oxygen to generate nitrite ions that can be measured using Griess reagent. All agents that can scavenge NO compete with oxygen, resulting in decreased NO generation [6]. The assay was carried out by incubating 500 µL of 10 mM sodium nitroprusside in sodium phosphate buffer (pH 7.4) and 500 µL of different extract concentrations (15–240 µg/mL) at 37 °C for 2 hours. Thereafter, 500 µL of Griess reagent was transferred to the reaction mixture. Diazotization of nitrite with sulphanilamide produce a chromophore that can be measured at 546 nm. The percentage inhibition of NO generated was measured by comparing with the absorbance value of a control (10 mM sodium nitroprusside in phosphate buffer).

All assays were carried out in triplicate. The scavenging activities of the plant extracts in the case of DPPH, hydroxyl and nitric oxide radicals scavenging assays were calculated by using the following formula:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

Where A_s is the absorbance in the presence of the sample and A_c is the absorbance of the control

2.5.6 Gas chromatography-mass spectrometric (GC-MS) analysis

Based on the results of anti-oxidative assays, the most active extract from each part of the plants (except *Z. mucronata*) were subjected to GC-MS analysis. The GC-MS analysis was conducted with an Agilent technologies 6890 GC coupled with (an Agilent) 5973 mass selective detector and driven by Agilent chemstation software. A HP-5MS capillary column was used (30 m x 0.25 mm internal diameter x 0.25 µm film thickness). The carrier gas was ultra-pure helium at a flow rate of 1.0 mL/minute and a linear velocity of 37 cm/second. The injector temperature was set at 250 °C. The initial oven temperature of 60 °C which was programmed to 280 °C at the rate of 10 °C/minute with a hold time of 3 minutes. Injections of 1 µL were made in a split mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 4 minutes and scan range 50-700 amu. Compounds were identified by direct comparison of the retention times and mass fragmentation pattern data with those in the National Institute of Standards and Technology (NIST) library.

2.6 *In vitro* anti-oxidative and anti-diabetic activities of solvent fractions derived from the crude extracts

2.6.1 Solvent fractionation of the most active crude extracts from each plant

Based on the results of the preliminary anti-oxidative assays (described above), the most active crude extract from each plant was selected for further fractionation (**Figure 2.1**). The selection was made in relation to the crude extracts of the same plant (intra) and not across the plants (inter). The selected extracts were ethanolic extract of the root (*Z. mucronata*), ethanolic extract of the leaves (*P. biglobosa*), ethanolic extract of the root (*K. senegalensis*), ethyl acetate extract of the stem bark (*C. singueana*) and ethanolic extract of the leaves (*V. doniana*). For each crude extract, a 10 g of the extracts was dissolved in a 200 mL of distilled water : methanol (9:1) and successively partitioned with hexane (2 x 200 mL), dichloromethane (2 x 200 mL), ethyl acetate (2 x 200 mL) and n-butanol (2 x 200 mL). However, the n-butanol was replaced with acetone during the fractionation of the ethyl acetate extract of the stem bark of *C. singueana* and it was also observed during the fractionation of the ethanolic extract of the leaves of *V. doniana* that the n-butanol completely dissolved the remaining aqueous fraction after ethyl acetate partitioning and therefore the fraction was regarded as aqueous fraction (**Figure 2.2**). All the resulting fractions were evaporated to dryness at 40 °C under reduced pressure whereas the remaining aqueous fractions were dried on a water bath. The fractions in each case were weighed, transferred to micro tubes and stored in a refrigerator at 4 °C until needed.

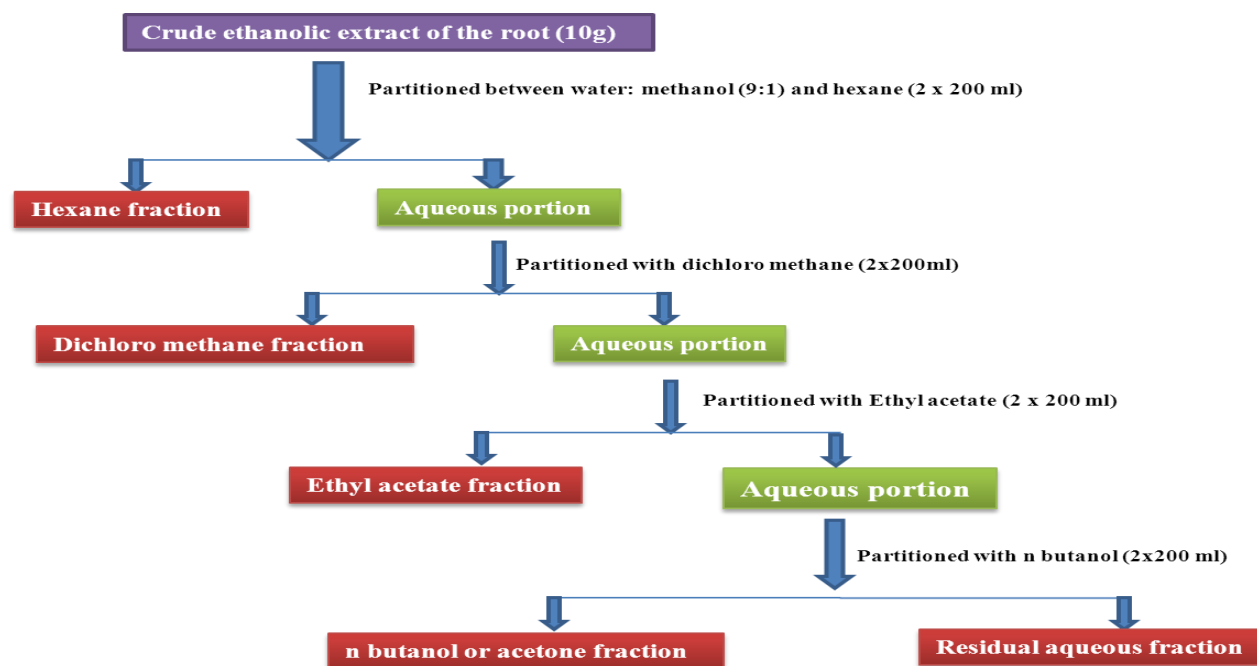


Figure 2.2: Protocol for the solvent-solvent fractionation of the crude extracts

2.6.2 Determination of α -glucosidase inhibitory activity of solvent fractions

The α -glucosidase inhibitory activity was measured according to the method described by Ademiluyi and Oboh [7] with slight modifications. Briefly, 250 μ L of each fraction or acarbose at different concentrations (30-240 μ g/mL) was incubated with 500 μ L of 1.0 U/mL α -glucosidase solution in 100 mM phosphate buffer (100mM, pH 6.8) at 37 °C for 15 minutes. Thereafter, 250 μ L of pNPG solution (5 mM) in phosphate buffer (100 mM, pH 6.8) was added and the reaction mixture was further incubated at 37 °C for 20 minutes. The absorbance of the released p-nitrophenol was measured at 405 nm and the inhibitory activity was expressed as percentage of the control without the inhibitors.

2.6.3 Determination of α -amylase inhibitory activity of the solvent fractions

The procedure described by Shai et al. [8] with slight modifications was used to determine the α -amylase inhibitory activity of the fractions. A volume of 250 μ L of each fraction or acarbose at different concentrations (30-240 μ g/mL) was incubated with 500 μ L of porcine pancreatic amylase (2 U/mL) in phosphate buffer (100 mM, pH 6.8) at 37 °C for 20 minutes. Thereafter, 250 μ L of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the reaction mixture and incubated at 37° C for 1 hour. Dinitrosalicylate colour reagent (1 mL) was then added and boiled for 10 minutes. The absorbance of the resulting mixture was read at 540 nm and the inhibitory activity was expressed as percentage of a control without the inhibitors.

All assays were carried out in triplicate. The inhibitory activities of the fractions on the α -glucosidase and α -amylase were calculated by using the following formula:

$$\text{Inhibitory activity (\%)} = \left(1 - \frac{A_s}{A_c}\right) \times 100$$

Where A_s is the absorbance in the presence of the sample and A_c is the absorbance of the control.

2.6.4 Mechanism of α -glucosidase and α -amylase inhibition

Based on the results of α -glucosidase and α -amylase inhibitory assays, the most active fraction from the solvent fractions of each plant was further subjected to kinetic experiments to determine the type of inhibition exerted on α -glucosidase and α -amylase. The experiment was conducted according to the protocols as described above at a constant concentration of the sample fraction with a variable concentration of substrate as described below. For the α -glucosidase inhibition assay, 0.625-5 mM of pNPG was used and 0.125-1% of starch was used for the α -amylase inhibition assay. The initial rates of the reactions were determined from calibration curves constructed using varying concentrations of p-nitrophenol and maltose for the α -glucosidase and α -amylase inhibition assays respectively. The initial velocity data obtained were used to construct Lineweaver-Burke's plot to determine the K_M (Michaelis constant) and V_{max} (maximum velocity) of the enzyme and the type of inhibition for both enzymes.

2.6.5 Anti-oxidative activities of the solvent fractions

All the solvent fractions were investigated for anti-oxidative activities using the four models described in sub chapters 2.5.2 – 2.5.5 above.

2.6.6 GC-MS analysis of the most active fractions

The phytochemical components of the most active fraction from the solvent fractions of each plant were analysed using the GC-MS protocol described in 2.5.6 above.

2.7 *In vivo* antidiabetic activity of the most active fractions in a type 2 diabetes rats model

Based on the results of the anti-oxidative activities as well as α -glucosidase and α -amylase inhibitory activities, butanol fractions from the aforementioned extracts (see 2.6.1 above) of *Z. mucronata*, *P. biglobosa* and *K. senegalensis* and the acetone fraction from *C. singueana* (see 2.6.1 above) were the most active and therefore selected for *in vivo* studies. The selection was made by comparing the activities of solvent fractions derived from a plant and not across plants (see **Figure 2.1** above). The aforementioned most active fractions from each plant was re-prepared as described in subchapters 2.4 and 2.6.1 above but the starting material was three kilograms (3 kg) because of the large quantity of the fractions needed for the *in vivo* studies. Unfortunately, due to the a low yield and unavailability (for

further extraction) of the leaves of *V. doniana* when needed, the most active (aqueous) fraction derived from the ethanolic extract of the leaves of this plant was not subjected to the *in vivo* study.

2.7.1 Experimental animals

Male Sprague-Dawley (six-week-old) rats were obtained from the Biomedical Resource Unit (BRU) located at the University of KwaZulu-Natal (Westville Campus), South Africa with initial mean body weight (BW) 207.60 ± 4.27 g. The rats were maintained as 2 in one medium size polycarbonated cage in a humidity and temperature controlled room with a 12 hour light–dark cycle. A standard rat pellet diet was supplied *ad libitum* for the whole duration of the experiment. The rules and regulations of the Committee for Experimental Animal Ethics of the University of KwaZulu-Natal, South Africa (Ethical approval number: 022/12/Animal) were followed in maintaining the rats.

2.7.2 Animal grouping and induction of type 2 diabetes

The rats were randomly divided into six groups of eight animals each namely:

Normal Control (NC) : Normal rats (non diabetic and not treated)

Diabetic Control (DBC): These are diabetic and untreated control

Diabetic + fraction low dose (DFL): diabetic rats treated with 150 mg/kg BW of the fraction.

Diabetic + fraction high dose (DFH): diabetic rats treated with 300 mg/kg BW of the fraction.

Diabetic + metformin (DMF): diabetic rats treated with 300 mg/kg BW of metformin

Non-diabetic fraction control (NFT): non-diabetic rats treated with 300 mg/kg BW of the fraction.

The same procedure and arrangement was done for the four fractions derived from the four plants (**Figure 2.3**). After one-week adaptation period, the animals in DBC, DFL, DFH and DMF groups were supplied with a 10% fructose solution *ad libitum* for two weeks to induce insulin resistance which was followed by an overnight fast and subsequently, a single injection (i.p.) of streptozotocin (40 mg/kg BW) dissolved in citrate buffer (pH 4.5) was injected to the animals in DBC, DFL, DFH and DMF groups to induce partial pancreatic beta-cell dysfunction (**Figure 2.3**). The rats in NC and NFT groups were given normal drinking water and injected with citrate buffer instead of 10% fructose and STZ, respectively [9]. One week after the STZ injection, the nonfasting blood glucose (NFBG) levels of all the rats were measured in the blood collected from the tail vein by using a portable glucometer (Glucoplus Inc., Saint-Laurent, Quebec, Canada). The rats with NFBG level > 17 mM were considered as diabetic [10] while rats with NFBG level < 17 mM were excluded from the study.

2.7.3 Intervention trial

After the confirmation of diabetes, a respective dose of the fraction was orally administered five days in a week by using a gastric gavage needle to the rats in DFL and DFH and NFT groups while the rats in

controls (NC and DBC) and DMF groups were treated with a similar volume of the vehicle and metformin respectively for a 4 week experimental period. During this period, daily food and fluid intakes as well as weekly body weight changes and NFBG concentrations were measured in all animal groups.

2.7.4 Oral glucose tolerance test (OGTT)

The OGTT was conducted in the last week of the 4-week intervention period to measure the glucose tolerance ability of each animal. To perform this test, a single dose of glucose solution (2 g/kg BW) was orally ingested into each animal and the levels of blood glucose were measured at 0 (just before the ingestion of glucose), 30, 60, 90 and 120 minutes after the dose of glucose.

2.7.5 Collection of blood and organs

The rats were euthanized by halothane anesthesia at the end of the experimental period and blood and organ samples were collected (**Figure 2.3**). Cardiac puncture was used to collect the whole blood of each rat which was immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 minutes and serum from each blood sample was separated and kept at -30°C for subsequent analysis. The liver, kidney and heart were collected from each rat, rinsed with normal saline, wiped with filter paper, weighed and kept at -30°C until further analysis. A small portion of the pancreas sample from each rat was cut and immersed into a 10% neutral buffered formalin and stored at room temperature for histopathological analysis. The neutral buffered formalin of each samples was changed weekly during the entire preservation period.

2.7.6 Analytical methods

The concentrations of serum insulin were measured by an enzyme-linked immunosorbent assay (ELISA) method using an ultrasensitive rat insulin ELISA kit (Mercodia, Uppsala, Sweden) in multi-plate ELISA reader (Biorad-680, BIORAD Ltd., Japan). The serum lipid profile (total cholesterol, HDL- and LDL-cholesterols, and triglycerides), fructosamine, urea and creatinine concentrations as well as liver function enzymes; aspartate and alanine aminotransferases (AST and ALT) and alkaline phosphatase (ALP) were measured with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial assay kits for these analyses from the same company. Computation of the homeostatic model assessment (HOMA-IR and HOMA- β) using were conducted using serum insulin and FBG concentrations measured at the end of the experimental period using the formula below:

$$\text{HOMA} - \text{IR} = \frac{\text{Serum insulin in U/L} \times \text{Blood glucose in mmol/L}}{22.5}$$

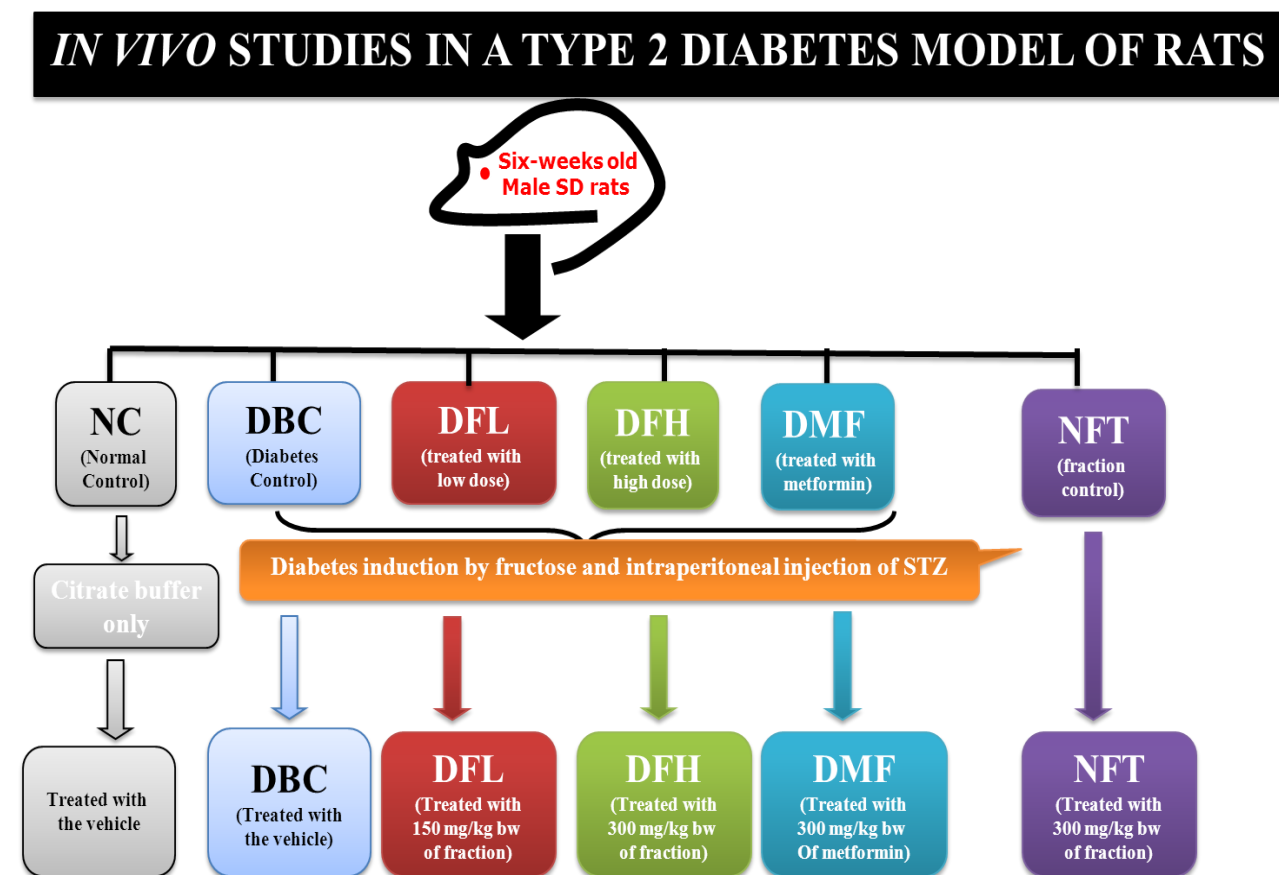
$$\text{HOMA} - \beta \text{ cell function} = \frac{20 \times \text{Serum insulin in U/L}}{\text{Blood glucose in mmol/L} - 3.5}$$

Conversion factor: insulin (1U/L = 7.174 pmol/L)

The liver glycogen concentrations were determined using a phenol-sulfuric acid procedure of Lo et al. [11].

2.7.7 Histopathological analysis of the pancreas

A standard laboratory protocol for paraffin embedding was followed in processing the formalin preserved pancreatic tissues. Sections were cut to a size of 4 μ m. Thereafter, *p*-xylene was used to deparaffinize the slides which were then rehydrated in ethanol concentration gradient (100%, 80%, 70%, 50%) and rinsed with tap water. Subsequently, the hematoxylin was used to stain the slides for 5 minutes which was rinsed with water. This is followed by counterstaining using eosin, Finally, the slides were mounted in DPX, cover-slipped and viewed with Leica slide scanner (SCN 4000, Leicabiosystems Germany).



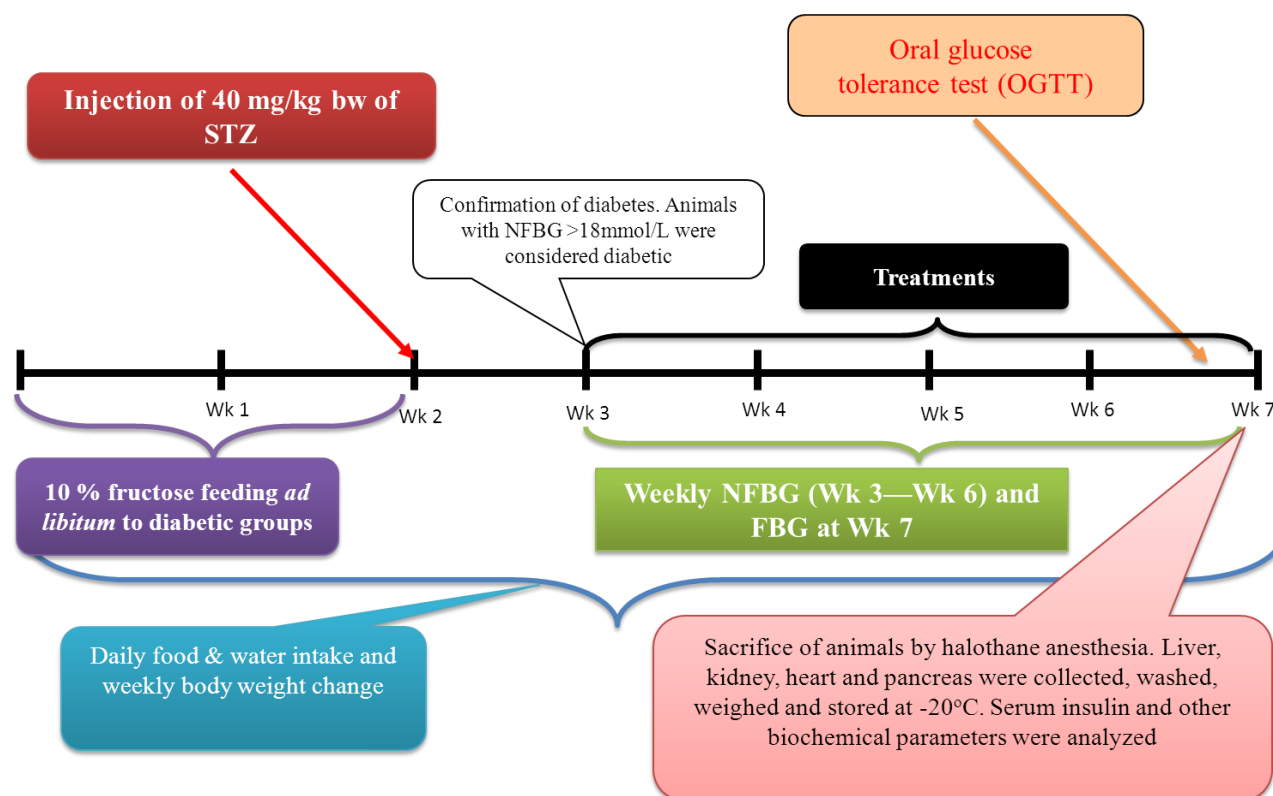


Figure 2.3: Flow charts to describe the overall experimental flow for the *in vivo* anti-diabetic activities of the selected solvent fractions from the four plants

2.7.8 *In vivo* anti-oxidative studies

In order to understand the possible contributory role of anti-oxidative activity to the observed anti-diabetic activity, the *in vivo* anti-oxidative activities of the fractions were investigated in the serum and organs of the animals using four *in vivo* oxidative stress markers namely; reduced glutathione, thiobarbituric acid reactive substances (TBARS) levels, catalase and superoxide dismutase activities.

To perform these assays, homogenates of the organs (liver, kidney, heart and pancreas) were initially prepared by homogenising 0.5 g of the sample in 4 mL of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5) using a portable tissue homogenizer. Thereafter, the mixture was transferred to a microtube and spinned for 15 minutes at 15000 rpm in a microcentrifuge. The supernatant was then collected in another micro centrifuge and stored at -20 °C for further analysis.

2.7.8.1 Determination of reduced glutathione

The reduced glutathione levels were determined using the procedure described by Ellman [12] with slight modifications. A 0.5 mL of the serum or tissue homogenate was added to 0.5 mL of 10% TCA and centrifuged at 5000 rpm for 10 minutes. Thereafter, 0.5 mL of the supernatant was mixed with 0.5 mL of

Ellman's reagent (19.80 mg of DTNB dissolved in 100 mL of 0.1% NaNO₃) and 3 mL of phosphate buffer (200 mM, pH 8.0). The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at 412 nm. The concentration of the reduced glutathione in the samples was calculated from a standard glutathione curve.

2.7.8.2 Determination of thiobarbituric acid reactive substance (TBARS) concentration as malondialdehyde (MDA) equivalent

Thiobarbituric acid reactive substances, expressed as MDA concentration were measured to determine the extent of lipid peroxidation using the protocol described by Fraga et al. [13]. A 200 µL of the sample or MDA standards were thoroughly mixed with 200 µL of 8.1% SDS solution, 750 µL of 20% acetic acid, 2 mL of 0.25% TBA and 850 µL of distilled water. Subsequently, the resulting mixture was heated in a water bath at 95 °C for 1 hour and allowed to cool to room temperature. The absorbance of the resulting solution was then read at 532 nm and the concentration of TBARS in the samples was calculated from the MDA standard curve.

2.7.8.3 Determination of catalase activity

This assay was carried out as described by Aebi [14]. A 340 µL of 50 mM sodium phosphate buffer (pH 7.0) was mixed with 150 µL of 2 M H₂O₂. Thereafter, 10 µL of the sample was added and the decrease in absorbance at 240 nm was monitored for 3 minutes at 1 minute interval.

2.7.8.4 Determination of superoxide dismutase activity

This assay was carried out by transferring 1 mL of 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) and 90 µL of the sample into a quartz cuvette. Then a 90 µL of 1.6 mM 6-hydroxydopamine (6-HD) was added and the mixture was quickly mixed. Absorbance of the resulting mixture was recorded at 492 nm for 3 minutes at 1 minute interval .

2.8 Isolation of the bioactive anti-diabetic compounds from the fractions

A bioassay guided isolation protocol was used to isolate the pure bioactive anti-diabetic agent(s) from each of the most active fraction (from *in vitro* studies described in sub chapter 2.6.2 - 2.6.5) but the approach slightly differs from one fraction to another.

2.8.1 Isolation of the bioactive compound from the butanol fraction of ethanolic extract of *Ziziphus mucronata* root

The fraction was separated on a silica gel column using dichloromethane with an increasing gradient of methanol as the solvent system to give fifty seven fractions of 15 mL each. Fractions were monitored by

thin layer chromatography (TLC) and fractions with similar TLC profiles were pooled together and tested for α -glucosidase inhibitory activity. However, fraction 29 was found to have the best α -glucosidase inhibitory activity but it was a mixture of a chromone and polar aliphatic alcohol, which co-eluted in the same fraction and was unable to be separated despite several attempts. The chromone was further characterized by ^1H NMR, ^{13}C NMR and two dimensional (2D) NMR. The sample was dissolved in deuterated chloroform (CDCl_3 , Merck South Africa) and transferred to 5 mm NMR tubes.

2.8.2 Isolation of the bioactive compound from the butanol fraction of ethanolic extract of *Parkia biglobosa* leaves

The fraction was subjected to silica gel column chromatography using a solvent system of dichloromethane with an increasing gradient of methanol to give fifty seven fractions of 15 mL each. Fractions were monitored by TLC and fractions with similar TLC profiles were pooled together and tested for α -glucosidase inhibitory activity. Fraction 11 however, was found to be a pure α -glucosidase inhibitory compound which was characterized by ^1H and ^{13}C NMR. The sample was dissolved in deuterated chloroform (CDCl_3 , Merck South Africa) and transferred to 5 mm NMR tubes.

2.8.3 Isolation of the bioactive compound from the butanol fraction of ethanolic extract of *Khaya senegalensis* root

The fraction was subjected to a column chromatography on a silica gel column (100 mesh) eluted with ethyl acetate: methanol (8:2, 7:3, 6:4 and 1:1) as the mobile phase to obtain forty fractions of 20 mL each. Fractions were monitored by TLC and fractions with similar TLC profiles were pooled together and tested for α -glucosidase inhibitory activity. A combined fraction 10-14 displayed the highest α -glucosidase inhibitory activity and was further subjected to silica gel column chromatography using methanol in dichloromethane (2%-10%) as the mobile phase. Thirty five fractions (I-XXXV) of 15 mL each were collected and fraction XIV was found to be a pure α -glucosidase inhibitory compound which was characterized by ^1H , ^{13}C and two dimensional (2D) NMR as well as mass spectroscopy. The sample was dissolved in deuterated methanol and transferred to 5 mm NMR tubes.

2.8.4 Isolation of the bioactive compound from the acetone fraction of the ethyl acetate extract of *Cassia singueana* stem bark

This fraction was also subjected to column chromatography on a silica gel column (100 mesh) eluted with hexane : ethyl acetate (8:2, 7:3 and 1:1) as the mobile phase to obtain fifteen fractions (I-XV). The fractions were monitored by TLC and were subsequently pooled according to their TLC profiles. Combined fractions XI-XIII displayed the highest α -glucosidase inhibitory activity and was further fractionated using methanol in dichloromethane (0%-10%) as the mobile phase. Sixty fractions of 15 mL

were collected. Fraction 34 was found to be the best α -glucosidase inhibitory compound which was further characterized by ^1H and ^{13}C NMR. The sample was dissolved in deuterated chloroform (CDCl_3 , Merck South Africa) and transferred to 5 mm NMR tubes.

All the NMR experiments were performed on a Bruker AvanceIII 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) at room temperature with all chemical shifts (δ) values recorded against the internal standard, tetramethylsilane (TMS). Chemical shifts for all NMR spectra are reported in parts per million (ppm). The NMR system was controlled by TopSpin 2.0 software.

The α -glucosidase and α -amylase inhibitory activities as well as the mechanism of action of the pure compounds were determined as described in sub chapters 2.6.2, 2.6.3 and 2.6.4 above

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CHAPTER 3

3.0 THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *Ziziphus mucronata* Willd *IN VITRO* AND *IN VIVO*

3.1 *Ziziphus mucronata* Willd. (Rhamnaceae)



Figure 3.1: *Ziziphus mucronata* Willd. (Rhamnaceae); Common names: buffalo thorn (English), Magarya (Hausa, Nigeria), Umlahlankosi (Zulu, South Africa), Chinanga (Shona, Zimbabwe), Omukekete (Oshiwambo, Namibia). Photo: Mr. Umar Gallah (2012), Zaria, Nigeria

3.1.2 Background

Ziziphus mucronata is a deciduous tree with an irregular spiky canopy mostly supported by a single trunk but sometimes contains minor branches. The plant is usually 8 – 10 m in height and the leaves are shiny and light green while the thorns are located at the base of the leaves. The flowers are yellowish star-shaped while the fruits are circular, about 2.5 cm in diameter, sub-globose drupe and green at younger stage but becomes shiny reddish-brown when ripened. The plant is distributed throughout West and East African countries as well as some Central and Southern African countries [1].

3.1.3 Ethnomedicinal uses

The high adaptability of this plant to the climates of most sub-Saharan countries makes it prone to exploitation for medicinal purposes by the traditional medicine practitioners of a number of African countries. The powdered leaf and bark are used as for curing chest pain and cough by the Zulu tribe of South Africa. Furthermore, warm aqueous extract of the root, bark and leaf are applied topically for the treatment of sores, boils and swellings [2]. The roots are also used in the local treatment of dysentery, diarrhoea and stomach ulcers. In Namibia, the leaf and root are used in the folkloric medicine for the

treatment of skin allergies and sores [3]. Indeed, all the parts including the fruits are used in different forms such as food, poultice and drinks for the treatment of so many tropical diseases [1]. However, more relevant to this dissertation, is that the stem bark of the plant is commonly used in the North Western region of Nigeria for the traditional management of diabetes mellitus [4]. Furthermore, decoction of the leaves is used for diabetes management by the traditional healers of South Africa [2].

3.1.4 Biological activities

The first study on the scientifically investigated biological activity of *Z. mucronata* was antihelminthic effects [5]. Aqueous extract of the root bark of *Z. mucronata* was reported to be highly active against schistosomiasis (tapeworms) [5]. This finding was further confirmed in another antihelminthic studies where aqueous and organic extracts of the various parts of the plant were found possess strong activity against the levamisole strain of *Caenorhabditis elegans*. In a genotoxicity study, a 90% methanolic extract of *Z. mucronata* leaves was reported to be mutagenic in the presence of metabolic activation (6). Also, the anti-sickling activity of the aqueous and ethanolic extracts of different parts of the plant was demonstrated when a UV lamp and solar irradiations were used to induce photodegradation [7]. In order to validate the folkloric use of the plant for treating bacterial diseases, Cooposamy et al. [8] studied the activity of various solvent extracts of the different parts of the plant against some pathogenic bacteria and reported that the leaves extracts had the greatest inhibitory properties on both gram positive and gram negative bacteria. In a related study, Olajuyigbe and Afolayan [9] reported that the ethanolic extract of the bark possessed strong synergistic effect with antibiotics against clinically important bacteria. The anticholinesterase and antioxidant activity of the root part of the plant was investigated [10] where the methanol and ethyl acetate extracts were reported to display good anticholinesterase and radical scavenging activities. Furthermore, the antioxidant activity of the bark extracts of the plant was investigated where the ethanolic extract was found to have the highest antioxidant activity among other solvent extracts of the plant [11].

3.1.5 Phytochemistry

Information on the phytochemical contents of different parts of this plant is very scanty in the literature. Preliminary quantitative analysis revealed that the bark of the plant contain phenolics, flavonoids and proanthocyanidin [11] but the phenolics content was significantly higher than the flavonoids and proanthocyanidins. The only isolated group of phytochemicals reported in *Z. mucronata* is the cyclopeptide alkaloids, predominantly mucronines. Auvin et al. [12] described the isolation and characterization of mucronine J along with abyssenine A and mucronine D from the dichloromethane extract of the root bark. Furthermore, franguloline (sanjoinine A) with strong sedative property was isolated from all the parts of the plant [13].

The aim of this study is to comprehensively investigate the anti-diabetic activity of this plant using *in vitro* and *in vivo* models. To accomplish this, various solvent crude extracts of the stem bark, root and leaves of the plant were subjected to detailed *in vitro* anti-oxidative studies (using four models) from where the ethanolic extract of the root was found to have the best activity. Subsequently, the ethanolic extract of the root was fractionated across various solvents and the fractions were subjected to the anti-oxidative and α -glucosidase and α -amylase inhibitory studies where the butanol fraction was found to have the best anti-oxidative and enzymes inhibitory activities. Thus, the butanol fraction was investigated for *in vivo* anti-diabetic activity in type 2 diabetic rats as well as the possible mechanism of action. A pure bioactive anti-diabetic compound was also isolated from the butanol fraction.

3.2 Anti-oxidative activities of various extracts of stem bark, root and leaves of *Ziziphus mucronata* (Rhamnaceae) *in vitro*

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Preface: The *in vitro* anti-oxidative activities of the stem bark, root and leaves of the plant were investigated in this article. The article has already been accepted published in the Journal of Medicinal Plants Research, 2012, Vol 6 number 25 pages 4176-4184.

3.2.1 Abstract

The present study examined the anti-oxidative activities of extracts from different parts of *Ziziphus mucronata*. Stem bark, root and leaves samples were sequentially extracted with solvents of increasing polarity and tested for *in vitro* anti-oxidative activity using various models. Our results indicated that all the extracts had potent electron donating and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activities. However, the ethanolic extracts exhibited a significantly ($P < 0.05$) more potent DPPH radical scavenging activity than other extracts and possessed lower IC_{50} values than ascorbic acid and trolox. The organic extracts of all the parts demonstrated hydroxyl radical scavenging activities but the ethyl acetate extracts of the stem bark and root as well as the ethanolic extract of the leaves displayed more powerful anti-OH^{*} activity than trolox. All aqueous extracts were found to possess pro-oxidative activities in the hydroxyl radical scavenging method. Nitric oxide (NO) scavenging activities were observed in all the extracts tested except the aqueous extract of the roots that was found to be pro-oxidative at higher concentrations. Furthermore, a non-dose dependent NO scavenging activities were observed in the ethanolic extract of the stem bark and ethyl acetate extracts of the root and leaves as well as aqueous extract of the leaves. Data from this study suggest that the different parts of *Z. mucronata*, especially the ethanolic extracts possessed potent anti-oxidative activities that warrant further studies to identify the active key principles.

3.2.2 Introduction

The use of herbs and natural products in the treatment of various diseases is as old as human existence and continues to be an important component of health care delivery system, especially in African countries. Africa has a rich diversity of plants and about 25% of the total numbers of higher plants in the

world are found in Africa where more than 5400 medicinal plants were reported to have over 16,300 medicinal uses [14]. Recent pharmaceutical and biological research findings suggest plants as good sources of compounds that could provide chemical leads for the development of new generation of drugs to treat various diseases [15], especially non-communicable chronic diseases such as diabetes.

Diabetes mellitus is one of the major global health problems whose prevalence is currently on the increase at an alarming rate [16]. Mainly two types of diabetes are identified such as type 1 or insulin dependent diabetes mellitus (IDDM) and type 2 or non-insulin dependent diabetes mellitus (NIDDM). Among these types, type 2 is the most prevalent one and > 95% of the total diabetic patients are suffers from it. Type 2 diabetes (T2D) is a complex heterogeneous disorder that is associated with a gradual decline in the action of insulin (insulin resistance), followed by the failure of pancreatic β -cells to account for the insulin resistance (β -cell dysfunction) [17]. A number of mechanisms are involved in the β -cell damage but oxidative stress is considered as one of the major contributor [18-20].

Oxidative stress refers to the presence of free radicals and reactive oxygen species (ROS) that are generated in normal body processes but become harmful when not being quenched by the endogenous antioxidants systems. This usually occurs when there is an excess generation of ROS or when the antioxidants (AO) are inactivated, thereby altering the ROS/AO equilibrium in favor of stress [21, 22]. In T2D, various free radicals, including ROS, hydroxyl and nitric oxide radicals [23-25] are involved in the induction of oxidative stress induced pancreatic β -cell destruction [26] as well as the activation of all major pathways underlying the different components of vascular diabetic complications such as glycation, sorbitol pathways among others [27]. The foregoing therefore makes research on diabetes therapy and prevention to focus a lot of attention on the search for alternative agents with anti-oxidative properties that could be used to ameliorate the complications associated with T2D.

Ziziphus mucronata (Rhamnaceae), commonly known as buffalo thorn, is native to northern Nigeria. The plant decoction is used traditionally in the treatment of diabetes mellitus [4] among the rural populace of northern Nigeria, however scientific report(s) on any pharmacological activity from this plant is scanty in the literature. Hence, this study was designed to evaluate the anti-oxidative activities of various extracts of stem bark, root and leaves of *Z. mucronata* initially *in vitro*.

3.2.3 Materials and methods

Please refer to the chapter 2; sub sections 2.3 – 2.5 pages 35-37 for details of the methods used for *Z. mucronata*

3.2.4 Results

The percentage yield of the various extracts collected from the plant indicated that higher yields are obtained from the leaves extracts compared to roots and stem bark (**Table 3.2.1**). Furthermore, the ethanolic extracts of the stem bark and root contained significantly ($P<0.05$) higher amount of total polyphenols than other extracts from these parts of the plant whereas ethyl acetate extract of the leaves contained significantly ($P<0.05$) higher amount of the total polyphenols than other extracts in this part (**Table 3.2.1**).

Table 3.2.1: Percentage yield and total phenolic content of various extracts of *Z. mucronata* parts

Plant Extracts	% Yield	Total phenolic (mg/g GAE)
Stem bark		
Ethyl acetate	0.15	3.10 ± 0.13^a
Ethanolic	0.38	87.43 ± 2.87^f
Aqueous	1.46	25.01 ± 0.97^b
Root		
Ethyl acetate	0.34	2.38 ± 0.58^a
Ethanolic	0.29	77.41 ± 0.50^e
Aqueous	0.93	36.62 ± 0.46^c
Leaves		
Ethyl acetate	3.65	75.66 ± 1.21^e
Ethanolic	1.09	71.38 ± 1.81^d
Aqueous	1.35	27.81 ± 0.60^b

Results are expressed as mean \pm SD of triplicate determinations. ^{a-f}Different superscripts alphabets within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The total reducing power (in terms of percentage gallic acid equivalent) of the various extracts of *Z. mucronata* was compared to ascorbic acid and trolox and presented in **Figure 3.2.1**. According to the results, the total reducing power of all extracts was found to increase steadily with increasing concentrations of the samples (**Figure 3.2.1**). However, the ethanolic extracts from the different parts of the plant demonstrated a significantly ($P<0.05$) higher Fe^{3+} to Fe^{2+} reductive ability, at least at higher concentrations, than other extracts and trolox.

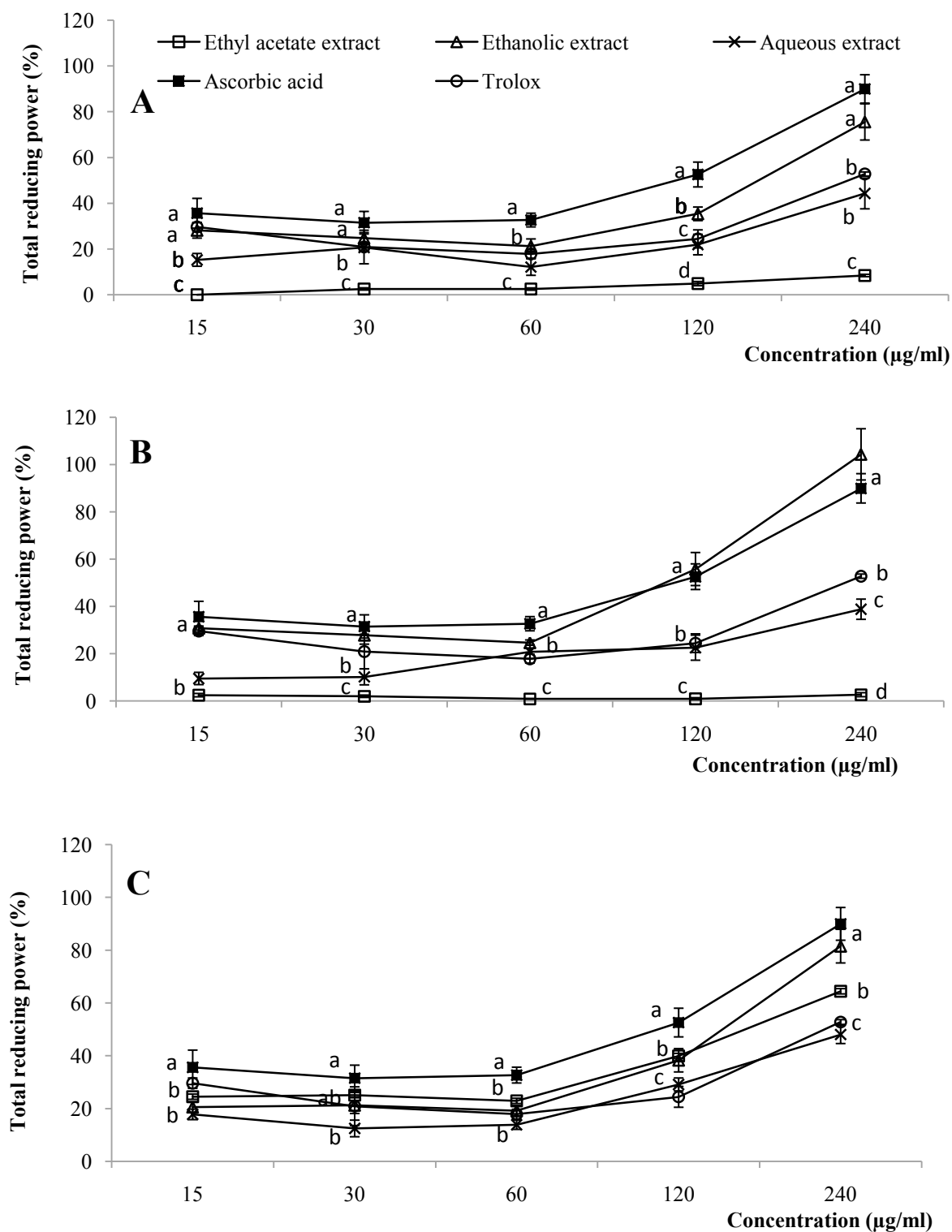


Figure 3.2.1: Total reducing power (relative to gallic acid) of stem bark (A), root (B) and leaves (C) extracts of *Z. mucronata*. Results are expressed as mean±SD of triplicate determinations. ^{a-d}Different alphabets presented for a given concentration for each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The DPPH radical scavenging activities of *Z mucronata* stem bark, root and leaves extracts are presented in **Figure 3.2.2**. The stem bark and root ethanolic extracts demonstrated powerful free radical scavenging activity (100% radical scavenging activity at 120-240 µg/ml) which were statistically similar with all the standard antioxidants used and significantly ($P < 0.05$) higher than other extracts from the other plant parts. The IC_{50} values of 1.99, 1.38 and 1.68 µg/ml were obtained for stem bark, root and leaves ethanolic extracts respectively whereas 2.56, 1.27 and 5.04 µg/ml were found for ascorbic acid, gallic acid and trolox respectively (**Table 3.2.4**).

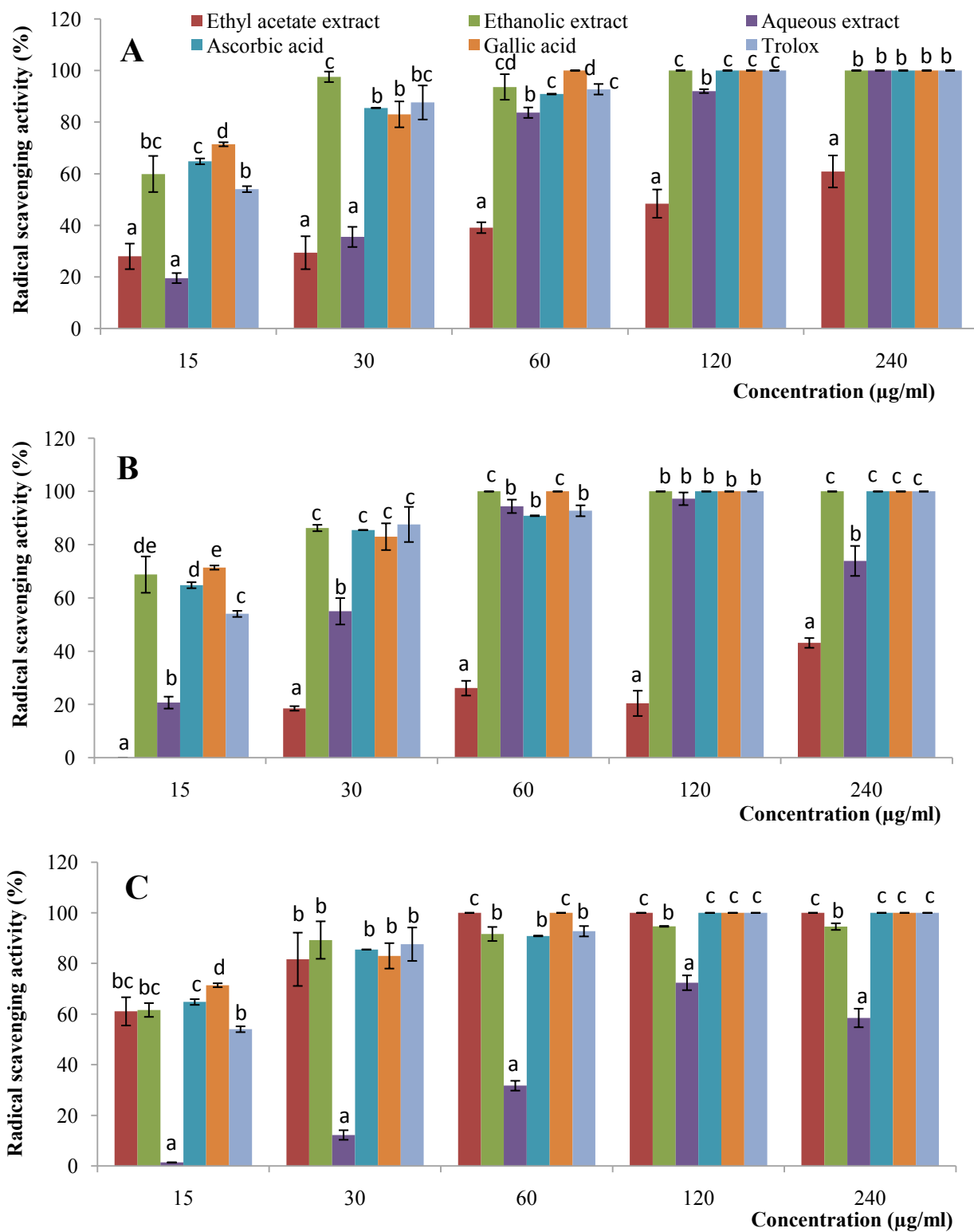


Figure 3.2.2: DPPH radical scavenging activity of stem bark (A), root (B) and leaves (C) extracts of *Z. mucronata*. Results are expressed as mean±SD of triplicate determinations. ^{a-e}Different alphabets over the bars for a given concentration for each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The results of the hydroxyl radical scavenging effects of extracts from *Z. mucronata* (Table 3.2.2) indicate that the ethyl acetate extracts derived from stem bark (IC₅₀ 1.07 µg/ml) and root (IC₅₀ 1.36 µg/ml) samples exhibited a concentration dependent anti-OH activities which were also significantly (P<0.05) higher than the activities observed with the corresponding ethanolic extracts and trolox (IC₅₀ 2.61 µg/ml) at all concentrations tested. Stem bark ethanolic extract demonstrated an anti-OH activity that inversely correlates with the concentration used while a non-dose dependent activity was observed with leaf ethanolic extract (Table 3.2.2). However, the aqueous extracts from all the plant's parts showed pro-oxidative rather than anti-oxidative activities at least in this model.

Table 3.2.2: Percentages of hydroxyl radical scavenging activity of extracts from various parts of *Z. mucronata*

Samples	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
Ethyl acetate	74.48 ± 0.50 ^g	79.85 ± 1.78 ^g	83.87 ± 0.18 ^h	94.20 ± 0.12 ^h	98.72 ± 0.42 ^h
Ethanolic	45.06 ± 1.02 ^b	38.65 ± 0.75 ^c	23.64 ± 3.69 ^c	16.45 ± 1.39 ^d	1.09 ± 0.50 ^d
Aqueous	-70.19 ± 2.37 ^a	-100.00 ± 0.00 ^a	-100.00 ± 0.00 ^a	-100.00 ± 0.00 ^a	-100.00 ± 0.00 ^a
Root					
Ethyl acetate	68.35 ± 1.39 ^f	80.78 ± 0.57 ^g	89.12 ± 1.71 ⁱ	93.26 ± 1.09 ^h	94.66 ± 0.64 ^g
Ethanolic	43.27 ± 3.41 ^b	38.16 ± 3.71 ^c	30.82 ± 0.22 ^d	20.45 ± 0.93 ^e	16.76 ± 7.54 ^e
Aqueous	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	-10.63 ± 1.99 ^b	-23.58 ± 1.43 ^c	-51.14 ± 1.60 ^b
Leaves					
Ethyl acetate	49.42 ± 0.56 ^c	47.10 ± 0.27 ^d	48.30 ± 4.86 ^e	28.81 ± 1.26 ^f	16.24 ± 0.47 ^e
Ethanolic	60.89 ± 0.60 ^d	67.52 ± 1.86 ^e	80.67 ± 0.94 ^g	82.41 ± 0.65 ^g	78.52 ± 1.06 ^f
Aqueous	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	-7.93 ± 1.36 ^b	-11.86 ± 0.86 ^b	-20.48 ± 3.22 ^c
Trolox	57.32 ± 2.95 ^d	73.11 ± 1.44 ^f	76.04 ± 2.05 ^f	80.09 ± 3.93 ^g	79.82 ± 3.50 ^f

Results are expressed as mean±SD values of triplicate determinations. ^{a-i}Different alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 3.2.3 shows the NO radical scavenging activities of the various extracts of *Z. mucronata*. A non-dose dependent NO inhibition effects with root and leaf ethyl acetate extracts as well as stem bark ethanolic extract were observed while root aqueous extract was found to be pro-oxidative in this model. On the other hand, dose dependent NO inhibition activities were observed for ethyl acetate and ethanolic extracts of stem bark section of this plant which were significantly higher (P<0.05) than the results of gallic acid standard.

Table 3.2.3: Percentage of nitric oxide scavenging activities of extracts from various parts *Z. mucronata*

Samples	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
Ethyl acetate	12.70 ± 0.62 ^c	54.00 ± 9.41 ^{ef}	64.55 ± 4.74 ^f	72.66 ± 4.80 ^h	80.60 ± 1.26 ^h
Ethanollic	31.33 ± 1.27 ^d	51.24 ± 4.93 ^e	43.96 ± 1.86 ^e	41.24 ± 0.94 ^e	40.60 ± 0.80 ^e
Aqueous	25.06 ± 6.91 ^d	26.87 ± 3.23 ^d	32.35 ± 2.80 ^d	41.59 ± 6.64 ^{ef}	53.07 ± 2.09 ^c
Root					
Ethyl acetate	8.30 ± 3.12 ^b	15.81 ± 2.95 ^c	21.48 ± 8.92 ^{bcd}	15.61 ± 3.65 ^c	38.75 ± 2.29 ^e
Ethanollic	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	15.17 ± 0.78 ^b	44.36 ± 1.05 ^f	48.10 ± 1.93 ^f
Aqueous	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	-66.70 ± 3.55 ^a	-100.00 ± 0.00 ^a
Leaves					
Ethyl acetate	62.45 ± 2.30 ^g	60.99 ± 8.60 ^{ef}	69.94 ± 4.99 ^a	24.00 ± 3.89 ^d	14.24 ± 2.30 ^b
Ethanollic	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^b	18.05 ± 1.66 ^c
Aqueous	52.99 ± 1.47 ^f	32.06 ± 3.60 ^d	29.66 ± 1.09 ^d	28.36 ± 9.70 ^{de}	12.60 ± 0.97 ^b
Ascorbic acid	47.81 ± 0.51 ^e	50.97 ± 1.35 ^e	52.82 ± 1.35 ^f	56.72 ± 3.69 ^g	64.08 ± 4.25 ^g
Gallic acid	4.43 ± 2.69 ^b	7.52 ± 1.11 ^b	25.74 ± 0.44 ^c	27.66 ± 1.91 ^d	31.70 ± 2.51 ^d
Trolox	66.30 ± 1.23 ^h	65.51 ± 1.27 ^f	63.39 ± 1.84 ^f	59.53 ± 1.81 ^g	52.21 ± 2.47 ^f

Results are expressed as mean±SD values of triplicate determinations. ^{a-h}Different alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 3.2.4: IC₅₀ values of various extracts of *Z. mucronata* parts in different anti-oxidative models

Samples	IC ₅₀ (µg/ml)		
	DPPH scavenging activity	Hydroxyl radical scavenging activity	Nitric oxide radical scavenging activity
Stem bark			
Ethyl acetate	122.16	1.07	43.34
Ethanollic	1.99	12.18	56431.12
Aqueous	35.36	P	239.10
Root			
Ethyl acetate	553.33	1.36	1818.82
Ethanollic	1.38	8.19	242.17
Aqueous	24.64	P	P
Leaves			
Ethyl acetate	3.61	22.06	48.85
Ethanollic	1.68	2.09	425039.14
Aqueous	106.73	P	12.52
Ascorbic acid	2.56	ND	26.28
Gallic acid	1.27	ND	1026.45
Trolox	5.04	2.61	599.21

ND means not determined and P means the extract showed pro-oxidative properties in the experimental model

3.2.5 Discussion

Ziziphus mucronata has been reported to be used in the traditional management of diabetes mellitus, a disease whose pathogenesis has, in part, been linked to the development of oxidative stress in affected humans. As per our knowledge, the antioxidant activities of all the parts of the plants within the *Ziziphus* genus have not been reported until now. This study investigated the anti-oxidative activities of various extracts from different parts of *Z. mucronata* as a prelude to find agent(s) that could be used to ameliorate T2D-associated abnormalities. We found in this study that some of the *Z. mucronata* extracts possess exceptionally very high anti-oxidative activities, at least with experimental models used in this study.

Different models for *in vitro* anti-oxidative studies were used because a single method cannot give a comprehensive prediction of the anti-oxidative capacities of the different extracts under investigation. It was proposed that the electron donating ability which reflects the reducing power of phytochemicals is linked with antioxidant activity [28, 29]. Antioxidants can be reductants which can inactivate oxidants and the reaction can be described as a redox reaction in which one reaction species is reduced at the expense of the oxidation of the other. Thus, the presence of antioxidant substances in the samples causes the conversion of the Fe^{3+} /ferricyanide complex to the Fe^{2+} form which can be measured at 700 nm [30]. The reducing power of the extracts increased with increasing concentration which suggests that the electron donating ability of the extracts is concentration dependent. The higher reducing power of the ethanolic extracts from different parts of this plant at almost all concentrations used suggest that the ethanol extractable phytochemical constituents of this plant has strong redox potentials that could act as reducing agents, hydrogen donors and singlet oxygen quenchers [31].

The model of scavenging the stable DPPH radical is a widely used method to evaluate the antioxidant activity of extracts or pure compounds. DPPH is a free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Hence, DPPH is usually used as a substrate to evaluate the free radical scavenging activity of antioxidative agents *in vitro* [32]. Using this model, the ethanolic extracts of all the parts of *Z. mucronata* also demonstrated remarkable anti-radical activities with IC_{50} values lower than those of ascorbic acid and trolox. Although, a number of reports on the DPPH radical scavenging activity of extracts from plants belonging to different families and from parts of the world exist in the literature but only a few authors [33] reported such a low IC_{50} value as observed with the ethanolic extracts of this plant. This further suggests that these extracts contain powerful free radical scavenging phytochemicals that could have the ability to inhibit free radical upsurge as well as oxidative stress which consequently might ameliorate T2D-associated complications and indeed other oxidative stress associated metabolic disorders.

The hydroxyl radical is a highly potent free radical in living organism and has been implicated as a highly damaging species in free radical pathology that can damage all life essential biomolecules [34]. The hydroxyl radical scavenging activity is thus measured as the percentage of inhibition of these radicals generated in the Fenton's reaction mixture (Fe^{3+} /EDTA/ascorbate/ H_2O_2) by studying the competition between deoxyribose and the plant extracts [35]. In this study, all the organic extracts from *Z. mucronata* contain phytochemicals with hydroxyl radical inhibition activity. However, the stem bark and root ethyl acetate extracts were found to be more effective in hydroxyl radical inhibition than the corresponding ethanolic extracts whereas leaves ethanolic extract was more effective in hydroxyl radical inhibition than the ethyl acetate extract. The reciprocal hydroxyl radical inhibition pattern found in the ethanolic extracts of the stem bark and root samples as well as in the ethyl acetate extract of the leaves could be due to hormesis phenomenon exhibited by these extracts. Hormesis is a dose-response relationship for a single endpoint that is associated with reversal of response between high and low doses of biological molecule, chemicals, physical stressors, or any other agent that can initiate a response [36] and its occurrence has been documented in numerous biological, toxicological and pharmacological investigations [37]. The phenomenon could also account for the non-dose dependent response of the ethanolic extract of the leaves of this plant. Thus, these *Z. mucronata* extracts possess optimal points for effective inhibition of hydroxyl radical. On the other hand, the pro-oxidative tendencies of the aqueous extracts in the hydroxyl radical based anti-oxidative model further indicate the need to use a multi method approach before a definite statement can be made on the anti-oxidative effects of a plant extract.

Nitric oxide is a very unstable species that has been implicated in the pathology of cancer, type 2 diabetes and several other diseases [23, 25, 38]. Ethyl acetate extracts of the stem bark and leaves of this plant displayed greater inhibitions against NO radical than the corresponding ethanolic extracts. This could indicate that the anti-oxidative principles extracted by the two solvents act via different antioxidant mechanisms. Further, the hormetic responses shown by some extracts towards scavenging NO indicate that the anti-NO principles have some optimal points of effective inhibitions and/or antagonism occur with other phytochemicals at certain concentrations. Considering all the anti-oxidative models used in this study, it is possible to surmise that the quantitative difference in the anti-oxidative activities among the plant parts could be attributed to the variation(s) in the concentrations and compositions of the anti-oxidative principles in the different parts since distinct function(s) is performed by the parts and hence tend to produce slightly different chemical constituents.

We therefore concluded that the various parts of *Z. mucronata* possessed anti-oxidative activities that could account for the folkloric use of the plant in the treatment of diabetes mellitus. Our results also suggest that the ethanolic extracts from different parts of the plant contain the most active anti-oxidative principles. Bioassay guided fractionation and evaluating the anti-T2D effects of the most active anti-oxidative fraction from the ethanolic extracts will be the subject of our future investigations.

Postscript : From the above experiment, the ethanolic crude extract from the root was found to have relatively higher anti-oxidative activity that cuts across various types of radicals than other extracts. It was therefore selected for further investigations.

3.3 Butanol fraction of *Ziziphus mucronata* (Willd) root ethanolic extract contains anti-oxidative agents and potent inhibitors α -glucosidase and α -amylase

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Preface: In this sub-chapter, solvent-solvent fractionation of the crude ethanol extract of the root was carried out and the solvent fractions were investigated for anti-oxidative and α -glucosidase and α -amylase inhibitory activities. This article has been submitted for publication to Indian Journal of Experimental Biology and is currently under review

3.3.1 Abstract

The *in vitro* anti-oxidative as well as the α -glucosidase and α -amylase inhibitory effects of various solvent fractions derived from the crude ethanol extract of *Ziziphus mucronata* root were investigated. Our findings indicated that butanol fraction contained a significantly higher ($P < 0.05$) amount of total phenolics, FRAP and DPPH radical scavenging activity than other solvent fractions. Furthermore, the butanol fraction elicited a significantly higher ($P < 0.05$) inhibitory effects on α -glucosidase ($IC_{50}=1.41 \pm 0.16 \mu\text{g/ml}$) and α -amylase ($IC_{50}=105.86 \pm 5.21 \mu\text{g/ml}$) activities than other fractions. Steady state kinetic analysis revealed that the butanol fraction is a mixed inhibitor of both α -glucosidase and α -amylase altering both K_M and V_{max} of the enzymes. The computed index of physiological efficiency (K_{cat}) of the enzymes was also reduced in the presence of the fraction. Analysis of the butanol fraction using GC-MS indicated that the fraction contains mainly phenolics such as catechol, pyrogallol, 2,6-dimethoxy phenol and 2-methoxyhydroquinone. Data from the study suggest that the butanol fraction contains potent anti-oxidative agents and inhibitors of α -glucosidase and α -amylase which could be exploited for developing holistic therapeutic agents for the control of postprandial blood glucose levels, type 2 diabetes and chronic vascular complications.

3.3.2 Introduction

Oxidative stress refers to the presence of free radicals and reactive oxygen species (ROS) that are generated in normal physiological processes. However, when there is an excess generation of ROS or the endogenous antioxidants (AO) systems are inactivated, the ROS/AO equilibrium is altered in favor of

stress and consequently the species become harmful [21, 22]. At present, the concept of oxidative stress as a major pathogenic phenomenon for a number of non-communicable chronic diseases and their associated complications is a favored hypothesis [39, 40]. Furthermore, oxidative stress is believed to be the most important biological phenomenon for both macrovascular and microvascular complications associated with type 2 diabetes (T2D) [41]. Thus, diabetes therapy targeted to reduce oxidative stress would be beneficial to the patients and other risk populations.

On the other hand, the control of postprandial hyperglycemia is also considered as an important strategy for treatment of T2D and preventing its associated complications such as nephropathy, neuropathy, retinopathy and cardiomyopathy. Among the different therapeutic strategies to reduce postprandial hyperglycemia, delaying the digestion and absorption of carbohydrates by inhibiting carbohydrate-hydrolyzing enzymes, such as α -amylase and α -glucosidase, in the digestive organs holds a strong potential [42]. α -Amylase hydrolyzes starch and other complex polysaccharides to oligosaccharides which are further hydrolyzed by intestinal α -glucosidase to liberate glucose which is then absorbed into the intestinal epithelium and enter blood circulation. Consequently, postprandial rise in blood glucose concentration could be retarded by inhibitors of these enzymes [43]. Thus, the amelioration of oxidative stress and the control of postprandial hyperglycemia offer a unique therapeutic strategy for the treatment of T2D and for reducing its associated complications [44]. Therefore, the search for alternative agents with potent anti-oxidative properties that could also decrease postprandial hyperglycemia via inhibiting carbohydrate digesting enzymes is an important therapeutic strategy that would provide a holistic avenue to control hyperglycemia and other diabetic complications resulting from oxidative stress.

Traditional herbal medicines have been used throughout the world for the treatment of various diseases and are still considered to be an important component of health care delivery system, especially in Africa [14]. The World Health Organization (WHO) has also recommended herbal medicines as targets for scientific investigation in order to search for novel agents to treat different diseases [45]. This is because most modern medicines exert serious side effects. A typical example is the clinically used glucosidase inhibitors, acarbose and miglitol, mostly found to cause gastrointestinal disturbances such as diarrhea, other intestinal pain and flatulence [46]. Interestingly, glucosidase inhibitors derived from plants sources were reported to be more acceptable because they are cheap and relatively safer, including a lower tendencies of causing serious gastrointestinal discomfort [47, 48]. This makes the search of glucosidase inhibitors from plants an appealing alternative.

Ziziphus mucronata Willd (Rhamnaceae), commonly referred to as “buffalo thorn”, is native to northern Nigeria. A decoction of the plant is used traditionally in the treatment of diabetes mellitus among the rural inhabitants of northern Nigeria [4]. In a recent study, we subjected the various crude solvents extracts

from the stem bark, root and leaf samples of the plant to a series of *in vitro* anti-oxidative assays and found that the ethanolic extract of the root had the most potent anti-oxidative activities that reasonably cut across various models [49]. In this study, we further fractionated the ethanolic extract of the root by solvent-solvent partitioning and then conducted a comprehensive investigation on the *in vitro* anti-oxidative as well as the α -glucosidase and α -amylase inhibitory effects of the fractions.

3.3.3. Materials and Methods

Please refer to the chapter 2 sub-sections 2.6.1-2.6.6 pages 38-40 for detailed material and methods that involve *Z. mucronata*

3.3.4 Results

The total reducing power of the butanol fraction was significantly higher ($P < 0.05$) than all other fractions and the standard antioxidants used (**Figure 3.3.1**). All the fractions were found to scavenge the DPPH radical in a dose dependent fashion (**Figure 3.3.2**) but judging from the IC_{50} values, the butanol fraction also displayed a significantly ($P < 0.05$) more powerful DPPH radical scavenging activity than other fractions (**Table 3.3.2**).

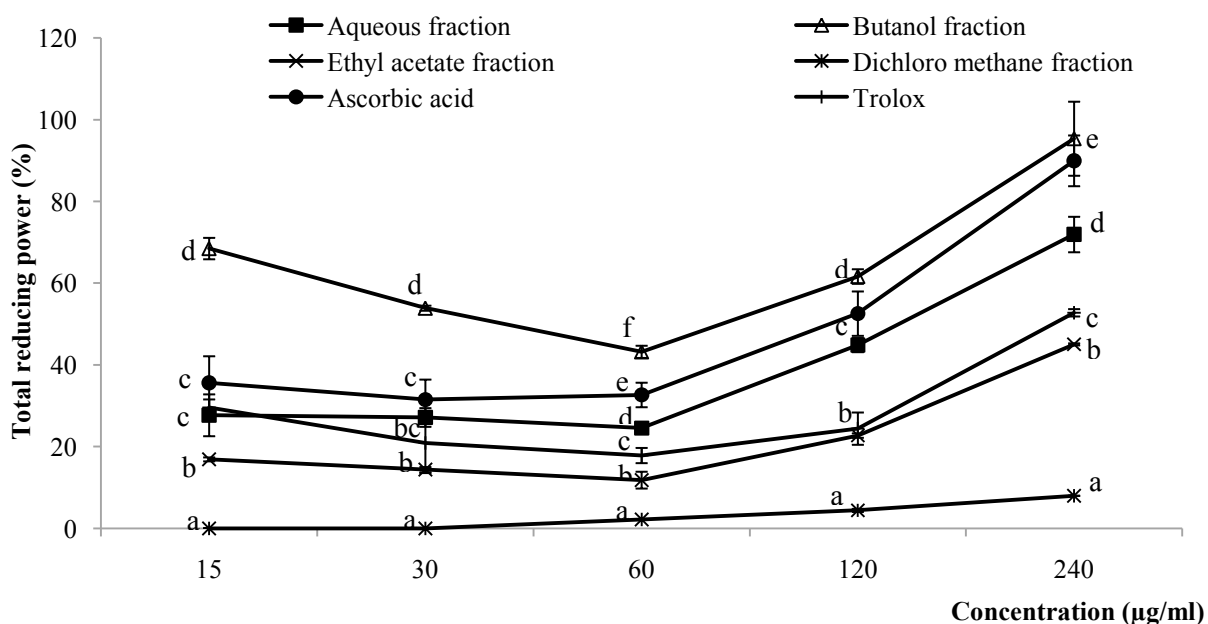


Figure 3.3.1: Total reducing power (relative to gallic acid) of different solvent fractions of *Z. mucronata* root ethanolic extract. The results are expressed as mean \pm SD of triplicate determinations. ^{a-f}Different alphabets for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

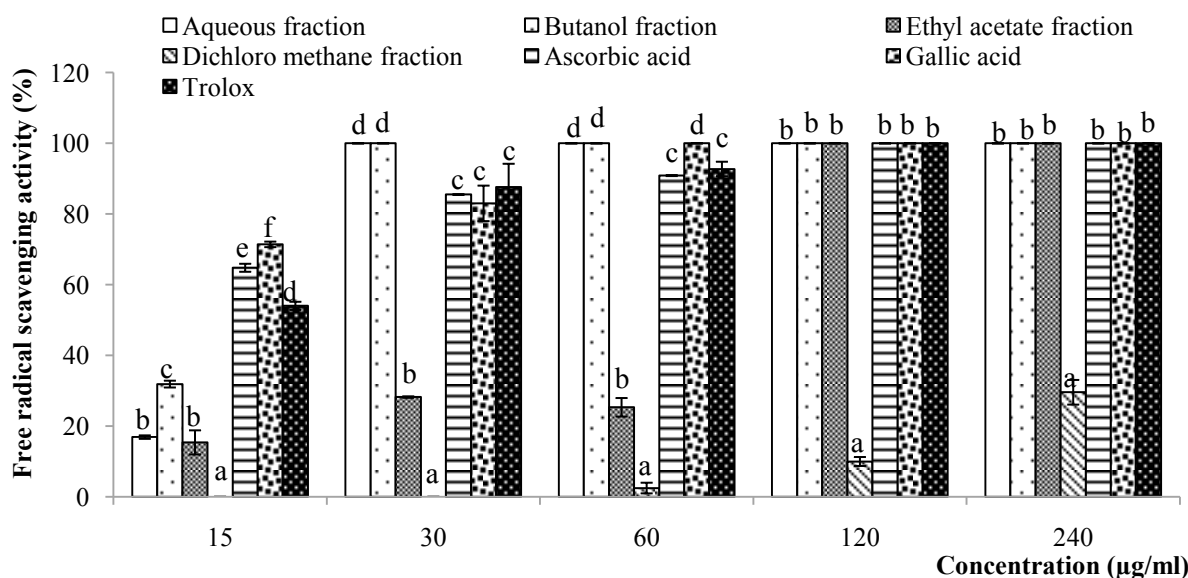


Figure 3.3.2: Free radical scavenging activities of different solvent fractions of *Z. mucronata* root ethanolic extract. The results are expressed as mean \pm SD of triplicate determinations. ^{a-f}Different alphabets over the bars for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The results of the HRS assay also showed that all the fractions contained phytochemicals that could scavenge hydroxyl radicals produced by Fenton's reaction in a dose dependent manner (**Table 3.3.1**). However, the less polar fractions (ethyl acetate and dichloromethane) showed a significantly higher ($P < 0.05$) HRS activity than the more polar fractions (butanol and water) as shown by the IC_{50} values (**Table 3.3.2**). On the other hand, analysis of the total phenolic content of the fractions indicated that the butanol fraction had a significantly higher ($P < 0.05$) phenolic content than other fractions (**Table 3.3.2**).

Table 3.3.1: Percentage HRS activity of fractions from the ethanolic extract of *Z. mucronata* root

Fractions	Concentration ($\mu\text{g/ml}$)				
	15	30	60	120	240
Aqueous	35.15 ± 7.55^b	51.63 ± 1.24^b	63.59 ± 1.98^b	71.36 ± 0.52^b	74.96 ± 0.41^a
Butanol	20.12 ± 1.24^a	42.60 ± 1.47^a	56.59 ± 2.85^a	68.95 ± 1.56^a	75.61 ± 1.39^a
Ethyl acetate	52.28 ± 0.74^c	64.11 ± 0.71^c	73.40 ± 1.14^c	78.55 ± 0.30^c	78.94 ± 0.56^b
Dichloromethane	49.73 ± 1.67^c	$62.21 \pm .08^c$	71.30 ± 1.14^c	71.04 ± 0.68^a	74.63 ± 0.30^a
Trolox	57.32 ± 2.95^d	73.11 ± 1.44^d	76.04 ± 2.05^d	80.09 ± 3.93^c	$79.82 \pm 3.50^{a,c}$

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-d}Different superscript alphabets within a column are significantly different (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 3.3.2 : IC₅₀ values of various solvent fractions of ethanolic extract of *Z. mucronata* root in different anti-oxidative models

Fractions	Total phenolics (mg/g GAE)	IC ₅₀ (µg/ml)	
		DPPH	Hydroxyl radical
Aqueous	143.06 ± 0.96 ^c	14.57 ± 0.18 ^d	34.00 ± 0.42 ^c
Butanol	150.39 ± 1.15 ^d	9.17 ± 0.33 ^c	52.31 ± 2.00 ^d
Ethyl acetate	95.56 ± 0.78 ^b	53.01 ± 1.70 ^e	8.00 ± 0.26 ^b
Dichloromethane	3.91 ± 0.80 ^a	3.29 ± 0.63 ^{f*}	9.11 ± 1.63 ^b
Ascorbic acid	-	2.56 ± 0.26 ^b	ND
Gallic acid	-	1.40 ± 0.43 ^a	ND
Trolox	-	8.47 ± 2.88 ^c	3.23 ± 0.49 ^a

Results are expressed as mean ± SD values of triplicate determinations. ^{a-d}Different superscript alphabets within a column for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05).

*Unit is presented in mg/ml.

The α-glucosidase and α-amylase inhibitory activities of the fractions are shown in **Figure 3.3.3**. All the solvent fractions inhibited α-glucosidase activity *in vitro* in a dose dependent pattern (**Figure 3.3.3A**). The more polar fractions demonstrated significantly higher (P < 0.05) α-glucosidase inhibitory activity than the less polar fractions and acarbose. However, within the more polar fractions, the inhibitory activity demonstrated by the butanol fraction was significantly higher (P < 0.05) than aqueous fraction as shown by the IC₅₀ values (**Table 3.3.3**). Pancreatic α-amylase activity was also inhibited *in vitro* by all the fractions in a dose dependent fashion (**Figure 3.3.3B**) and the butanol fraction showed a significantly higher (P < 0.05) α-amylase inhibitory effect than other fractions and acarbose.

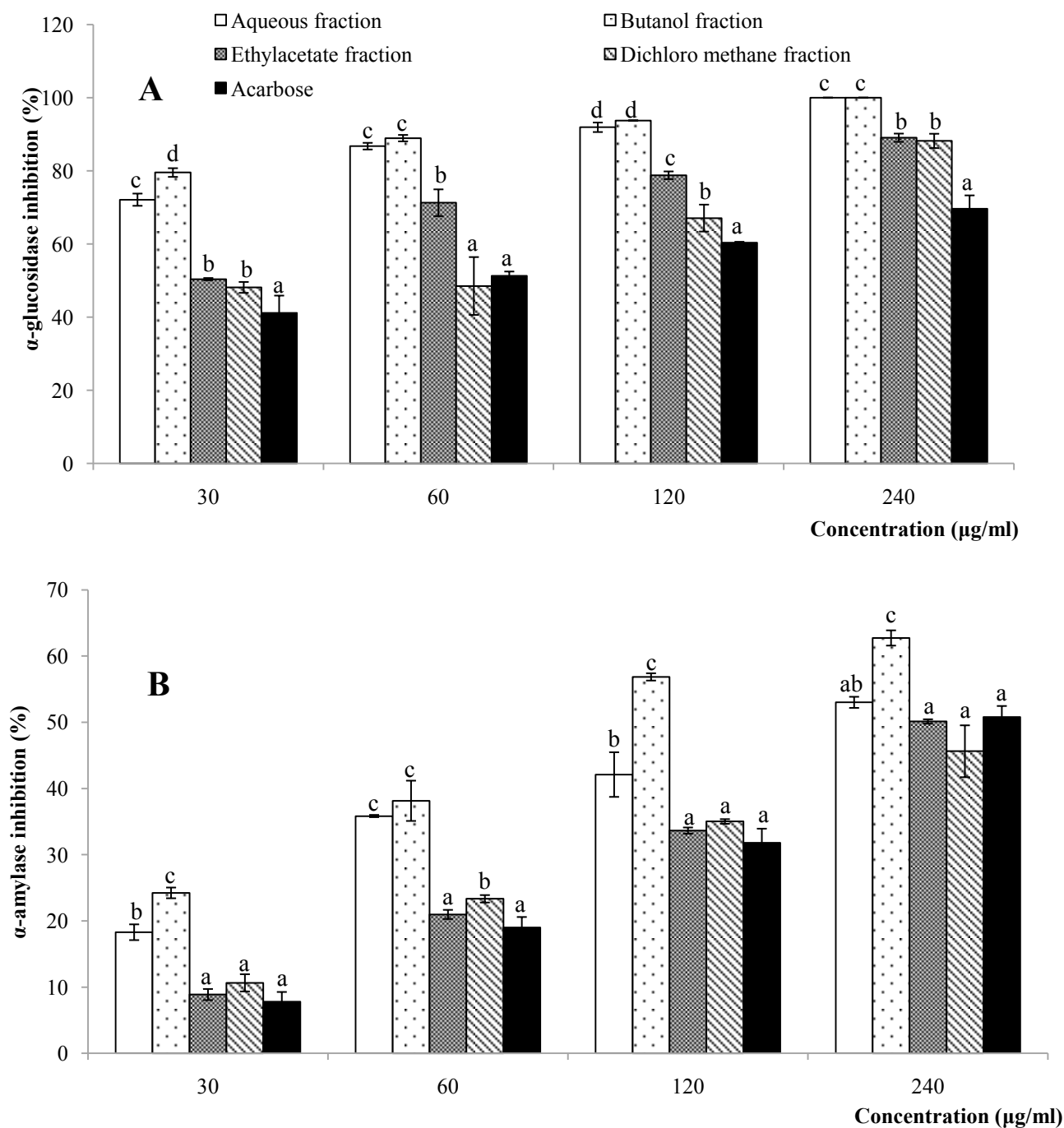


Figure 3.3.3: α -glucosidase (A) and α -amylase (B) inhibitory effects of different solvent fractions of ethanolic extract of *Z. mucronata* root. The results are expressed as mean \pm SD of triplicate determinations. ^{a-d} Different alphabets over the bars for a given concentration of each fraction indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 3.3.3: IC₅₀ values for the inhibition of α -glucosidase and α -amylase by various solvent fractions of ethanolic extract of *Z. mucronata* root

Fractions/standard	IC ₅₀ (μ g/ml)	
	α -glucosidase	α -amylase
Aqueous	4.38 \pm 1.08 ^b	187.31 \pm 15.99 ^b
Butanol	1.41 \pm 0.16 ^a	105.86 \pm 5.21 ^a
Ethyl acetate	23.60 \pm 2.33 ^c	252.48 \pm 2.56 ^c
Dichloromethane	43.91 \pm 6.99 ^d	306.97 \pm 57.81 ^c
Acarbose	55.59 \pm 5.22 ^d	256.66 \pm 20.52 ^c

The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different superscripts alphabets within a column for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05).

Furthermore, a steady state kinetic analysis of both α -glucosidase and α -amylase at varied substrate concentrations in the presence and absence of 30 μ g/ml of the butanol fraction was conducted. From the experiment, the butanol fraction exerted mixed inhibition pattern on both α -glucosidase and α -amylase with altered the K_M and V_{max} of both enzymes (**Table 3.3.4**). Interestingly, the computed index of physiological efficiency (K_{cat}) of both enzymes was reduced in the presence of the butanol fraction (**Table 3.3.4**).

Table 3.3.4: Effect of butanol fraction (30 μ g/ml) of ethanolic extract of *Z. mucronata* root on some kinetic parameters of α -glucosidase and α -amylase

Kinetic parameters	α -glucosidase		α -amylase	
	Control	+ butanol fraction	Control	+ butanol fraction
K _M	2.00*	1.17*	0.25 [#]	6.66 [#]
V _{max} (μ mol/min)	655.09	19.64	33.70	223.21
K _{cat} (min ⁻¹)	327.54	16.78	134.80	33.51

The units for K_M was mM (*) and % ([#])

Results from the GC-MS analysis of the butanol fraction revealed that it contains phenolic compounds such as catechol, pyrogallol, 2,6-dimethoxy phenol and 2-methoxyhydroquinone, sugars such as levoglucosan trimethyl ether and ethyl α -D-glucopyranoside, fatty acids and esters as well as other aromatics (**Figure 3.3.4** and **Table 3.3.5**) which were identified by their fragmentation pattern in conjunction with the NIST library. The chemical structure of the identified components is presented in **Figure 3.3.5**.

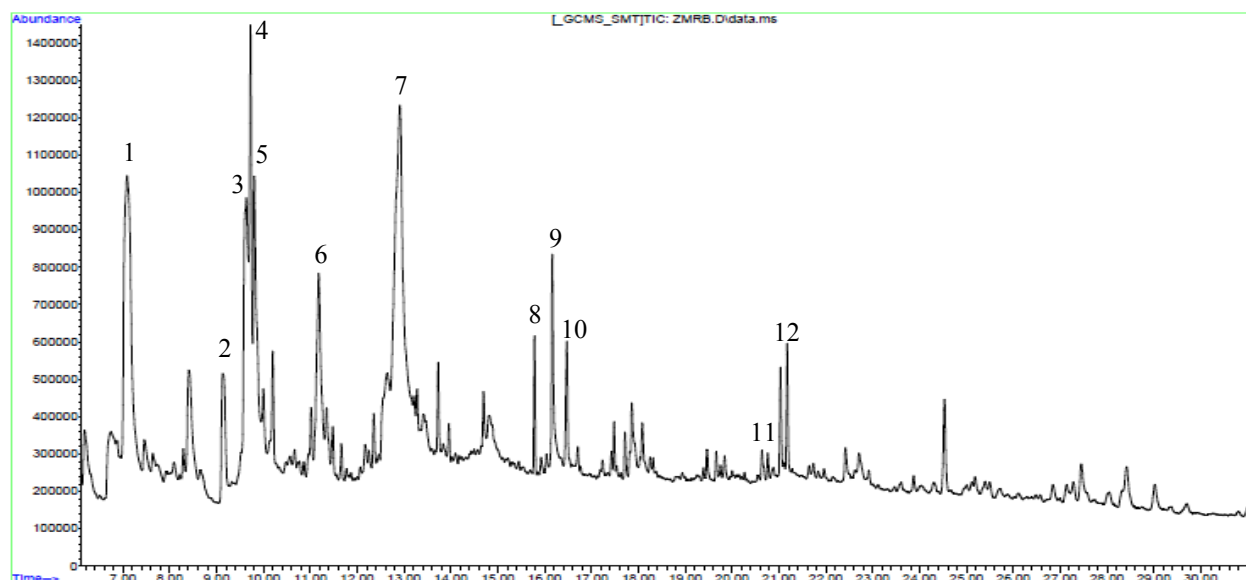


Figure. 3.3.4: GC-MS chromatogram of the butanol fraction from the ethanolic extract of *Z. mucronata* root

Table 3.3.5: Identified components of butanol fraction of *Z. mucronata* root ethanolic extract by GC-MS

§	Compound	Retention time (min)	Molecular mass
1	catechol	7.08	110.1
2	2,6-dimethoxy phenol	9.13	154.1
3	pyrogallol	9.63	126.0
4	2,4-diisopropenyl-1-methyl-1-vinylcyclohexane	9.72	204.4
5	2-methoxyhydroquinone	9.80	140.1
6	levoglucosan trimethyl ether	11.18	204.2
7	ethyl α -D-glucopyranoside	12.92	208.2
8	palmitic acid, methyl ester	15.78	270.3
9	palmitic acid	16.16	256.3
10	2-(2-chlorobenzylidene)cyclohexanone	16.47	220.1
11	[1-cyano-2-(3-methoxyphenyl)-vinyl]-phosphonic acid diethyl ester	21.03	295.1
12	Phthalic acid mono -(2-ethylhexyl) ester	21.17	279.2

§ represents annotated peak numbers

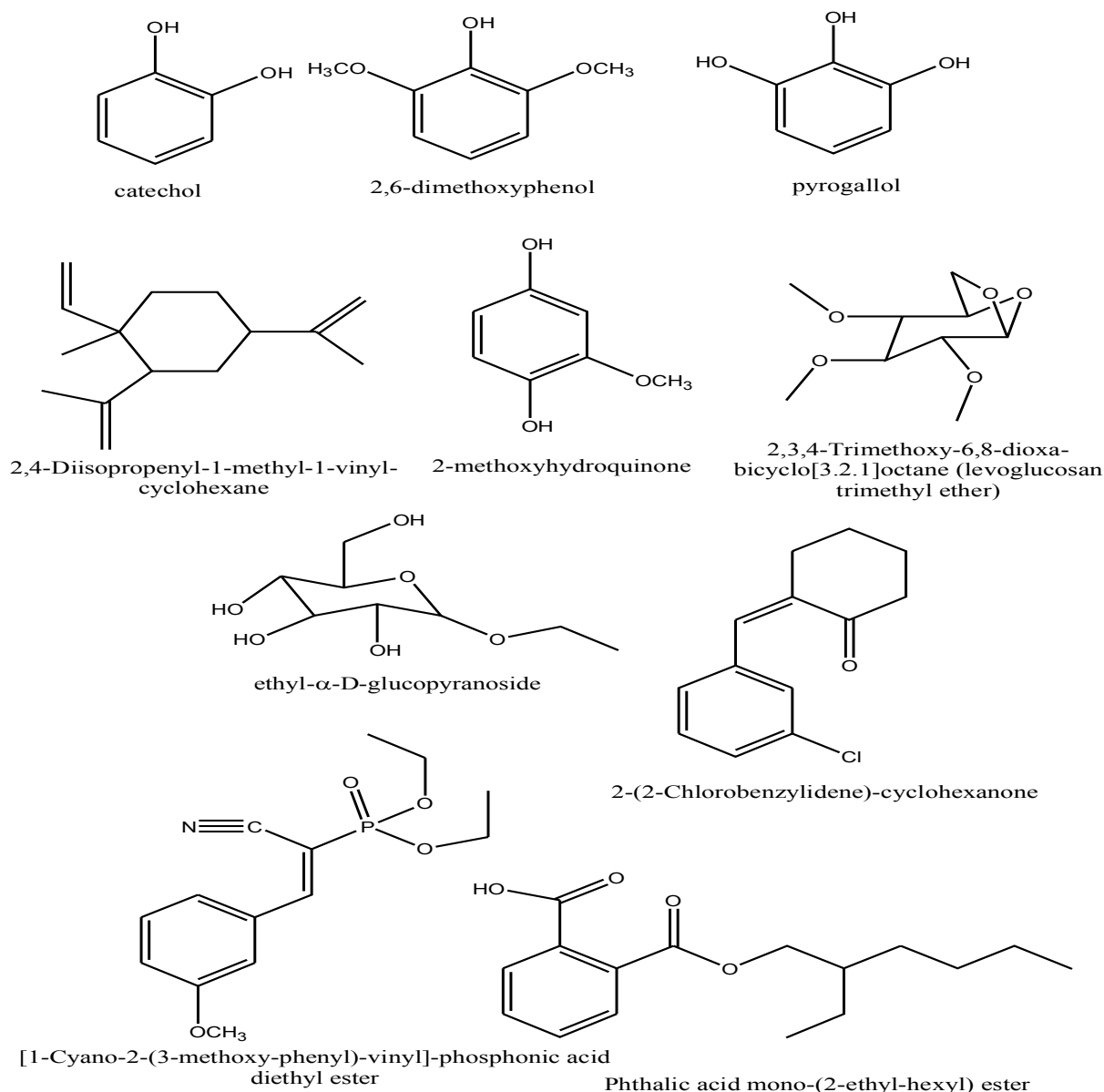


Figure 3.3.5 : Structures of some of the compounds identified in the butanol fraction of *Z. mucronata* root ethanolic extract by GC-MS.

3.3.5 Discussion

A viable strategy for T2D management is via the inhibition of α -glucosidase and α -amylase as well as the use of potent anti-oxidative agents [41, 42]. This is because the approach provides a holistic therapeutic option for the amelioration of hyperglycemia and chronic vascular complications associated with oxidative stress. Our findings revealed that the butanol fraction derived from the crude ethanolic extract of *Z. mucronata* root contained powerful anti-oxidative agents as well as α -glucosidase and α -amylase inhibitors.

Plants produce various classes of phytochemicals that could be polar or non-polar. Thus, the crude ethanolic extract of the root was sequentially fractionated to achieve good separation of all non-polar as well as polar components and thereby separately involving all components in the study. Different models for *in vitro* anti-oxidative studies were used because a single method cannot give a comprehensive prediction of the anti-oxidative capabilities of the different fractions tested. It was proposed that the Fe^{3+} to Fe^{2+} reducing ability of phytochemicals is associated with their anti-oxidative activity [29]. Using this model, the butanol fraction displayed consistently higher reducing power than other fractions. Free radicals are important contributory factors to a number of biological damages and DPPH is used to investigate the free radical scavenging activity of phytochemicals. Hydroxyl radicals are also extremely reactive species that could damage any biological molecule found in living systems [34]. All these radicals are implicated in the pathogenesis of T2D [41]. From the present study, the butanol fraction also demonstrated remarkable anti-radicals activities which could further suggest that the fraction contains powerful radical scavenging phytochemicals that could have the ability to inhibit free radical upsurge as well as oxidative stress which consequently might ameliorate T2D-associated complications and indeed other oxidative stress associated metabolic disorders.

The process of digestion of carbohydrates in mammals involves rapid conversion of digestible starch into monosaccharide units (glucose) which are absorbed into the intestinal epithelium. Pancreatic and intestinal α -glucosidases perform disaccharides hydrolysis and in T2D, an increase in the rate of hepatic glycogenolysis and gluconeogenesis is linked with low glucose utilization by tissues and this is a vital mechanism underlying hyperglycemia [50]. Thus, inhibitors of liver α -glucosidase could inhibit α -1,6-glucosidase of glycogen-debranching enzymes, reduce the glycogenolytic rate and consequently decrease hyperglycemia [50] whereas inhibitors of intestinal α -glucosidase are effective in delaying intestinal glucose uptake and consequently decrease postprandial hyperglycemia. In this study, we found that the butanol fraction exhibited the most powerful inhibitory activity against the α -glucosidase through a mixed inhibition pattern. Thus, the fraction contains powerful (mixed) inhibitors that are able to bind both at the active site and at an allosteric site of the enzyme [51] which consequently decreased the computed index of physiological efficiency of the enzyme. These observations could further imply that the fraction is capable of binding to the different isozymes of α -glucosidase at saturation of the enzyme and cause a reduction in the hydrolysis of disaccharides/oligosaccharides to liberate glucose and consequently lower the postprandial blood glucose levels.

The butanol fraction had the highest α -amylase inhibitory activity which is mediated through a mixed type of inhibition. In humans, two α -amylase isozymes (salivary and pancreatic) exist which are encoded by five genes, three for the salivary isozyme and two for the pancreatic isozyme. Both salivary and

pancreatic α -amylases are made up of 496 amino acids in a single polypeptide chain with an overall sequence similarity of 97% and 92% similarity in the catalytic domain [42]. These similarities connote that the butanol fraction could interact with both isozymes through a similar pattern to slow down the breakdown of complex carbohydrates to oligosaccharides, thereby diminishing the postprandial effect of carbohydrates consumption on blood glucose. Even though our study was carried out *in vitro* but the findings could be relevant in an *in vivo* situation. Some plant extracts were demonstrated to inhibit α -glucosidase and α -amylase activities in both *in vitro* and *in vivo* models [52]. Furthermore, the *in vitro* and *in vivo* α -glucosidase and α -amylase inhibitory activities of some flavonoids and triazoles have been documented [53, 54].

Phenolic compounds are the main plant's constituents responsible for anti-oxidative activity [55]. These phytochemicals also interact with proteins and inhibit enzyme actions including α -glucosidase and α -amylase activity [43]. The butanol fraction contained the highest amount of phenolics in addition to the observed high anti-oxidative and enzymes inhibitory activities. It could thus imply, although without a correlation analysis, that phenolics play a vital role in the α -glucosidase and α -amylase inhibitory activities of this fraction. Previous studies indicated that the α -amylase and α -glucosidase inhibitory effects of *Ocimum basilicum*, *Citrus maxima*, cinnamon and tea extracts are mediated by phenolics [43, 56, 57]. Indeed, α -amylase inhibitory activities of some phenolics have been proposed to be through the hydrogen bonds formation between the -COO^- groups of Asp197 and Glu233 in the active site of the enzyme and the hydroxyl groups of the phenolics [58]. Interestingly, our GC MS analysis also revealed that the butanol fraction contained an array of phenolics which includes catechol, 2,6-dimethoxy phenol, 2-methoxyhydroquinone and pyrogallol. Thus, while not discounting the possible contribution of other components, it is possible to suggest that these phenolics act individually or synergistically to bring about the observed inhibitory effects.

In conclusion, our data suggest that the butanol fraction of *Z. mucronata* root ethanolic extract contains potent anti-oxidative agents and inhibitors of α -glucosidase and α -amylase which could be exploited for the development of holistic therapeutic strategy for the control of postprandial blood glucose levels, T2D and chronic vascular complications. While phenolics appear to play a vital role on the observed activity, the possible contribution of other detected phytochemicals should not be discounted. Our future work will involve a detailed study on the antidiabetic activity of the butanol fraction in a T2D diabetes model of rats as well as isolating some pure compounds from it.

Postscript: From the above studies, the butanol fraction had the best α -glucosidase and α -amylase effects and therefore it was selected for detailed *in vivo* anti-diabetic study

3.4 Effects of butanol fraction of *Ziziphus mucronata* root on glucose homeostasis, serum insulin, hepatic glycogen metabolism and diabetes-related complications in a type 2 diabetes model of rats

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Preface: This article reports the detailed *in vivo* anti-diabetic studies of the acetone fraction and it is currently awaiting revision before submission to a journal for publication.

3.4.1. Abstract

Ziziphus mucronata is currently used in the traditional treatment of diabetes mellitus in Nigeria. The present study investigated the effects of the butanol fraction of *Z. mucronata* root (ZMBF). Chromatographic investigation of the fraction led to the isolation of a chromone, 2,7-dihydroxy-4H-1-benzopyran-4-one which strongly inhibited α -glucosidase and α -amylase using uncompetitive and non-competitive inhibition patterns respectively. Subsequently, the effects of ZMBF treatments on polyphagia, polydipsia, body weight gain, glucose homeostasis, insulin secretion, β cell function (HOMA- β), insulin resistance (HOMA-IR), serum fructosamine level, liver glycogen metabolism, serum lipid profile and biomarkers of hepatic and renal functions in a type 2 diabetes (T2D) model of rats. The ZMBF treatment, at 300 mg/kg bw, significantly ($P < 0.05$) lowered blood glucose level, improved oral glucose tolerance ability, stimulated insulin secretion and hepatic glycogen synthesis. However, polyphagia, polydipsia, body weight gain, β cell function, insulin resistance, serum fructosamine level and hepatic and renal functions were not significantly ($P > 0.05$) affected by the treatment. Findings from this study suggest that ZMBF treatment, at 300 mg/kg b.w, possess anti-diabetic activity but could slightly ameliorate some diabetes-associated complications in a T2D model of rats.

3.4.2 Introduction

The International Diabetes Federation (IDF) estimates that more than 366 million people worldwide are affected by the diabetes and this figure is projected to reach a staggering 552 million by 2030 [59] while type 2 diabetes (T2D) accounts for >90% of all the diabetic cases. T2D is a complex and polygenic metabolic disorder characterized mainly by insulin resistance and pancreatic β cell dysfunction [60] which leads to chronic hyperglycemia. Several mechanisms have been proposed for the β -cell destruction

including oxidative stress [41]. Currently, T2D is predominantly managed with sulfonylureas and biguanides but the use of α -glucosidase inhibitors is also considered as an important approach for the treatment of the disease [61]. Unfortunately, the use of these synthetic anti-diabetic agents is beset with a number of side effects such as diarrhea, nausea and liver failure [46].

For some few years back, research interest in traditional herbal medicine has tremendously grown especially for the care and management of diabetes and this happens in both developing and developed countries, due to their natural origin and lesser side effects [62, 63]. Indeed, traditional medicinal plants were listed by the World Health Organization (WHO) expert committee on diabetes as one of the methods for the treatment of diabetes which should be further investigated [64] to provide potential chemical leads for the development of novel anti-diabetic agents. In this context, African medicinal plants may provide the much needed chemical leads because of their multiple health benefits.

Ziziphus mucronata Willd (Rhamnaceae), commonly referred to as “buffalo thorn” is available in most parts of Africa. A decoction of the plant is used traditionally in the treatment of diabetes mellitus [4] among the rural inhabitants of northern Nigeria. In spite of this report, the anti-diabetic effects of the plant have not been scientifically validated either in humans or experimental animals. In fact, apart from few reports [7, 10, 65], information on the pharmacological actions of the plant is scanty in the literature. In a recent study [49], we subjected the various crude solvents extracts from the stem bark, root and leaf samples of the plant to a series of *in vitro* anti-oxidative assays and reported that the ethanolic extract of the root had the most potent anti-oxidative activities that reasonably cuts across various models. Hence, in the present study, we isolated a chromone from the butanol fraction of the root and subjected it to an *in vitro* α -glucosidase and α -amylase inhibitory activity assays. Subsequently, we conducted a comprehensive investigation on the *in vivo* activity of the butanol fraction on T2D which is an oxidative stress-related metabolic disorder.

3.4.3 Materials and methods

Please refer to the chapter 2 sub-sections 2.3-2.4 (page 35-36); 2.6.1 (page 38); 2.7.1-2.7.7 (pages 40-43) and 2.8.1 (pages 45-46) for detailed material and methods that involve *Z. mucronata*. However, the group codes DFL, DFH and NFT used in the chapter two are replaced with DZL, DZH and NZT respectively. ZMBF refers to the butanol fraction derived from the crude ethanol extract of *Z. mucronata* root.

3.4.4 Results

The ^1H NMR spectrum of fraction 29 indicated that it contained a mixture of an aromatic compound (1) and polar aliphatic chains. The aromatic resonances were contained in the region of δ 6.50 to δ 7.30 and the polar aliphatic chains had resonances between 0.80 and 1.60 as well as between δ 3.40 and 4.10, the latter indicating alcoholic functionality in the aliphatic chains. It was clear that the side chains were not

part of the chromone as part of an ester as the integration of the respective resonances was not in agreement. While it was not possible to identify the aliphatic chains, it was possible to elucidate a structure for the chromone based on its NMR data.

With regard to the aliphatic chains, it was difficult to ascertain the number of chains that co-eluted with the chromone, but alcoholic functionality could be identified by the methylene hydroxy peaks in the ^1H NMR spectrum at δ 3.46 (2H, dd, $J = 6.5, 4.1$ Hz), 3.51 (2H, dd, $J = 4.8, 4.8$ Hz), 3.70 (2H, brt, $J = 4.0$ Hz), coupled resonances at δ 3.92 (1H, d, $J = 9.0$ Hz), 4.00 (1H, d, $J = 9.0$ Hz) and a methine hydroxy peak at δ 4.08 (s). Their corresponding oxygenated carbon resonances further indicated alcoholic groups at δ 71.1, 71.7, 61.9 and 76.3 for the oxygenated methylene carbon atoms and at δ 75.8 for the methine hydroxyl carbon. The methyl and methylene resonances of the aliphatic chains were present between δ 0.80 and 1.60 in the ^1H NMR spectrum and between δ 13.9 and 32.0 in the ^{13}C NMR spectrum.

The ^1H NMR spectrum of compound **1** showed a characteristic NMR pattern for an A ring chromone system with *ortho* and *meta* coupled protons at δ 6.57 (d, $J = 8.2$ Hz, H-5), δ 7.04 (dd, $J = 8.2, 2.4$ Hz, H-6) and δ 7.27 (d, $J = 2.4$ Hz, H-8). The singlet resonance at δ 6.97 is attributed to H-3 on the pyranone ring. The 3-hydroxy option was ruled out since an H-2 proton would appear as a singlet in the region of δ 8.00 [66]. Furthermore, the NMR data were not consistent with Lai et al. [66] for 3,7-dihydroxy-4*H*-1-benzopyran-4-one. Using the HSQC spectrum, C-5 was identified at δ 115.9, C-6 at δ 123.5, C-8 at δ 124.1 and C-2 at δ 124.8. The oxygenated aromatic carbon resonances were seen at 151.8, 155.6 and 159.2 with the carbonyl resonance, C-4 being present at δ 177.2. The remaining carbon resonance of C-10 could be seen at δ 130.9. Based on the above, the compound (**1**) was identified as 2,7-dihydroxy-4*H*-1-benzopyran-4-one (**Figure 3.4.1**).

The enzyme inhibitory studies revealed that 2,7-dihydroxy-4*H*-1-benzopyran-4-one inhibited α -glucosidase and α -amylase catalyzed reactions with IC_{50} values of 39.01 ± 4.17 and 93.73 ± 5.43 $\mu\text{g/ml}$ respectively. Using kinetics approach, the compound was found to be an uncompetitive inhibitor of α -glucosidase thereby decreasing both the V_{max} and K_{M} of the enzyme. The V_{max} decreased from 655.09 $\mu\text{mol/min}$ to 48.16 $\mu\text{mol/min}$ while the K_{M} decreased from 2 mM to 0.9 mM. On the other hand, 2,7-dihydroxy-4*H*-1-benzopyran-4-one inhibited α -amylase in a non-competitive fashion with V_{max} decreasing from 33.70 $\mu\text{mol/min}$ to 1.53 $\mu\text{mol/min}$ while the K_{M} remained unchanged at 0.25%.

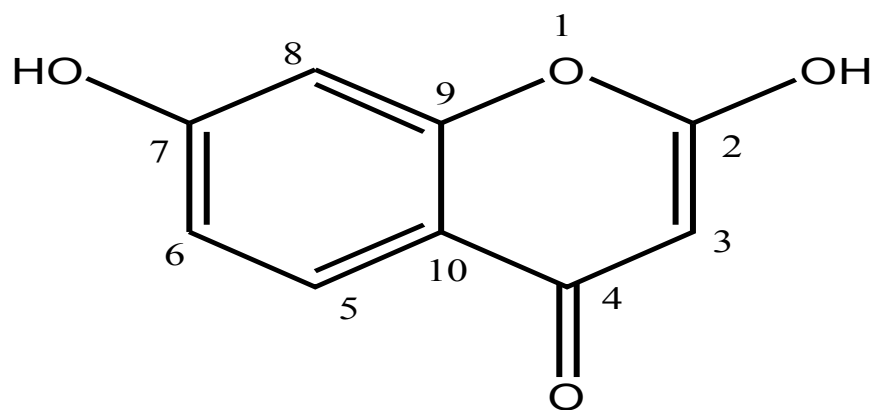


Figure 3.4.1: Structure of 2,7-dihydroxy-4*H*-1-benzopyran-4-one (**1**) isolated from ZMBF

During the 4-week intervention period, feed and fluid intakes as well as body weight gain were not significantly ($P < 0.05$) different between DBC, DZL and DZH groups (**Figures 3.4.2 and 3.4.3**). The ZMBF treatment also did not affect feed and fluid intakes of normal rats. Also, there was no significant difference between the NFBG levels of DZL and DBC groups throughout the experiment except for a sharp decline in the NFBG level of DZL group after the first week of treatment which was not subsequently sustained. On the other hand, the DZH group recorded a significantly lower ($P < 0.05$) NFBG level than the DBC group during the entire experimental period (**Figure 3.4.4**).

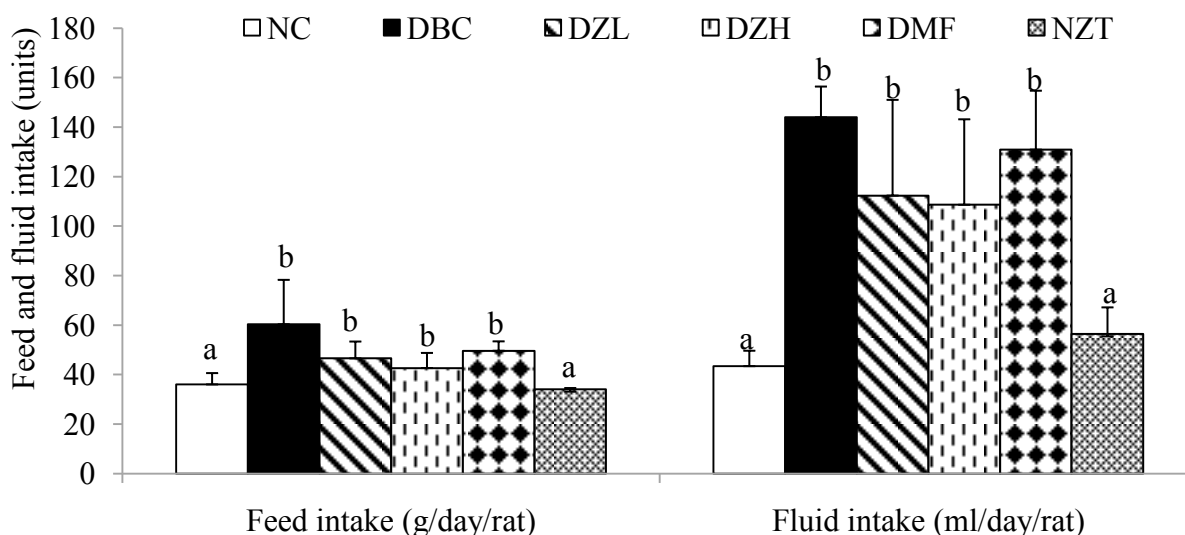


Figure 3.4.2: The effects of oral treatment of butanol fraction of *Z. mucronata* root on feed and fluid intakes of type 2 diabetic rats. The results are expressed as mean \pm SD of eight rats. ^{a-b}Different alphabets over the bars for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

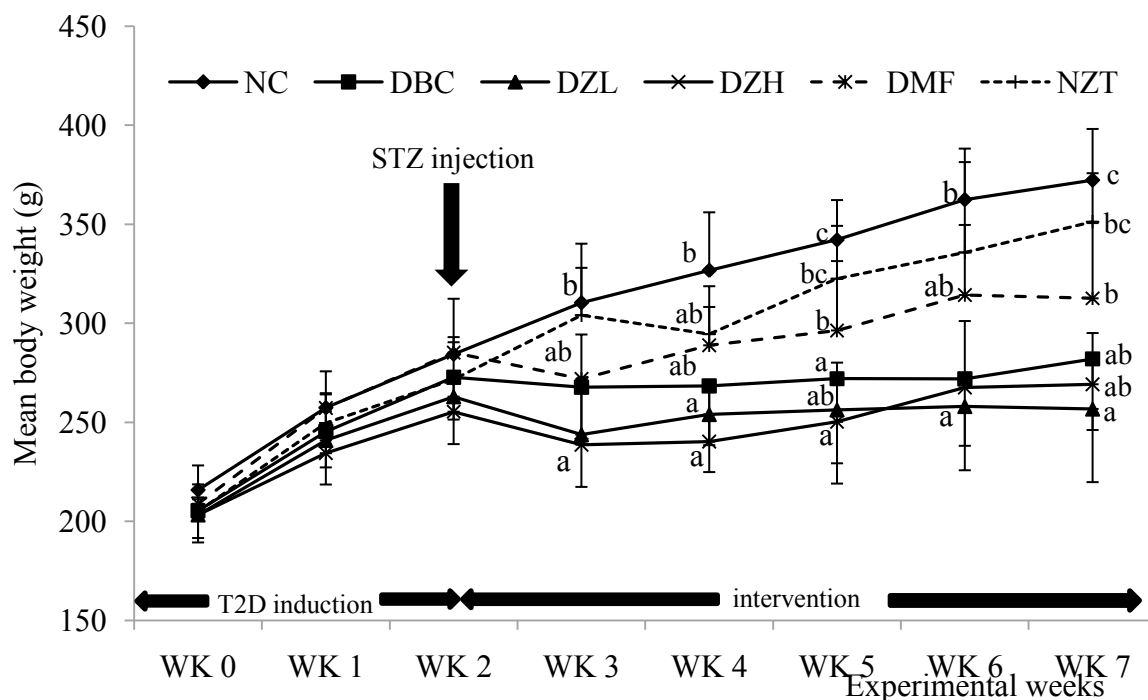


Figure 3.4.3: The effects of oral treatment of butanol fraction of *Z. mucronata* root on mean body weight gain of type 2 diabetic rats. The results are expressed as mean \pm SD of eight rats. ^{a-b}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

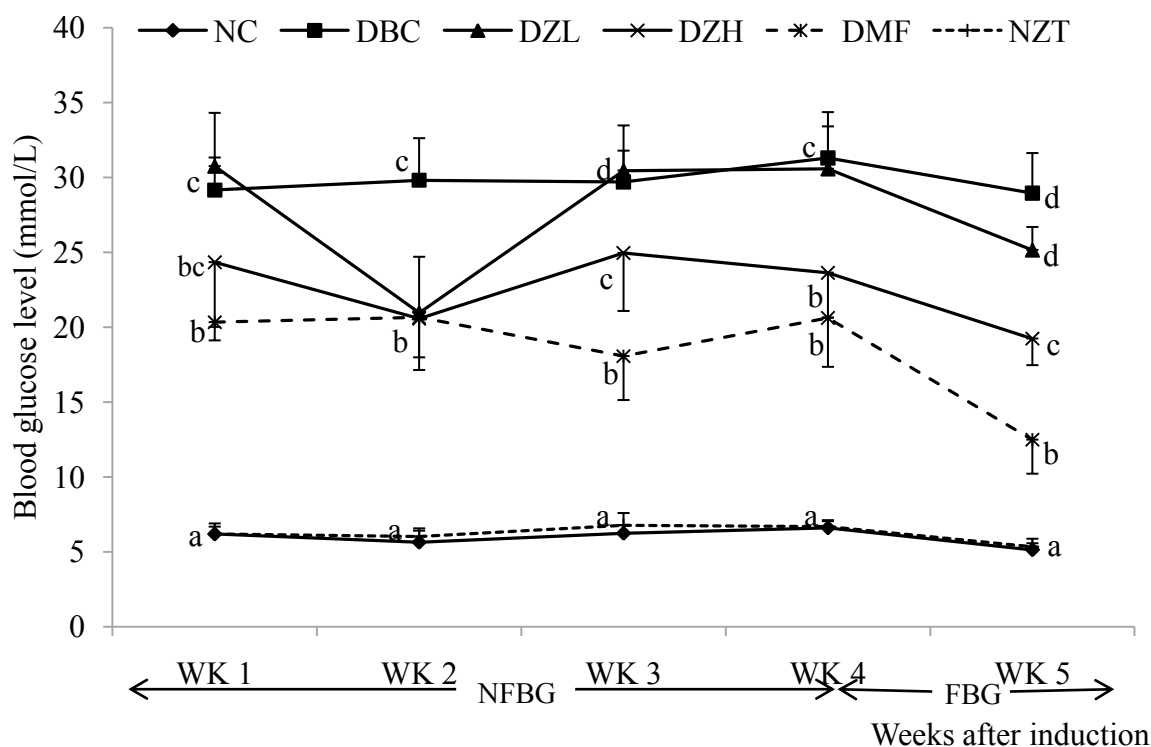


Figure 3.4.4: The effects of ZMBF treatment on weekly blood glucose concentrations (post induction) of type 2 diabetic rats. The results are expressed as mean \pm SD eight rats. ^{a-d}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The data for the OGTT are shown in **Figure 3.4.5**. Although the results were not significantly different ($P<0.05$) but better glucose tolerance ability was recorded in the DZL group than the DBC group. Furthermore, DZH group had a significantly ($P<0.05$) better glucose tolerance ability compared to DBC and DZL groups.

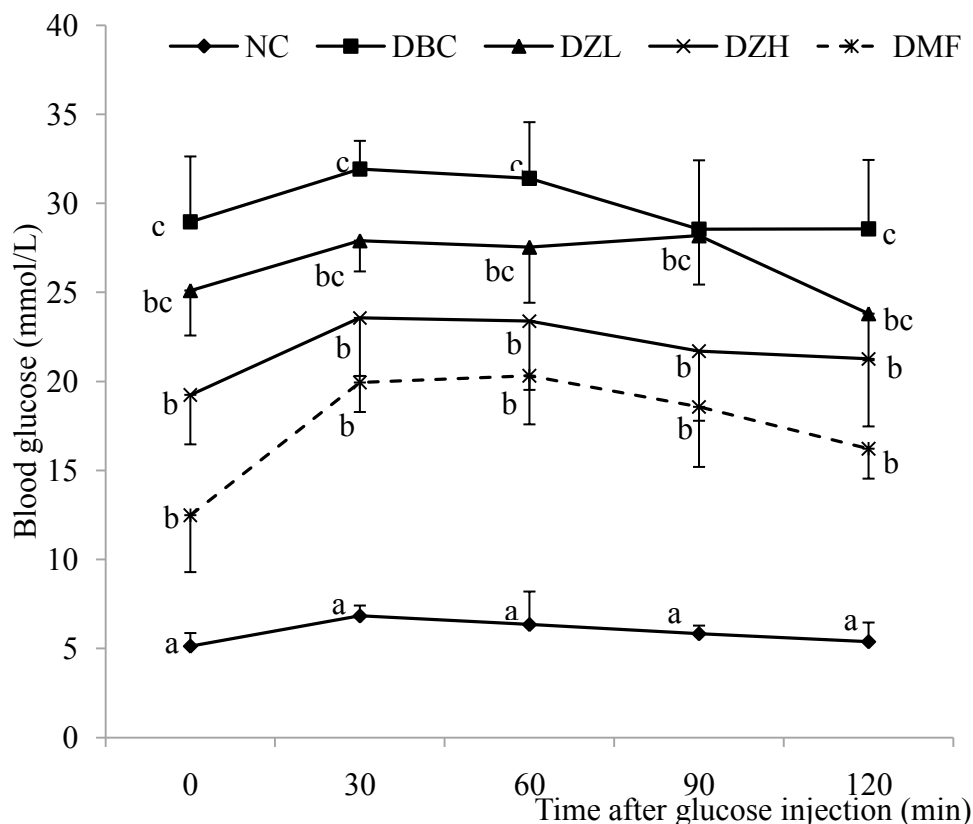


Figure 3.4.5: Oral glucose tolerance test (OGTT) for all groups of animals in the last week of experimental period. The results are expressed as mean \pm SD of eight rats. ^{a-c}Different alphabets for a given time indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Serum insulin levels and β -cell functions (HOMA- β) were significantly lower ($P<0.05$) while serum fructosamine concentrations and peripheral insulin resistance (HOMA-IR) were significantly ($P<0.05$) elevated in the DBC group than the NC group (**Table 3.4.1**). However, compared to the DBC group, the ZMBF treated diabetic groups had significantly higher ($P<0.05$) serum insulin concentrations, relatively higher β -cell functions but relatively lower insulin resistance and serum fructosamine concentrations. The serum level of AST was neither affected by the T2D nor the ZMBF treatments but serum ALT, ALP and urea levels were significantly elevated ($P<0.05$) in the DBC group compared to the NC group. However, ZMBF treatments did not statistically affect the serum ALT level but reduced the serum ALP and urea levels in diabetic rats (**Table 3.4.1**). No significant differences were observed between the NC and NZT groups for all these parameters. Relatively lower serum creatinine concentration was observed in the DBC group which was relatively increased in the DZL and DZH groups.

Table 3.4.1: Serum insulin and fructosamine concentrations, indices of hepatic and renal damages as well as computed HOMA-IR and HOMA- β scores for different animal groups at the end of the experiment

	NC	DBC	DZL	DZH	DMF	NZT
Serum insulin (pmol/L)	153.1 \pm 9.9 ^c	64.2 \pm 29.9 ^a	148.2 \pm 33.0 ^{bc}	131.4 \pm 35.1 ^{bc}	119.2 \pm 13.0 ^b	165.7 \pm 18.9 ^c
Serum fructosamine (μ mol/L)	196.3 \pm 5.6 ^a	258.4 \pm 10.9 ^b	258.6 \pm 19.2 ^b	234.2 \pm 20.0 ^b	253.8 \pm 30.4 ^b	189.8 \pm 61.0 ^{ab}
HOMA-IR	4.9 \pm 1.1 ^a	16.2 \pm 5.65 ^b	10.8 \pm 5.7 ^b	9.8 \pm 4.5 ^b	9.8 \pm 3.8 ^b	5.12 \pm 0.70 ^a
HOMA- β	304.1 \pm 53.7 ^c	9.7 \pm 5.2 ^a	24.9 \pm 14.5 ^{ab}	13.6 \pm 7.9 ^a	54.9 \pm 27.5 ^b	253.4 \pm 30.9 ^c
AST (U/L)	88.00 \pm 14.88	73.6 \pm 7.6	91.2 \pm 26.3	71.8 \pm 6.6	88.7 \pm 17.8	89.16 \pm 15.49
ALT (U/L)	56.25 \pm 12.55 ^a	77.8 \pm 4.6 ^{ab}	81.8 \pm 30.0 ^{ab}	88.3 \pm 12.8 ^b	63.3 \pm 18.2 ^a	57.3 \pm 15.9 ^a
ALP (U/L)	188.8 \pm 19.8 ^a	898.6 \pm 174.4 ^c	578.0 \pm 163.4 ^{bc}	510.5 \pm 94.3 ^b	472.0 \pm 74.9 ^b	214.4 \pm 78.2 ^a
Urea (mg/dl)	48.00 \pm 6.16 ^a	93.0 \pm 16.4 ^b	67.8 \pm 12.7 ^b	59.3 \pm 22.9 ^{ab}	42.0 \pm 11.8 ^{ab}	47.7 \pm 9.0 ^{ab}
Creatinine (μ g/dl)	582.0 \pm 70.4 ^{ab}	485.0 \pm 40.4 ^a	563.3 \pm 51.6 ^{ab}	550.0 \pm 74.3 ^{ab}	675.0 \pm 95.2 ^b	471.4 \pm 49.1 ^a

Results are expressed as mean \pm SD of eight rats. ^{a-c}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The data for the liver weights and liver glycogen concentration of the experimental animals are presented in **Table 3.4.2**. Relative liver weight was significantly higher in the DBC, DZL and DZH groups than the NC group with no significant difference between the DBC and ZMBF treated diabetic groups. Conversely, liver glycogen was significantly (P<0.05) depleted in the DBC group but the ZMBF treatment significantly (P<0.05) boosted the liver glycogen reserves in the DZH group only.

Table 3.4.2: Liver weights and liver glycogen concentrations in different animal groups at the end of the experimental period

	NC	DBC	DZL	DZH	DMF	NZT
Liver weight (g)	11.8 \pm 2.4	10.9 \pm 1.9	10.3 \pm 1.9	10.3 \pm 1.2	10.4 \pm 1.7	11.9 \pm 1.9
Relative liver weight (%)	3.3 \pm 0.3 ^a	3.9 \pm 0.2 ^b	4.0 \pm 0.3 ^b	3.8 \pm 0.1 ^{ab}	3.7 \pm 0.1 ^a	3.4 \pm 0.4 ^a
Liver glycogen (mg/g tissue)	3.5 \pm 0.8 ^b	1.5 \pm 0.1 ^a	2.2 \pm 0.9 ^{ab}	2.4 \pm 0.5 ^b	2.7 \pm 0.4 ^b	2.9 \pm 1.0 ^b

Results are presented as mean \pm SD of eight rats. ^{a-b}Values with different alphabets along a row are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 3.4.3 presents the effects of the ZMBF treatment on serum lipid profile of the experimental rats. Serum total and LDL-cholesterols were not significantly different among the animals groups. However, the DBC group recorded a significantly lower level of HDL-cholesterol which was increased in the ZMBF treated diabetic groups. Serum triglyceride concentration was significantly higher in the DBC group compared to the NC and NZT groups but the DZL group had a relatively lower and the DZH group had a relatively higher serum triglycerides compared to the DBC group.

Table 3.4.3: The effects of ZMBF treatments on serum lipid concentrations of type 2 diabetic rats

Serum lipids	NC	DBC	DZL	DZH	DMF	NZT
	(mg/dl)					
Total cholesterol	66.25±6.39	76.25±10.62	61.00±9.45	79.25±17.25	74.63±12.10	80.66±11.28
HDL cholesterol	31.00±9.55 ^b	19.80±1.64 ^a	28.50±5.24 ^{ab}	26.00±9.27 ^{ab}	28.14±5.39 ^{ab}	32.42±8.48 ^b
LDL cholesterol	19.85±5.35	27.45±4.34	18.60±8.47	16.50±6.90	25.98±7.43	30.57±8.48
Triglycerides	102.5±27.5 ^a	184.8±40.9 ^{ab}	113.5±30.1 ^a	198.4±7.5 ^b	120.13±27.4 ^a	92.00±14.22 ^a

Results are expressed as mean ± SD of eight rats. ^{a-b}Different superscript alphabets along a row indicate are significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Histopathological examination of the pancreatic sections revealed a reduction in the size of pancreatic islets as well as the number of cells per islet count in the DBC group compared to the NC group. No much difference was detected between DZL group and the DBC group. However, the DZH group had higher number of cells per islet than the DBC group (**Figure 3.4.6**). The NZT group had similar pancreatic islets and the number of cells per islet with the NC group.

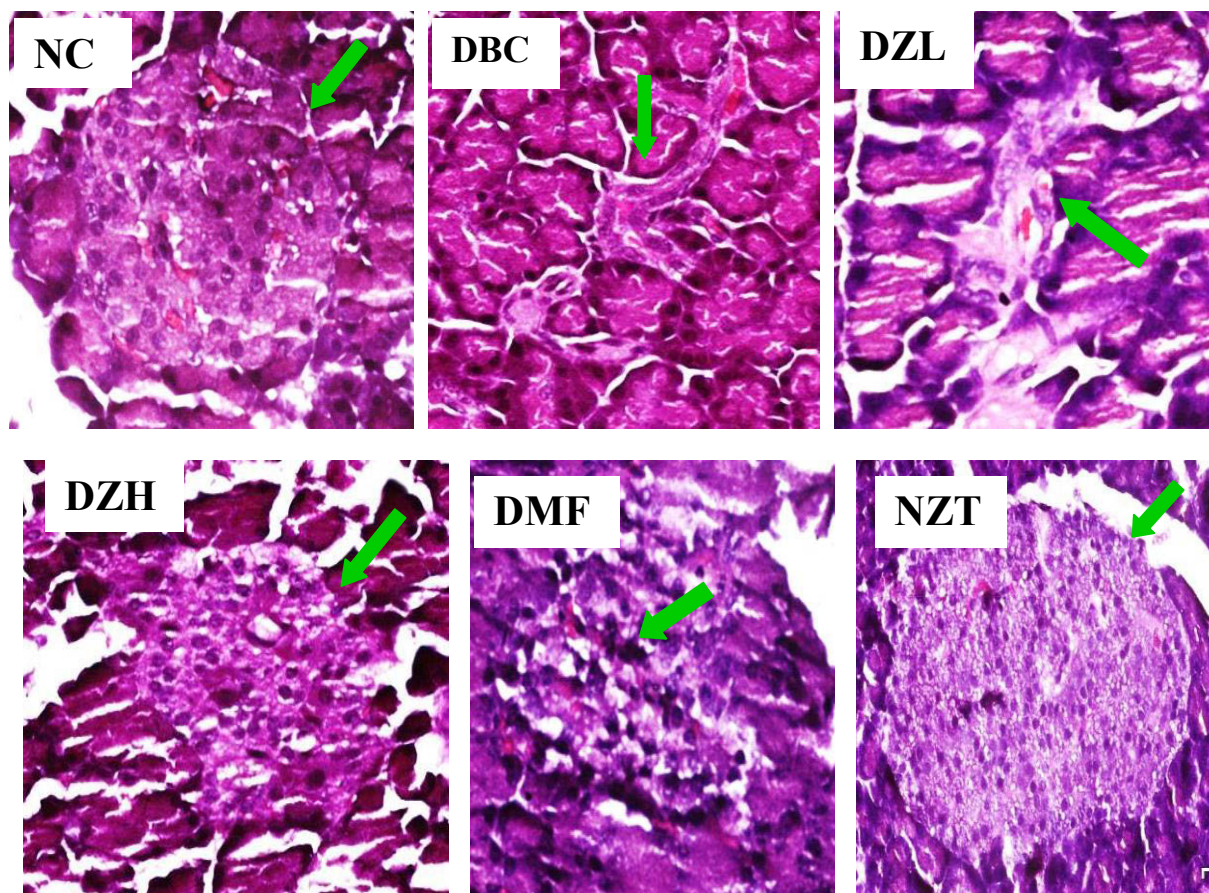


Figure 3.4.6: Representative sections of histopathological examinations of the pancreas from different animal groups. The NC group had high number cells per islet while the DBC and DZL group had smaller pancreatic islets with very few cells. The DZH had relatively higher number of cells per islet compared to DBC and DZL groups but far lower than the NC group.

3.4.5 Discussion

The role of medicinal plants in the health care delivery systems of many parts of the world has been duly recognized [15]. However, the traditional use of a plant in the traditional management of a disease might not necessarily be a reflection of its efficacy. This study reveals that the butanol fraction of *Z. mucronata* root, a plant traditionally used for the treatment of diabetes [4], contains potent α -glucosidase and α -amylase inhibitory compound and elicited anti-hyperglycemic effects but slightly ameliorated the T2D-associated complications in a rat model.

The potent anti-oxidative activity of ethanolic extract of the root of *Z. mucronata* [49] prompted us to subject it to further studies. Solvent-solvent fractionation of the ethanolic extract led us to a butanol fraction with potent α -glucosidase and α -amylase inhibitory activity (see manuscript 3.3 above) and subsequent phytochemical analysis of the butanol fraction led to the isolation of a chromone, 2,7-dihydroxy-4*H*-1-benzopyran-4-one. Apart from few reports on cyclopeptide alkaloids isolated from this plant [1, 12], no much information on the isolation and characterization of other phytochemical compounds from this plant have been reported in the literature. Interestingly, the chromone isolated in

this study was found to be a dual inhibitor of the two enzymes (α -glucosidase and α -amylase) in an *in vitro* model which suggests that this compound could lead to delayed carbohydrate digestion and glucose absorption with concomitant reduction in postprandial hyperglycemic shoot up [67]. In previous studies, hydroxyl groups have been implicated as the vital structural features responsible for the inhibitory effects of most phytochemicals on α -glucosidase and α -amylase proteins [58, 68]. In our study, kinetic delineation of α -glucosidase and α -amylase inhibitions by 2,7-dihydroxy-4H-1-benzopyran-4-one revealed uncompetitive and non-competitive patterns respectively. These observations could suggest that one or more hydroxyl groups of the chromones interact individually or synergistically with the enzyme-substrate complexes to inhibit the α -glucosidase and α -amylase.

After confirming the *in vitro* anti-hyperglycemic potentials of the chromone isolated from the ZMBF, we subjected the fraction to an *in vivo* study in a T2D model of rats. Polyphagia and polydipsia with concomitant reduction of body weight are major symptoms of diabetes mellitus [69] which were also evidently observed in the diabetic groups of our experiment. These parameters are usually linked to stable and prolong diabetic condition [70]. In our study, the treatment with ZMBF did not ameliorate the T2D-induced polyphagia, polydipsia and weight loss possibly because other diabetic complications, especially metabolic parameters, were not significantly affected by the fraction.

Elevated fasting or postprandial hyperglycemia causes life-threatening complications linked to diabetes. Consequently, maintaining glucose homeostasis is essential in preventing the detrimental effects of hyperglycemia and its associated complications [71]. At a dose of 300 mg/kg bw, the ZMBF displayed appreciable anti-hyperglycemic and insulintropic effects, improved glucose tolerance but slightly prevented the T2D-induced β -cell dysfunction (HOMA- β) and the damage to pancreatic architecture. Taken together, it appears that the fraction slightly protected the β cell from destruction but remarkably potentiated the residual β -cells to secrete more insulin which consequently might promote more glucose uptake by the cells. However, the failure of the fraction to significantly decrease peripheral insulin resistance and at the same time, achieved significant anti-hyperglycemia suggests that, in addition to the observed insulintropism, another mechanism probably an extrapancreatic retardation of intestinal glucose production could be involved. This hypothesis is supported by the potent α -glucosidase and α -amylase inhibitory actions displayed by the ZMBF and the isolated chromone from it. On the other hand, the ability of the fraction to slightly protect pancreatic damage caused by the disease could be linked to its potent anti-oxidative activity (see manuscript 3.3 above) because the protection of pancreatic damage is usually mediated through an anti-oxidative dependent mechanism since oxidative stress is an important biological phenomenon in the induction of pancreatic β -cell damage in T2D [72].

Another important feature of experimentally induced diabetes is a reduction in the liver glycogen level [70] which is caused by the modulation of glycogen synthase and glycogen phosphorylase activities. Previous studies have demonstrated that a number of plant materials [73, 74] and isolated flavonoids [75] elicited an anti-diabetic activity partly through stimulation of hepatic glycogenesis. Thus, the significantly higher liver glycogen content recorded in the DZH group than the DBC group (**Table 3.4.2**) indicated that the anti-hyperglycemic activity of the ZMBF at 300 mg/kg bw might be mediated not only by stimulating insulin secretion and/or retarding carbohydrates digestion but also by increasing hepatic glycogen synthesis.

Lipids are vital to the pathogenesis of experimentally induced diabetes and the level of serum lipids is usually elevated at diabetic state which is a risk factor for cardiovascular diseases associated with T2D [76]. Although the results were not significant, an increasing tendency in the serum total cholesterol and LDL-cholesterol was observed in the DBC group compared to NC group (**Table 3.4.3**). On the other hand, significant HDL-cholesterol level and hypertriglyceridemia were recorded in the DBC group compared to NC group. The ZMBF treatments displayed appreciable ameliorative effects towards the disease-induced alterations LDL- and HDL-cholesterols but not towards the other forms of lipids. Although a number of plant derived phytochemicals have been shown to possess hypolipidemic activity in experimental diabetes [77-79] but findings from our study revealed that ZMBF did not contain powerful hypolipidemic phytochemicals. Results from other indices of diabetic complications also indicated that the T2D caused a significant increase in serum levels of ALT, ALP and urea. However, despite the potent anti-hyperglycemic effects of the fraction, a close look at the results of these biomarkers of hepatic and renal functions also indicated that ZMBF treatments slightly ameliorated the hepatic and renal damages caused by diabetes. Therefore, a further study is required in order to understand the basis of these observations. Apart from assessing renal damage, creatinine was reported to be a predictor for T2D and insulin resistance and previous studies have demonstrated lower serum creatinine level in T2D human subjects compared to normal individuals [80, 81]. It is thus plausible to suggest that the relatively lower insulin resistance (HOMA-IR) of the ZMBF treated diabetic groups compared to DBC group is responsible for the relatively elevated serum creatinine levels recorded in these groups.

On a general note, we observed that ZMBF treatment at 150 mg/kg bw was not able to show anti-diabetic activity for most of the parameters measured in this study. This might indicate that the amount of ZMBF has to reach a threshold level before the anti-diabetic activity could be manifested.

We conclude that ZMBF, at a dose of 300 mg/kg, had anti-hyperglycemic activity but slightly alleviate most of the T2D-associated complications. Furthermore, 2,7-dihydroxy-4*H*-1-benzopyran-4-one could play a role in the anti-glycemic activity of the fraction. Our future work will focus on detailed studies on

the molecular mechanism(s) for the observed results as well as a more detailed phytochemistry of the fraction.

Postscript: In order to further explore the possible mechanism of anti-diabetic action of the butanol fraction, the serum, liver, kidney, heart and pancreas were investigated for *in vivo* anti-oxidative parameters

3.5 Modulation of *in vivo* antioxidant status by the butanol fraction of *Ziziphus mucronata* root in a type 2 diabetic rats

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Preface: This article describes the *in vivo* anti-oxidative status of the serum and organs collected from the rats in the above experiment.

3.5.1 Abstract

The effects of oral administration of the butanol fraction of *Ziziphus mucronata* root (ZMBF) on antioxidant status of the liver, kidney, heart, pancreas and serum of type 2 diabetic animals were investigated. The *in vivo* oxidative stress markers measured were thiobarbituric acid reactive substances (TBARS), reduced glutathione, superoxide dismutase (SOD) and catalase. The ZMBF treatment significantly ($P < 0.05$) increased the reduced glutathione levels in the serum and all the investigated organs compared to diabetic controls. Hepatic TBARS were significantly lowered while SOD and catalase were significantly ($P < 0.05$) increased in the ZMBF treated rats compared to diabetic controls. However, diabetes-induced changes in kidney TBARS, SOD and catalase were not statistically affected by the ZMBF treatment. An insignificant ($P > 0.05$) increase in the heart catalase level and a dose dependent decrease in the heart TBARS were observed in the ZMBF treated rats compared to diabetic controls. Also, diabetes-induced changes in pancreatic and serum TBARS, SOD and catalase levels were ameliorated by the treatment. Results from this study indicate that ZMBF possess *in vivo* anti-oxidative effects in a type 2 diabetic rats.

3.5.2 Introduction

Hyperglycemia is a major pathogenic feature of diabetes mellitus and is implicated in the pathogenesis of most of the secondary complications associated with the disease [82]. In type 2 diabetes (T2D), hyperglycemia induces oxidative stress via multiple pathways which include glucose autooxidation, increased metabolic flux of the polyol (sorbitol) pathway, activation of protein kinase C, increased generation of advanced glycation end products and increased flux of hexosamine pathway [83, 84]. The oxidative stress is thus considered as an important unifying mechanism that plays major roles in all aspects of T2D pathology that include pancreatic β cell dysfunction, microvascular and macrovascular

complications [84-86]. Therefore, modulation of *in vivo* oxidative stress is an important therapeutic mechanism that could ultimately benefit diabetic patients.

In normal individuals, oxidative stress is suppressed by physiological enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (glutathione, vitamins C and E) anti-oxidative defense system *in vivo* but in diabetic condition significant alterations in the serum and tissue anti-oxidative defense system as well as, in lipid peroxidation occur [72]. Thus, anti-oxidative agents that could prevent these changes would offer protection against the long-term vascular complications associated with the disease. Indeed, some antioxidants have been reported to reduce the diabetic complications thereby affecting the overall outcome of the disease [87].

Ziziphus mucronata Willd (Rhamnaceae), commonly referred to as “buffalo thorn” is available in most parts of Africa. A decoction of the plant is used traditionally in the treatment of diabetes mellitus among the rural inhabitants of northern Nigeria [4]. In a previous study [49], we subjected the various crude solvents extracts from the stem bark, root and leaf samples of the plant to a series of *in vitro* anti-oxidative assays and reported that the ethanolic extract of the root had the most potent anti-oxidative activities that reasonably cuts across various models. Subsequently, we subjected the ethanolic extract of the root to solvent-solvent partitioning and found that the butanol fraction contain the most potent anti-oxidative and α -glucosidase and α -amylase inhibitory phytochemicals in *in vitro* models (see manuscript 3.3 above). Based on these findings, a follow up comprehensive investigation was conducted on the *in vivo* activity of the butanol fraction in a T2D model of rats where the fraction exhibited anti-hyperglycemic and insulinotropic effects but slightly ameliorate some diabetes-related complications (see manuscript 3.4 above). In order to further understand the biochemical basis for the observed seemingly unusual effects, in the present study, we investigated the effects of the fraction on the serum and tissues (liver, kidney, heart and pancreas) anti-oxidative status of T2D model of rats.

3.5.3 Materials and methods

Please refer to the chapter two sub sections 2.3-2.4 (pages 35-36); 2.7.1-2.7.3 and 2.7.5 (pages 41-42) and 2.7.8 (2.7.8.1-2.7.8.4) (pages 44-45) for material and methods that involve *Z. mucronata*. However, the group codes DFL, DFH and NFT used in the chapter two are replaced with DZL, DZH and NZT respectively.

3.5.4 Results

Diabetes was found to induce lipid peroxidation and deplete endogenous antioxidant reserves as manifested by the significantly higher ($P<0.05$) levels of TBARS with a corresponding lower levels of glutathione, SOD and catalase in the DBC group of all tissues compared to the NC group. However, the ZMBF treatments significantly ($P<0.05$) prevented the diabetes-induced lipid peroxidation in the hepatic

tissues. Also, the DBC group had significantly ($P<0.05$) decreased levels of hepatic glutathione, SOD and catalase which were significantly ($P<0.05$) boosted in the ZMBF-treated diabetic groups (**Table 3.5.1**).

Table 3.5.1: Effects of ZMBF on hepatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DZL	DZH	DMF	NZT
TBARS (μM)	10.6 \pm 3.0 ^a	33.9 \pm 4.9 ^c	21.5 \pm 4.8 ^b	21.3 \pm 4.5 ^b	35.9 \pm 9.5 ^c	14.2 \pm 5.4 ^{ab}
Glutathione (μM)	120.8 \pm 6.0 ^c	29.9 \pm 9.3 ^a	54.2 \pm 12.6 ^b	69.2 \pm 5.4 ^b	73.6 \pm 10.2 ^b	70.8 \pm 11.6 ^b
Superoxide dismutase (nmol/min)	25.5 \pm 5.3 ^b	12.4 \pm 1.6 ^a	20.8 \pm 5.3 ^b	21.8 \pm 4.3 ^b	17.5 \pm 5.2 ^{ab}	23.9 \pm 5.5 ^b
Catalase ($\mu\text{mol/min}$)	16.5 \pm 0.9 ^b	1.5 \pm 0.3 ^a	12.9 \pm 4.8 ^{bc}	8.4 \pm 2.7 ^b	1.7 \pm 0.6 ^a	9.2 \pm 2.0 ^b

Results are expressed as the mean \pm SD of five rats. ^{a-c}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

In the kidney, the TBARS levels in the DZL and DZH groups were lowered, though insignificantly ($P>0.05$) compared to the DBC group but the depletion in glutathione levels was significantly ($P<0.05$) ameliorated in the ZMBF-treated diabetic groups compared to DBC group (**Table 3.5.2**). However, the kidney SOD and catalase levels were higher in the DZL and DZH groups in comparison to DBC group.

Table 3.5.2: Effects of ZMBF on kidney lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DZL	DZH	DMF	NZT
TBARS (μM)	14.1 \pm 5.8 ^a	40.8 \pm 12.5 ^{bc}	42.8 \pm 9.8 ^c	30.1 \pm 10.2 ^{bc}	33.4 \pm 10.0 ^{bc}	26.6 \pm 5.0 ^b
Glutathione (μM)	68.2 \pm 20.8 ^{ab}	46.0 \pm 8.6 ^a	93.3 \pm 14.3 ^b	104.8 \pm 29.9 ^b	70.8 \pm 10.4 ^b	81.9 \pm 9.6 ^b
Superoxide dismutase (nmol/min)	16.1 \pm 3.4 ^b	6.8 \pm 1.5 ^a	9.7 \pm 2.7 ^a	8.3 \pm 2.8 ^a	11.5 \pm 4.4 ^{ab}	8.6 \pm 2.4 ^a
Catalase ($\mu\text{mol/min}$)	1.7 \pm 0.3 ^b	0.88 \pm 0.21 ^a	1.9 \pm 0.9 ^b	1.0 \pm 0.2 ^{ab}	1.6 \pm 0.4 ^b	1.6 \pm 0.5 ^b

Results are expressed as the mean \pm SD of five rats. ^{a-c}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

In the heart, the TBARS level was significantly lower ($P<0.05$), the glutathione level was significantly higher ($P<0.05$) while the SOD and catalase were relatively elevated in the DZH group than the DBC group (**Table 3.5.3**).

Table 3.5.3: Effects of ZMBF on lipid peroxidation and anti-oxidant status in the heart of type 2 diabetic rats

	NC	DBC	DZL	DZH	DMF	NZT
TBARS (μM)	9.4 \pm 2.6 ^a	24.6 \pm 8.8 ^b	20.9 \pm 8.8 ^b	11.5 \pm 4.7 ^{ab}	33.9 \pm 5.9 ^b	8.6 \pm 3.2 ^a
Glutathione (μM)	122.2 \pm 22.0 ^{bc}	48.5 \pm 14.6 ^a	108.7 \pm 12.2 ^b	134.2 \pm 7.1 ^c	79.2 \pm 18.3 ^{ab}	111.8 \pm 26.6 ^b
Superoxide dismutase (nmol/min)	15.2 \pm 4.0 ^b	5.8 \pm 1.2 ^a	14.4 \pm 2.4 ^b	6.4 \pm 2.2 ^a	12.8 \pm 2.0 ^{ab}	8.1 \pm 1.9 ^a
Catalase ($\mu\text{mol/min}$)	2.1 \pm 0.5 ^b	0.9 \pm 0.2 ^a	0.9 \pm 0.2 ^a	1.1 \pm 0.2 ^a	1.4 \pm 0.4 ^{ab}	2.3 \pm 0.2 ^b

Results are expressed as the mean \pm SD of five rats. ^{a-c}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The data for the pancreatic *in vivo* antioxidant status also revealed that the DZH group had significantly lower ($P < 0.05$) level of TBARS as well as significantly higher ($P < 0.05$) levels of glutathione, SOD and catalase than the DBC group. The low dose significantly ($P < 0.05$) attenuated these pancreatic parameters except glutathione (Table 3.5.4).

Table 3.5.4: Effects of ZMBF on pancreatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DZL	DZH	DMF	NZT
TBARS (μM)	6.00 \pm 1.16 ^a	35.73 \pm 9.13 ^c	29.38 \pm 5.12 ^c	23.09 \pm 4.83 ^{bc}	18.35 \pm 4.37 ^b	8.37 \pm 3.36 ^a
Glutathione (μM)	80.02 \pm 8.09 ^c	6.22 \pm 3.49 ^a	51.27 \pm 9.60 ^b	54.36 \pm 9.81 ^b	37.33 \pm 8.56 ^b	48.44 \pm 8.49 ^b
Superoxide dismutase (nmol/min)	2.61 \pm 0.45 ^c	0.37 \pm 0.16 ^a	3.12 \pm 1.36 ^c	2.66 \pm 0.80 ^c	1.03 \pm 0.46 ^b	1.89 \pm 0.54 ^{bc}
Catalase ($\mu\text{mol/min}$)	0.65 \pm 0.17 ^{bc}	0.35 \pm 0.039 ^a	0.55 \pm 0.08 ^b	0.50 \pm 0.06 ^b	1.11 \pm 0.20 ^c	0.98 \pm 0.18 ^c

Results are expressed as the mean \pm SD of five rats. ^{a-c}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The *in vivo* antioxidant status of the serum were also significantly ($P < 0.05$) affected by the diabetes induction but the ZMBF treatments significantly ($P < 0.05$) ameliorated the diabetes-induced depletion in glutathione, SOD and catalase but could not significantly ($P < 0.05$) alleviate the disease-induced elevation in TBARS (Table 3.5.5). It is noteworthy that the *in vivo* antioxidant parameters of NZT group was not significantly different ($P > 0.05$) from the NC group for most of the parameters measured in the different tissues.

Table 3.5.5: Effects of ZMBF on serum lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DZL	DZH	DMF	NZT
TBARS (μM)	63.0 \pm 15.1 ^a	201.1 \pm 19.3 ^c	194.9 \pm 19.8 ^{bc}	210.6 \pm 17.7 ^c	142.0 \pm 32.0 ^b	113.8 \pm 26.1 ^b
Glutathione (μM)	84.4 \pm 7.6 ^d	19.8 \pm 2.4 ^a	47.6 \pm 8.8 ^b	57.4 \pm 9.4 ^{bc}	65.2 \pm 6.7 ^c	64.5 \pm 16.0 ^{bcd}
Superoxide dismutase (nmol/min)	18.0 \pm 2.4 ^c	0.86 \pm 0.19 ^a	2.2 \pm 0.9 ^b	4.07 \pm 0.92 ^b	3.2 \pm 0.7 ^b	13.7 \pm 5.9 ^c
Catalase ($\mu\text{mol/min}$)	3.62 \pm 0.76 ^c	1.54 \pm 0.39 ^a	1.82 \pm 0.75 ^{ab}	2.84 \pm 0.36 ^{bc}	2.41 \pm 0.47 ^b	1.95 \pm 0.57 ^{ab}

Results are expressed as the mean \pm SD of five rats. ^{a-d}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

3.5.5 Discussion

A previous study from our lab has revealed that a high dose of ZMBF displayed significant anti-hyperglycemic and insulintropic effects but slightly prevented tissue damages caused by T2D. In the present study, we observed that modulation of *in vivo* anti-oxidant status could play a role in the previously observed effects and that the effect of the ZMBF on the antioxidant status of diabetic animals is organ-specific.

Decreased glutathione and antioxidant enzymes with a concomitant increase in lipid peroxidation in all organs clearly demonstrate the development of oxidative stress in the diabetic animals. Alterations in the biomarkers of hepatic damage and insulin resistance are recognized features of experimentally induced diabetes and oxidative stress has been reported to be an important contributor to the hepatic damage and mediating insulin resistance in diabetic state [88, 89]. Thus, the ability of the ZMBF to significantly ameliorate the T2D-induced changes in hepatic antioxidant status whilst achieving less potent results for the liver function enzymes and insulin resistance suggests that the ZMBF mainly functions through an anti-oxidative dependent mechanism, thereby sparing other biological players of hepatic damage and insulin resistance to proceed. It is also possible that the experimental period is short for the amelioration of hepatic oxidative stress to be translated in to a complete reversal of hepatic damage and insulin resistance.

Kidney plays an important in glucose homeostasis and therefore it is regarded as an important organ in diabetes management [90]. In the present study, oxidative stress was also induced in the kidney of the diabetic rats but the ZMBF treatments restore the depleted glutathione and slightly boosted the antioxidant enzymes reserves. It also slightly prevented lipid peroxidation in the kidney. Taken as a whole, it seems that ZMBF is not highly effective in modulating kidney-based oxidative stress during diabetes. Interestingly, this observation correlates with an insignificant reduction in the level of a biomarker of kidney function (serum urea) caused by ZMBF treatment in the diabetic rats (see manuscript

3.4 above). This might further suggest that ZMBF mediates its effects in the kidney of diabetic animals through an anti-oxidative dependent mechanism.

Cardiovascular complications are associated with T2D and are usually caused by alterations in serum lipids profile during diabetes [78]. Thus, exploration of the biochemical events occurring at cardiac tissues during diabetes may ultimately provide a clue to the management of cardiovascular diseases associated with T2D. At a dose of 300 mg/kg bw, the ZMBF ameliorated the diabetes-induced lipid peroxidation and depletion of endogenous antioxidant reserves. This observation might suggest that ZMBF could be beneficial in reducing the detrimental effects of hyperlipidemia because hypertriglyceridemia has been reported to be an important factor that enhances the formation of lipid peroxides [91]. Unfortunately however, ZMBF was found to cause a slight elevation in triglycerides in diabetic animals (see manuscript 3.4 above). Based on the above, we speculated that ZMBF was able to protect cardiac tissues from oxidative attack via an anti-oxidative mediated mechanism but at the same time could act elsewhere to stimulate lipids synthesis.

An important pathogenic mechanism for pancreatic β cell dysfunction in T2D is via the induction of oxidative stress [72] and ZMBF treatments were found to replenish the depleted pancreatic glutathione, SOD and catalase as well as moderately prevented lipid peroxidation in this organ which suggests that the fraction could reduce pancreatic β cell dysfunction in diabetic patients and consequently improve the disease condition. This observation could further explain, at least in part, the biochemical basis for the increased β cell function (HOMA- β) and serum insulin concentration as well as higher number of β cells in ZMBF-treated diabetic rats compared to diabetic untreated rats (see manuscript 3.4 above).

In conclusion, the results of this study demonstrate that ZMBF modulates T2D-induced oxidative stress in various organs and this mechanism plays a vital role in the overall anti-T2D effects of the fraction.

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CHAPTER 4

4.0 THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *Cassia singueana* Delile *IN VITRO AND IN VIVO*

4.1 *Cassia singueana* Delile (Caesalpiniaceae)



Figure 4.1: *Cassia singueana* Delile (Caesalpiniaceae); synonym: *Cassia goratensis*; common names: golden shower tree (English), Runhu (Hausa, Nigeria), Rumfuhi (Fulani, Nigeria), Isihaqa esincinyane (Ndebele, Zimbabwe), Mudyamhungu (Shona, Zimbabwe), Kalusapwe (Nyanja, Zambia). Photo: Mr. Umar Gallah (2012), Zaria, Nigeria

4.1.2 Background

It is a small tree 1-15 m high with trunks of up to 35 cm in diameter. The leaves are compound, 2.5-5 cm long with 4-10 pairs of oval leaflets. The flowers are deep yellow, fragrant and could be up to 15 cm long. The species is distributed in the drier tropical countries of West, East and Southern Africa which include Niger, Nigeria, Mali, Angola, Zambia, Zimbabwe, Ethiopia, Tanzania and Kenya. It is often found in the thickets, deciduous woodland and savannah belts of these countries.

4.1.3 Ethnomedicinal uses

Different African cultures use this plant for different ethnobotanical practices. In Niger, the stem bark of the plant is used in the treatment of stomach ulcers whereas in northern Nigeria the plant is highly utilized for bathing recently delivered nursing mothers and also for treating feverish conditions, acute malaria and conjunctivitis [1] as well as against microbial infections [2]. In Tanzania, the root is used against gonorrhea, bilhazia, heartburn, constipation and snake bites but in northern Kenya, it is used against stomach disorders, sore throats and elephantiasis. Reports from the Southern part of Africa indicate that the leaves or root decoction is used for treating abdominal pains in Zimbabwe (http://www.africamuseum.be/collections/external/prelude/view_plant?pi=02690 accessed on 27th July,

2013). An ethnobotanical survey carried out in the northwestern Nigeria revealed that a decoction of the leaves of the plant is prescribed by traditional medicine practitioners for the treatment of diabetes mellitus [3].

4.1.4 Biological activities

Most of the scientific investigation on the biological activities of *C. singueana* comes from Nigeria. In 2003, Adzu et al. [4] reported that the methanolic extract of the root exhibits significant antimalarial, antipyretic and antinociceptive activities but unfortunately, the same extract was found to contain potent neuropharmacological agents with significant sedative effects by reducing spontaneous motor activity and prolonging barbital hypnosis [5]. The traditional exploitation of the plant for the treatment of stomach ulcers prompted a research group to investigate the leaves of anti-ulcer activities. Findings from the studies revealed that the methanolic and ethanolic extracts of the leaves possess anti-ulcer effects in different rat models of gastric ulcer [6-8]. In another study, the methanol crude extract of the leaves demonstrated potent *in vitro* relaxant effects on histamine-induced pre-contracted rabbit jejunum [9]. Interestingly, the methanol extract of the leaves from this plant was found to be safe in a toxicological study [10]. Beside the above mentioned investigations, a study reported the preliminary *in vitro* antioxidant potential of the methanolic extract from the leaves of this plant [11] and the methanolic extract of the root bark was also reported to contain potent antioxidant, hepatoprotective and hypolipidemic agents [1].

4.1.5 Phytochemistry

The only available information on the phytochemistry of *C. singueana* appearing in the literature was reported by Mutasa et al. [12] who described the isolation and characterization of 7-methylphyscion, Chrysophanol, Cassiamin A and lupeol as well as a fraction with a mixture of β -sitosterol, stigmasterol and campesterol. Using TLC, the presence of an unidentified flavonoid was demonstrated by Ode et al. [9] but detailed characterization and elucidation was not carried out which makes the data not weighty.

In order to validate the folkloric claim for anti-diabetic activity of this plant (as earlier mentioned), a systematic and comprehensive investigation on the anti-diabetic activity of this plant using *in vitro* and *in vivo* models was carried out. Initially, various solvent crude extracts from the stem bark, root as well as the leaves of the plant were subjected to detailed *in vitro* anti-oxidative studies (using four models) from where the ethyl acetate extract of the stem bark had the best anti-oxidative activity. Subsequently, the crude stem bark ethyl acetate extract was fractionated across various solvents and the fractions were subjected to the anti-oxidative and α -glucosidase and α -amylase inhibitory studies where the acetone fraction was found to have the best anti-oxidative and enzymes inhibitory activities. Thus, the acetone fraction was investigated for *in vivo* anti-diabetic activity in a type 2 diabetic rats as well as the possible mechanism of anti-diabetic action. A pure bioactive anti-diabetic compound was also isolated from the acetone fraction.

4.2 *In vitro* anti-oxidative activities and GC-MS analysis of various solvent extracts of *Cassia singueana* parts

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Preface: The *in vitro* anti-oxidative activities of the stem bark, root and leaves of the plant were investigated in this article. The article has already been published in the journal ‘‘ Acta poloniae pharmaceutica – drug research’’ Vol 70 number 4; Pages 709-719.

4.2.1 Abstract

The present study was conducted to investigate the anti-oxidative activities of different solvent extracts of *Cassia singueana* parts. Our results indicate that all the extracts have reducing power (Fe^{3+} - Fe^{2+}) and DPPH radical scavenging abilities. However, the stem bark ethyl acetate extract has highest total reducing power whilst the ethanol extract of the stem bark has more potent free radical scavenging activity than all the other extracts. The ethyl acetate extract of the stem bark exhibited more powerful hydroxyl radical scavenging activity than other extracts whilst the aqueous extract of the leaves displayed more potent nitric oxide inhibition activity than other extracts. The GC-MS analysis of the ethyl acetate extract of the stem bark and the ethanol extract of the root and leaves indicated that several aromatic compounds, including phenolics, fatty acids, amino acids, triterpenoids were present in these extracts. Data from this study suggest that the parts of *C. singueana* possessed anti-oxidative activities and it can be used as a potential alternative medicine for oxidative stress related non-communicable chronic diseases. Further experimental and clinical studies in this regard are warranted.

4.2.2 Introduction

Oxidative stress refers to the presence of free radicals and reactive oxygen species (ROS) that are generated in normal physiological processes but become harmful when they are not neutralized by the endogenous antioxidants systems. This usually occurs when there is an excess generation of ROS or when the antioxidants (AO) are inactivated, thereby altering the ROS/AO equilibrium in favor of stress [13]. Oxidative damage plays a vital pathological role in several non-communicable chronic diseases and metabolic disorders such as arthritis, atherosclerosis, cirrhosis, cancer and diabetes [14].

Current research findings have provide strong evidences that anti-oxidative agents could be the most viable agents to neutralize the harmful effects of oxidative stress and therefore, can retard the progress of many diseases [15, 16]. For this reason, research on therapy and prevention of oxidative stress mediated

non-communicable chronic diseases and metabolic disorders has focused attention on the search for agents with anti-oxidative activities that could be used to ameliorate the complications associated with the disease.

The use of medicinal plants for the treatment of various diseases continues to be an important component of health care delivery systems, especially in Africa because the continent has a rich diversity of plants with about 25% of the total number of higher plants in the world where more than 5400 medicinal plants were reported to have over 16,300 medicinal uses [17]. The influence of these medicinal plants and natural products upon drug discovery is impressive because a number of clinically active drugs are either natural products or have a natural product pharmacophore [18].

Cassia singueana (Caesalpiniaceae), commonly called golden shower, is native to northern Nigeria. The plant leaves are commonly used in the traditional circle of northern Nigeria to treat diabetes mellitus [3] and to bathe newly delivered mothers. The antipyretic and antiplasmodial activities of the root extract [4] and the anti-ulcer activity of leaf extract [8, 19] has been reported. Further, the methanol extract of the leaves from this plant was found to be safe in a toxicological study [10]. Beside the above mentioned informations, a most recent study reported the preliminary *in vitro* antioxidant potential of the methanolic extract from the leaves of this plant [11]. However, in order to completely understand the profile of the antioxidant capacity of any plant especially as a guide to a future pharmacological study, different solvents extracts from the various parts of the plant need to be investigated for antioxidant activity.

We therefore conducted a comprehensive investigation of the stem bark, root and leaves of this plant for anti-oxidative activity by using several models with a view to finding compound(s) that could be used to ameliorate oxidative stress related metabolic disorders. We also analyzed the most highly anti-oxidative extracts by gas chromatography-mass spectrometric (GC-MS) analysis in order to identify the phytochemical components of these extracts.

4.2.3 Materials and methods

Please see chapter two; sub sections 2.3 – 2.5 pages 35-38 for details that affect *C. singueana*

4.2.4 Results

The yield of the various extracts collected from the plant indicated that higher yields are apparently obtained from the stem bark extracts. Furthermore, the stem bark extracts contained significantly ($P<0.05$) higher amount of total phenolics than extracts from other parts of the plant. Within the stem

bark, the ethyl acetate extract contained a significantly ($P<0.05$) higher amount of the total phenolics than the other solvents extracts (**Table 4.2.1**).

Table 4.2.1: Percentage yield and total polyphenol concentrations of various solvent extracts of *Cassia singueana* parts

Samples	% Yield	Total polyphenol (mg/g GAE)
<i>Stem bark</i>		
EtOAc	4.04	102.36 \pm 1.18 ^a
EtOH	6.90	91.53 \pm 0.96 ^b
Aqueous	5.29	81.45 \pm 0.15 ^c
<i>Root</i>		
EtOAc	0.63	75.65 \pm 2.07 ^d
EtOH	2.21	77.59 \pm 1.36 ^d
Aqueous	2.40	13.02 \pm 1.93 ^f
<i>Leaves</i>		
EtOAc	0.78	5.55 \pm 0.77 ^g
EtOH	1.34	28.84 \pm 0.72 ^e
Aqueous	0.48	6.94 \pm 0.33 ^g

Data are presented as mean \pm SD values of triplicate determinations. ^{a-g}Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The total reducing power (in terms of percentage gallic acid equivalent) of the various extracts of *C. singueana* was compared to ascorbic acid and trolox (**Table 4.2.2**). While all the solvent extracts from the various parts of *C. singueana* demonstrated Fe^{3+} - Fe^{2+} reductive ability but the EtOAc extract of the stem bark exhibited a consistently higher reducing ability compared to all other extracts as well as a standard, trolox, though the difference was not significant ($P>0.05$) compared to EtOH extract of the same plant part at lower concentrations. With the exception of the EtOAc extract of the root, the total reducing power of the other root and leaf extracts were significantly lower than the standards used in these assays (**Table 4.2.2**).

Table 4.2.2: Total reducing power (gallic acid equivalent) of solvent extracts from the different part of *Cassia singueana*

Samples	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
EtOAc	37.19 ± 2.39 ^h	31.38 ± 4.18 ^d	27.11 ± 4.58 ^{cd}	44.47 ± 6.08 ^d	69.03 ± 16.14 ^{ced}
EtOH	28.51 ± 3.30 ^g	27.83 ± 5.06 ^d	21.11 ± 2.18 ^c	29.94 ± 4.41 ^c	56.84 ± 1.62 ^e
Aqueous	0.00 ± 0.00 ^a	6.04 ± 0.97 ^a	3.46 ± 1.02 ^a	8.48 ± 1.91 ^b	21.56 ± 4.34 ^b
Root					
EtOAc	20.63 ± 2.28 ^f	17.21 ± 2.76 ^c	15.96 ± 1.16 ^b	26.45 ± 2.73 ^c	45.54 ± 4.04 ^c
EtOH	1.99 ± 0.55 ^b	8.14 ± 1.62 ^{ab}	15.27 ± 2.13 ^b	32.51 ± 3.68 ^c	56.19 ± 8.53 ^c
Aqueous	3.53 ± 0.97 ^c	10.00 ± 1.84 ^b	3.49 ± 1.56 ^a	6.82 ± 1.79 ^a	11.93 ± 2.10 ^a
Leaves					
EtOAc	4.43 ± 0.40 ^c	6.00 ± 1.01 ^a	4.44 ± 0.49 ^a	6.97 ± 0.93 ^{ab}	10.70 ± 0.65 ^a
EtOH	7.62 ± 0.56 ^d	6.62 ± 1.19 ^a	5.20 ± 1.02 ^a	9.82 ± 1.07 ^b	15.68 ± 3.59 ^{ab}
Aqueous	10.23 ± 0.86 ^e	5.65 ± 1.75 ^a	3.56 ± 0.56 ^a	4.57 ± 1.66 ^a	13.28 ± 1.83 ^a
Ascorbic acid	31.50 ± 4.92 ^g	32.66 ± 3.00 ^d	35.62 ± 5.51 ^d	52.56 ± 5.43 ^d	89.95 ± 6.21 ^f
Trolox	17.84 ± 1.86 ^f	20.88 ± 7.33 ^c	24.42 ± 3.95 ^c	29.61 ± 1.96 ^c	52.77 ± 0.90 ^d

Data are presented as mean ± SD values of triplicate determinations. ^{a-h}Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Figure 4.2.1 shows the DPPH radical scavenging activities of the various solvent extracts of *C. singueana* parts. All the extracts had a tendency to quench DPPH free radicals as manifested by the concentration dependent increase in the percentage inhibitions. The EtOH extracts of the stem bark and root had a consistently higher DPPH radical scavenging ability than other extracts in these parts, although difference in some cases was insignificant. There was no difference between the free radical scavenging activity of most of stem bark and root extracts and the standard antioxidant used (**Figure 4.2.1**). On the contrary, the leaves extracts showed significantly lower free radical scavenging activity compared to the standards used in this assay.

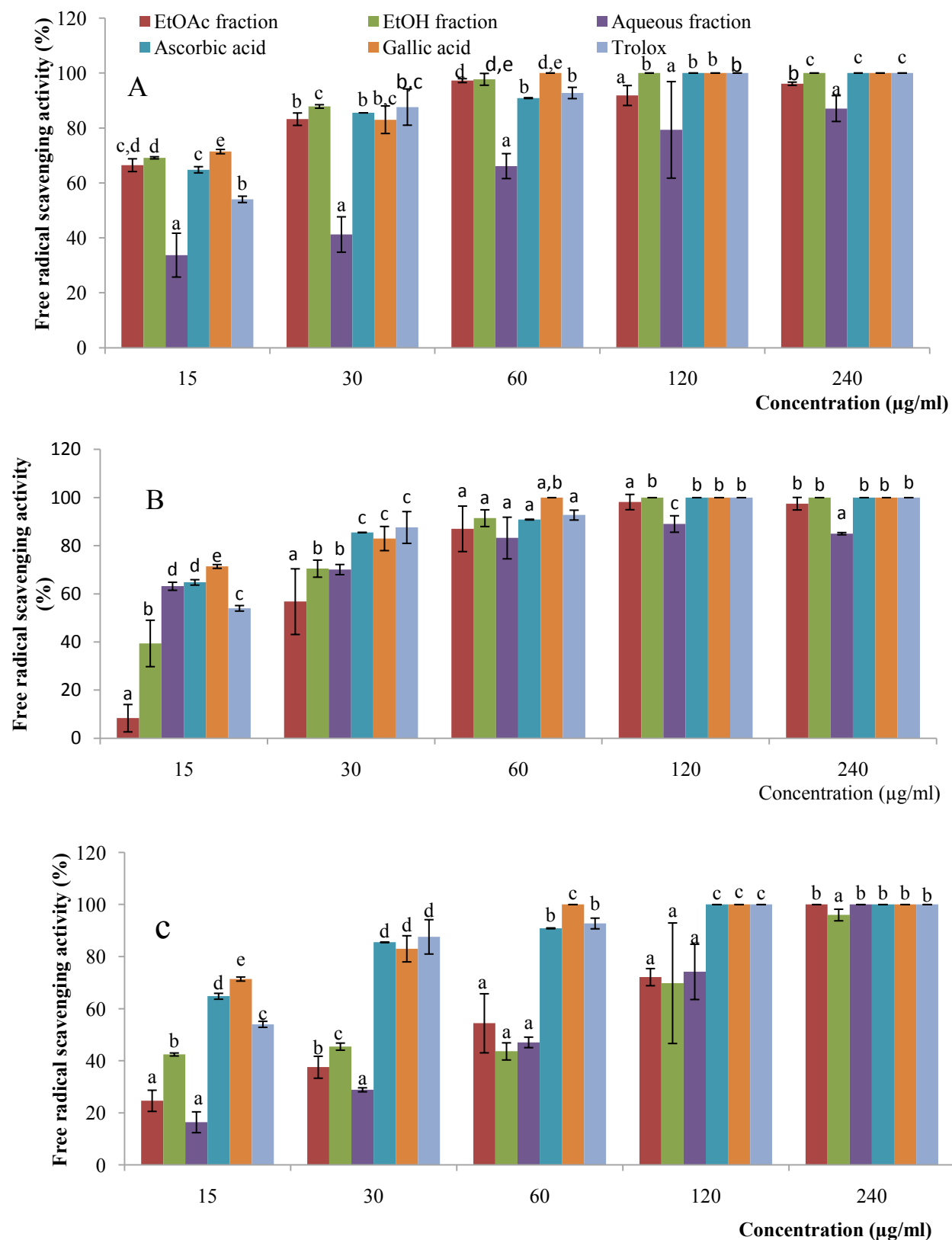


Figure 4.2.1: DPPH radical scavenging activities of various solvent fractions from stem bark (A), root (B) and leaves (C) of *Cassia singueana*. ^{a-c}Values with different letters over the bars for a given concentration for each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The results of the hydroxyl radical inhibition of the solvent extracts of *C. singueana* parts indicated that all the other extracts inhibited hydroxyl radicals generated by Fenton's reaction except the EtOH and aqueous extracts of the stem bark as well as the EtOAc extract of the root which showed pro-oxidative tendencies (**Table 4.2.3**). However, the EtOAc extract of the stem bark and EtOH extracts of the roots and leaves demonstrated significantly higher ($P<0.05$) anti-OH* activity than other extracts within the plant parts. The IC₅₀ values of 1.56, 3.12 and 6.47 µg/ml were obtained for EtOAc extract of the stem bark and EtOH extracts of the roots and leaves respectively whereas 2.61µg/ml was found for trolox (**Table 4.2.5**).

Table 4.2.3: Hydroxyl radical scavenging activity of extracts from various parts of *Cassia singuena*

Samples	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
EtOAc	59.30 ± 14.42 ^{fg}	78.06 ± 0.31 ^g	81.37 ± 1.46 ^g	84.07 ± 1.54 ^h	82.24 ± 0.53 ^f
EtOH	-5.81 ± 2.60 ^b	-15.20 ± 4.46 ^b	-30.67 ± 1.67 ^b	-52.47 ± 1.78 ^b	-100.00 ± 0.00 ^a
Aqueous	-25.14 ± 7.42 ^a	-45.39 ± 1.48 ^a	-66.61 ± 0.81 ^a	-100.00 ± 0.00 ^a	-100.00 ± 0.00 ^a
Root					
EtOAc	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	-13.94 ± 1.59 ^c	-33.08 ± 0.84 ^b
EtOH	62.23 ± 3.38 ^g	71.01 ± 2.04 ^f	74.47 ± 6.57 ^{fg}	84.56 ± 0.71 ^a	87.60 ± 0.56 ^g
Aqueous	42.50 ± 3.26 ^e	37.37 ± 0.38 ^e	35.18 ± 3.57 ^e	35.49 ± 2.98 ^f	29.17 ± 0.69 ^d
Leaves					
EtOAc	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.00 ± 0.00 ^c	0.00 ± 0.00 ^d	20.10 ± 4.62 ^c
EtOH	54.84 ± 1.17 ^f	68.48 ± 1.01 ^f	73.85 ± 0.76 ^f	77.53 ± 0.93 ^g	84.68 ± 2.23 ^f
Aqueous	11.85 ± 1.69 ^d	18.40 ± 2.82 ^d	21.37 ± 3.97 ^d	27.99 ± 0.75 ^e	35.77 ± 3.40 ^e
Trolox	57.32 ± 2.95 ^{fg}	73.11 ± 1.44 ^f	76.04 ± 2.05 ^{fg}	80.09 ± 3.93 ^{gh}	79.82 ± 3.50 ^f

Data are presented as mean ± SD values of triplicate determinations. ^{a-h}Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The nitric oxide inhibition activities of the different solvent extracts of *C. singuena* parts are presented in **Table 4.2.4**. Apart from the EtOH extracts of the stem bark and root, all other extracts were found to exhibit nitric oxide scavenging activities. The EtOAc and aqueous extracts of the root as well as EtOAc and EtOH extracts of the leaves exhibited a non-concentration dependent NO inhibition effect. Aqueous extracts were found to possess lower IC₅₀ values in the NO scavenging model than other extracts within the plant parts.

Table 4.2.4: Nitric oxide scavenging activities of extracts from various parts *Cassia singueana*

Samples	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
EtOAc	0.00± 0.00 ^b	0.00 ± 0.00 ^b	16.12 ± 0.85 ^c	23.70 ± 1.66 ^c	30.93 ± 4.20 ^c
EtOH	-46.61±5.34 ^a	-100.00±0.00 ^a	-100.00±0.00 ^a	-100.00±0.00 ^a	-100.00±0.00 ^a
Aqueous	8.99±1.31 ^c	12.70±0.59 ^d	37.56±0.75 ^e	44.91± 7.16 ^e	50.24±1.88 ^e
Root					
EtOAc	36.01±0.31 ^d	43.51±4.13 ^f	53.36± 7.82 ^{fg}	58.00± 1.08 ^f	44.36±4.24 ^{de}
EtOH	0.00± 0.00 ^b	0.00± 0.00 ^b	0.00± 0.00 ^b	0.00± 0.00 ^b	0.00± 0.00 ^b
Aqueous	6.89±0.89 ^c	17.20±2.09 ^e	77.83±5.26 ^h	80.87±2.89 ^h	34.07±1.31 ^c
Leaves					
EtOAc	56.01±2.30 ^f	52.93 ±13.42 ^f	61.67±2.48 ^{fg}	58.06 ± 1.84 ^f	65.39 ± 4.17 ^f
EtOH	46.51±1.20 ^e	37.66±10.97 ^f	67.60±0.39 ^h	65.33±2.60 ^g	44.15± 1.94 ^d
Aqueous	9.19 ± 2.91 ^c	16.36 ±0.89 ^d	24.97±2.81 ^d	26.03±0.37 ^d	56.22±4.69 ^e
Ascorbic acid	47.81 ± 0.51 ^e	50.97 ± 1.35 ^f	52.82 ± 1.35 ^f	56.72 ± 3.69 ^f	64.08 ± 4.25 ^f
Gallic acid	4.43 ± 2.69 ^c	7.52 ± 1.11 ^c	25.74 ± 0.44 ^d	27.66 ± 1.91 ^d	31.70 ± 2.51 ^c
Trolox	66.30 ± 1.23 ^g	65.51 ± 1.27 ^g	63.39 ± 1.84 ^g	59.53 ± 1.81 ^f	52.21 ± 2.47 ^e

Data are presented as mean ± SD values of triplicate determinations. ^{a-g}Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 4.2.5: IC₅₀ values of different extracts of *C. singueana* parts in different anti-oxidative models

Samples	IC ₅₀ (µg/ml)		
	DPPH	Hydroxyl	Nitric oxide
Stem bark			
EtOAc	1.76	1.56	1116.40
EtOH	1.20	P	P
Aqueous	34.05	P	188.78
Root			
EtOAc	31.78	P	115.51
EtOH	14.57	3.12	Nil
Aqueous	2.58	2.05	87.73
Leaves			
EtOAc	44.23	161016.65	4.57
EtOH	35.88	6.47	29.56
Aqueous	53.09	1439.25	2.81
Ascorbic acid	2.56	ND	26.28
Gallic acid	1.27	ND	1026.45
Trolox	5.04	2.610	599.21

ND means not determined and P means the extract showed pro-oxidative properties in the experimental model

Based on the high anti-oxidative activities, the EtOAc extract of the stem bark and the EtOH extracts of the root and leaves were analyzed by GC-MS analysis (**Figure 4.2.2**). The EtOAc extract of the stem bark was found to contain phenolic compounds such as resorcinol and 4-propylphenol, amino acids such as

dehydroxylevodopa, sterols such as 6-dehydroestradiol and aromatic esters such as the methyl benzoates. The ethanol extracts of the roots was found to contain polyphenols such as resorcinol, anthralin, and a polyhydroxylated flavonoid, precursors to polyphenol compounds such as hydroxyphenyl pyruvic acid and aromatics such as benzylidenexanthene and methyl chrysene. The EtOH extract of the leaves contained phytol along with benzyl alcohols, fatty acids and their esters and precursors to triterpenoids such as squalene (Table 4.2.6).

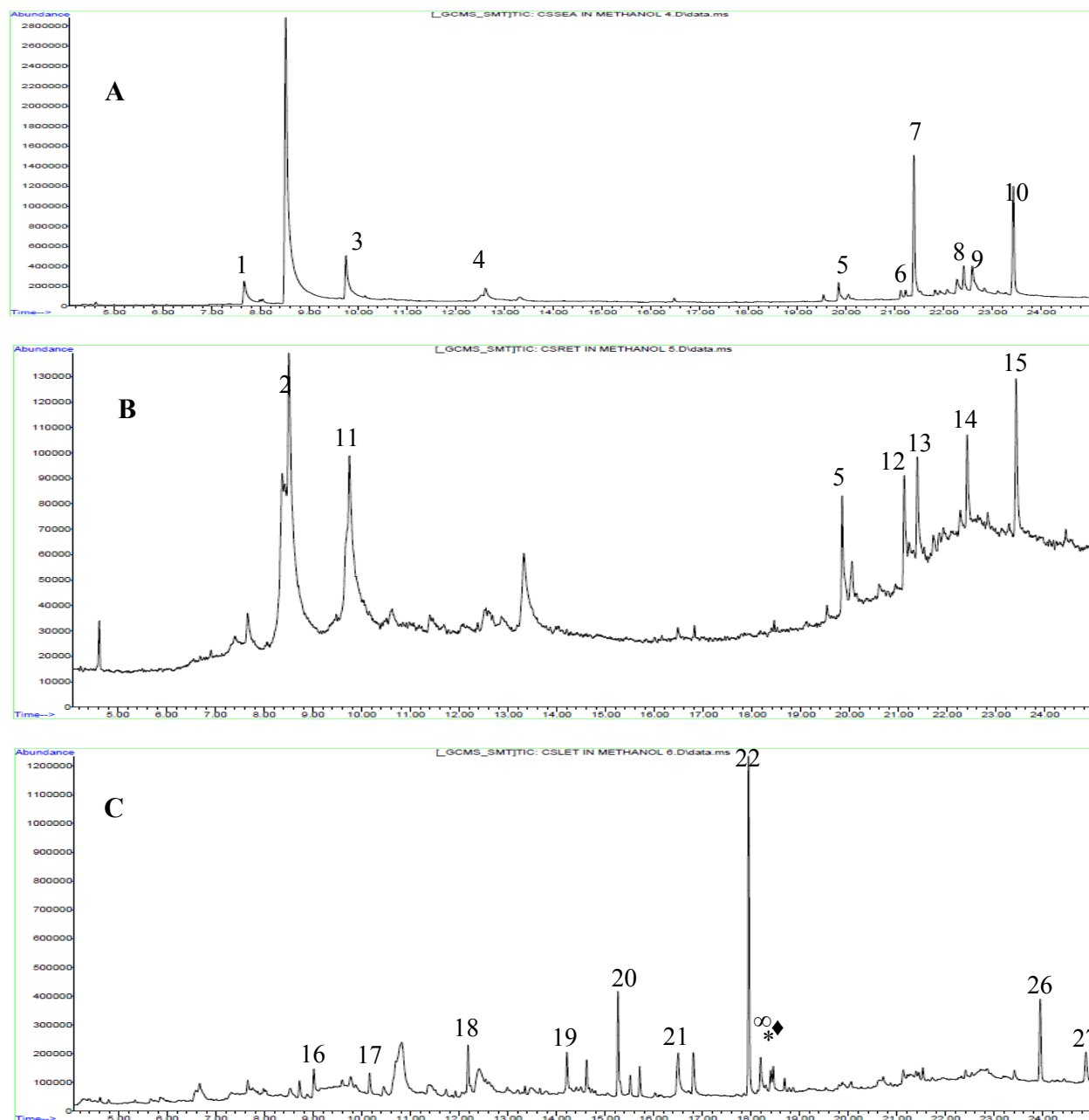


Figure 4.2.2: GC-MS chromatogram of EtOAc extract of the stem bark (A) and EtOH extracts of the root (B) and leaves (C) of *C. singueana*. ∞, * and ♦ refers to annotated peak numbers 23, 24 and 25 respectively.

Table 4.2.6: Identified compounds of EtOAc extract of stem bark and EtOH extracts of root and leaves of *Cassia singueana* by GC-MS

#	Compounds	RT (min)	Molecular mass	Relative abundance (%)
EtOAc extract of stem bark				
1	2,3-dihydro benzofuran	7.67	120.15	4.08
2	Resorcinol	8.52	110.10	54.03
3	4-propylphenol	9.74	136.19	7.24
4	Dehydroxylevodopa	12.59	179.10	ND
5	3-methylanthralin	19.85	240.05	1.71
6	2,3,5,7-tetramethylpyrrolo[2,3-f]quinolin-9-ol	21.12	240.00	ND
7	2,2'-(m-phenylene) dithiophene	21.40	242.10	ND
8	6-dehydroestradiol	22.41	270.36	2.62
9	1-(4-methoxyphenyl)-2-(2-hydroxy-4-methylphenyl)-diazene	22.58	242.10	ND
10	Methyl 3-(1-formyl-3,4-methylenedioxy) benzoate	23.42	284.06	9.28
EtOH extract of root				
2	Resorcinol	8.51	110.10	ND
11	P-hydroxyphenyl pyruvic acid	9.74	180.15	ND
5	3 methylanthralin	19.86	240.10	ND
12	5,6 dimethyl-4-phenyl-3-cyanopyridine-2-thione	21.13	240.10	ND
13	1-methyl chrysene	21.48	242.10	ND
14	9-benzylidenexanthene	22.41	270.10	ND
15	3',7-dihydroxy-4-methoxy-flavonol	23.43	284.10	ND
EtOH extract of the leaves				
16	2-methoxy-4-vinylphenol	9.02	150.06	2.67
17	1-(3,6,6-trimethyl-1,6,7,7-tetrahydrocyclopenta[c]pyran-1-yl) ethanone	10.16	191.10	ND
18	4-methyl-2,5-dimethoxybenzaldehyde	12.17	180.10	ND
19	Coniferyl alcohol	14.21	180.20	2.72
20	*Long chain aromatic ester	15.52	194.20	ND
21	Palmitic acid	16.50	256.42	5.50
22	Phytol	17.95	296.53	23.70
23	Linolenyl alcohol	18.20	264.44	3.92
24	Stearic acid	18.39	284.48	1.73
25	Linolenic acid ethyl ester	18.46	306.48	2.24
26	Squalene	23.94	410.72	7.28
27	Eicosane	24.88	282.55	3.32

The compounds presented in the table are those which matched similar compounds in the NIST library software and which contained the molecular ion of the matching compound. *Compound had the fragment pattern characteristics of long chain aromatic esters. The aliphatic chain had a good hit with the library. ND means not determined while # refers to annotated peak number.

4.2.5 Discussion

Cassia singueana is currently utilized in the traditional treatment of diabetes mellitus in Nigeria whose pathogenesis has, in part, been linked to the development of oxidative stress. Apart from now, only a few authors have reported the antioxidant activities of the plants within the *Cassia* genus [20-22]. This study investigated the complete anti-oxidative profile of various solvent extracts from different parts of this plant as a prelude to finding agent(s) that could be used to ameliorate oxidative stress-associated complications. We found some of the *C. singueana* extracts to possess exceptionally high anti-oxidative activities, at least with the experimental models.

Different anti-oxidative agents act via various mechanisms and therefore no single model can provide a full evaluation of the anti-oxidant potentials. Therefore, investigating the complex anti-oxidative activities mostly requires the use of multiple methods [23]. It was proposed that the ability to donate electrons by bioactive compounds which reflects their reducing power is linked to anti-oxidative activity [24, 25]. Antioxidants can be reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one species is reduced at the expense of the oxidation of the other. Thus, the availability of reductants, like anti-oxidative agents in the samples could cause the reduction of Fe^{2+} to Fe^{3+} which can be measured as the formation of Perl's prussian blue at 700 nm. The FRAP assay therefore provides a reliable method to study the antioxidant activity of various extracts and/or compounds. In our experiment, the higher reducing power of the EtOAc extracts of the stem bark and root in most of the concentrations used suggest that the phytochemical constituents with high redox potential are more extractable with EtOAc.

The DPPH radical is a widely used model to study the neutralizing effects of crude plant extracts, solvent fractions or pure compounds on free radicals. Antioxidants act on DPPH through their hydrogen donating ability [26]. Hence, DPPH is frequently used as a substrate to investigate the free radical scavenging activity of anti-oxidative agents *in vitro* [27]. The high DPPH radical scavenging activities of the various solvent extracts which are comparable to standard antioxidants used suggest that the extracts have compounds with high proton donating ability and could serve as free radical inhibitors. However, the organic solvents extract from the stem bark demonstrated a more remarkable anti-radical activity with IC_{50} values lower than those of ascorbic acid and trolox. Although many reports on the DPPH radical scavenging activity of extracts from plants belonging to different families and from various parts of the world exist in the literature but only few authors [28] reported low IC_{50} values as was observed with these extracts. This further suggests that these extracts contain powerful free radical scavenging phytochemicals that could have the ability to inhibit free radical upsurge as well as oxidative stress which consequently might ameliorate oxidative stress associated metabolic disorders.

Hydroxyl radicals are highly reactive species that causes damage to all life essential macromolecules [29]. These radicals are extremely detrimental because they initiate auto-oxidation, polymerization and fragmentation of biomolecules. Thus, compounds with excellent scavenging effects towards hydroxyl radicals would be important for treating diseases such as T2D where oxidative stress is important for the disease initiation and/or progression. The hydroxyl radical scavenging effects is thus monitored as the percentage of inhibition of hydroxyl radicals produced in the Fenton's reaction mixture by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+} /EDTA/ascorbate/ H_2O_2 system [30]. From the present study, the EtOAc extract of the stem bark and aqueous extract of the root are more effective in hydroxyl radical inhibitions, considering their lower IC_{50} values. The pro-oxidative tendencies showed by the EtOH and aqueous extracts of the stem bark as well as the EtOAc extract of the root in the hydroxyl radical based anti-oxidative model further indicate the need to use a multi method approach before a definite statement can be made on the anti-oxidative effects of any plant extract or bioactive compound.

Nitric oxide is a very unstable species that has been implicated in the pathology of cancer, T2D and several other disorders [31-33]. The lower IC_{50} values observed by the aqueous extracts in all the plant parts could indicate that the anti-oxidative principles extracted by the various solvents act via different antioxidant mechanism. On the other hand, the non-concentration dependent pattern of NO scavenging activity demonstrated by the EtOAc and aqueous extracts of the root as well as the organic solvent extracts of the leaves could be linked to a hormesis phenomenon exhibited by these extracts. Hormesis is a dose-response relationship for a single endpoint that is characterized by reversal of response between low and high doses of chemicals, biological molecules, physical stressors, or any other initiators of a response [34] and its occurrence has been documented in numerous biological, toxicological and pharmacological investigations [35]. Thus, these extracts possess optimal points for effective inhibition of NO radical and/or antagonism may occur with other phytochemicals at certain concentrations.

Phenolics are very important constituents among other natural antioxidants because of their various pharmacological actions and direct contribution to anti-oxidative activity [29]. Although a correlation analysis was not performed, our data tends to suggest strong correlations between the total phenolic content and anti-oxidative capacity because most extracts with the highest phenolic content within a plant part displayed high anti-oxidative activity in most of the experimental models used and consequently these extracts were selected for GC-MS analysis. While not discounting the possible contributions of the other detected phytochemicals, it is possible to surmise that the main anti-oxidative agents of these extracts are simple phenolics, especially resorcinol in the stem bark and root as well as phytol in the leaves. However, the differences in the anti-oxidative activities among the extracts could be due to different qualitative and quantitative composition of their phenolics as well as other phytochemicals. This

is because the antioxidant actions of phenolics and their derivatives depend on the number of hydroxyl groups in the molecule [36].

In conclusion, this work reveals that the various solvent extracts of *C. singueana* parts possessed strong anti-oxidative activities that could be relevant in the management of oxidative stress related non-communicable chronic diseases, however further experimental and clinical studies are warranted. Phenolic compounds were found to be the main phytochemicals with resorcinol being more prominent than other compounds in the stem bark and root and phytol being more prominent than other compounds in the leaves. Bioassay guided fractionation and evaluating the *in vivo* anti-oxidative effects of some of the extracts will be the subject of our future study.

Postscript: From the above experiment, the ethyl acetate crude extract from the stem bark was found to have relatively higher anti-oxidative activity that cuts across various types of radicals than other extracts. It was therefore selected for further investigations.

4.3 Acetone fraction of *Cassia singueana* stem bark ethyl acetate extract contains anti-oxidative agents and potent inhibitors of α -glucosidase and α -amylase

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Preface: In this sub-chapter, solvent-solvent fractionation of the crude stem bark ethyl acetate extract was carried out and the solvent fractions were investigated for anti-oxidative and α -glucosidase and α -amylase inhibitory activities. This article is yet to be submitted to the supervisors for comments and proof reading and eventual submission for publication

4.3.1 Abstract

The present study investigated the *in vitro* anti-oxidative as well as the α -glucosidase and α -amylase inhibitory activities of various solvent fractions derived from the crude ethyl acetate extract of *Cassia singueana* stem bark. Solvent-solvent fractionation was used to fractionate the crude ethyl acetate extract into hexane, dichloro methane, acetone and aqueous fractions. The acetone fraction had significantly higher ($P < 0.05$) total phenolics, total reducing power and DPPH radical scavenging activity than other solvent fractions. Furthermore, the acetone fraction elicited a significantly more potent ($P < 0.05$) inhibitory effects on α -glucosidase and α -amylase activities than other fractions. Steady state kinetic analysis revealed that the acetone fraction inhibited α -glucosidase in a non-competitive pattern with an inhibition binding constant (K_i) of 22.81 $\mu\text{g/ml}$ and inhibited α -amylase in a competitive pattern with K_i of 8.57 $\mu\text{g/ml}$. GC-MS analysis of the acetone fraction revealed that the main bioactive components are phenolics such as resorcinol and 2' 4'-dihydroxy chalcone. It was concluded that the acetone fraction contains potent inhibitors of α -glucosidase and α -amylase while resorcinol and 2' 4'-dihydroxy chalcone could be the main bioactive agents.

4.3.2 Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia with alterations in the metabolism of major macromolecules resulting from anomalies in the secretion of insulin or its action [37]. According to the International Diabetes Federation (IDF), about 366 million people are living with diabetes and this figure is projected to increase to 552 million by the year 2030 [38]. A number of hypotheses have been suggested to provide explanation on the different aspects of diabetes pathology among which oxidative stress, through a single unifying mechanism of reactive oxygen species

generation, is regarded as the common biological phenomenon that plays vital roles in all aspects of the disease pathogenesis such as insulin resistance, pancreatic β -cell dysfunction as well as macrovascular and microvascular complications [39-41]. Therefore, the use of antioxidants could be exploited in the treatment of T2D and prevention of its chronic vascular complications [42].

On the other hand, the control of postprandial hyperglycemia is also considered to be important in the management of diabetes and the prevention of its associated complications. At present, hyperglycemia is predominantly managed with sulfonylureas and biguanides but the use of α -glucosidase and α -amylase inhibitors is still considered as important line of treatment especially in the developing world [43]. α -Amylase hydrolyzes starch and other complex polysaccharides to oligosaccharides which are further hydrolyzed by intestinal α -glucosidase to liberate glucose which is then absorbed into the intestinal epithelium and enter blood circulation. Consequently, inhibitors of these enzymes will ultimately retard the release of glucose from dietary carbohydrates to the bloodstream, thereby impeding the postprandial hyperglycemia. Unfortunately, the clinically available α -glucosidase inhibitors, acarbose and miglitol, were frequently found to cause diarrhea and gastrointestinal side effects such as bloating, flatulence, cramping and abdominal pain [44]. These gastrointestinal disturbances have been reported to be the main reason for non-compliance and early withdrawal in randomized controlled trials [45]. Interestingly, glucosidase inhibitors derived from plants sources were reported to be more acceptable because they are cheap and relatively safer, including a lower tendency of causing serious gastrointestinal discomfort [46, 47]. Based on the above, research on diabetes therapy has focus attention on the search for plant based agents with potent anti-oxidative activity and α -glucosidase inhibitory activities, thereby providing a holistic therapeutic avenue to control hyperglycemia and other diabetic complications resulting from oxidative stress.

Cassia singueana Delile, also known as golden shower, is a member of the family Caesalpiniaceae and is commonly used by the traditional medicine practitioners of northern Nigeria to treat diabetes mellitus [3] as well as bathe nursing mothers. The antipyretic and antiplasmodial activities of the root extract [4] and the anti-ulcer activity of leaf extract [8, 19] have been reported. Furthermore, the methanol extract of the leaves from this plant was found to be safe in a toxicological study [10]. In a previous study, preliminary *in vitro* anti-oxidative potential of the methanolic extract from the leaves was reported [11]. However, in a most recent study, we subjected different solvent crude extracts of the stem bark, root and leaves of the plant to anti-oxidative activity assays using several models and reported that the ethyl acetate extract of the stem bark had the best anti-oxidative activity among all other extracts [48].

In the present study, we further fractionated the ethyl acetate extract of the stem bark across solvents of varying polarity and then conducted a comprehensive study on the *in vitro* anti-oxidative as well as the α -

glucosidase and α -amylase inhibitory activities of the fractions. Additionally, we subjected the most bioactive fraction to GC-MS analysis in order to identify the phytochemicals contained therein.

4.3.3 Materials and methods

Please see chapter two sub-sections 2.6.1-2.6.6 (pages 38-40) for detailed materials and methods that affect *C. singueana*

4.3.4 Results

The total phenolics content of the acetone fraction was significantly higher ($P<0.05$) than all other fractions. Also, the acetone fraction had a significantly higher ($P<0.05$) Fe^{3+} - Fe^{2+} reducing power (**Figure 4.3.1**) and DPPH radical scavenging activity (**Table 4.3.1**) than other fractions. The results of hydroxyl radical scavenging activity also showed that all the fractions contained phytochemicals that could scavenge hydroxyl radicals generated by Fenton's reaction but the aqueous fraction had the best activity in this model followed by the acetone fraction. NO radicals were also scavenged by all the fractions but the less polar fractions (hexane and dichloromethane) showed a significantly higher ($P<0.05$) NO radical scavenging activity than the more polar fractions (acetone and water) (**Table 4.3.1**).

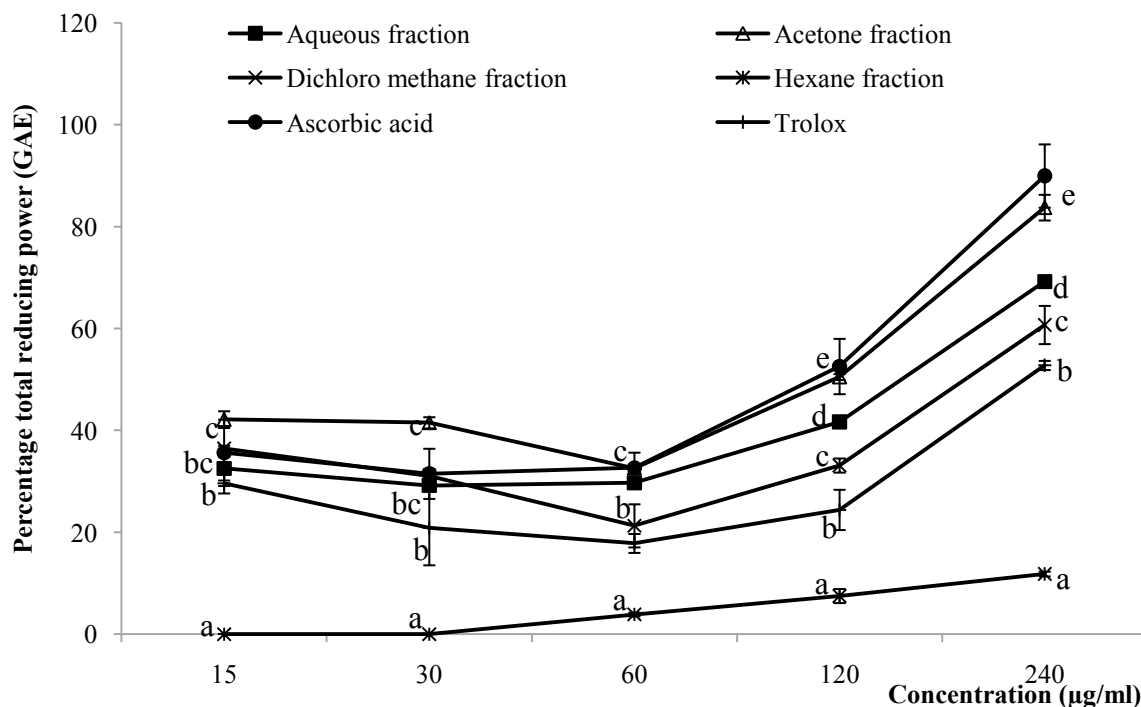


Figure 4.3.1: Total reducing power (relative to gallic acid) of different solvent fractions of the ethyl acetate extract of *C. singueana* stem bark. Data are presented as mean \pm SD of triplicate determinations. ^{a-} Values with different letters presented for a given concentration are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 4.3.1: IC₅₀ values of various solvent fractions of ethyl acetate extract of *C. singueana* stem bark in different anti-oxidative models

Fractions	Total phenolics (mg/g GAE)	IC ₅₀ (μg/ml)		
		DPPH	Hydroxyl radical	NO
Aqueous	167.49 ± 0.14 ^c	5.07 ± 0.09 ^c	169.58 ± 14.23 ^b	322.81 ± 20.49 ^c
Acetone	193.69 ± 3.78 ^d	1.95 ± 0.30 ^a	194.05 ± 8.78 ^c	346.44 ± 30.43 ^c
Dichloromethane	154.96 ± 11.06 ^b	31.46 ± 8.67 ^e	219.80 ± 14.21 ^d	226.75 ± 45.47 ^b
Hexane	7.77 ± 0.94 ^a	89.63 ± 0.44 ^f	272.65 ± 8.99 ^e	272.97 ± 47.08 ^{bc}
Ascorbic acid	-	2.56 ± 0.26 ^b	ND	26.40 ± 6.46 ^a
Gallic acid	-	1.40 ± 0.43 ^a	ND	1053.48 ± 239.92 ^e
Trolox	-	8.47 ± 2.88 ^d	3.23 ± 0.49 ^a	629.76 ± 63.98 ^d

Data are presented as mean ± SD values of triplicate determinations. ^{a-c}Different letters along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Figure 4.3.2 presents the α-glucosidase and α-amylase inhibitory effects of the fractions. With the exception of hexane fraction, all other fractions inhibited α-glucosidase in a dose dependent pattern (**Figure 4.3.2A**). The more polar fractions demonstrated significantly higher (P<0.05) α-glucosidase inhibitory effects than the less polar fractions. However, within the more polar fractions, the α-glucosidase inhibitory activity demonstrated by the acetone fraction was significantly higher (P<0.05) than aqueous fraction. Pancreatic α-amylase activity was inhibited by all the fractions in a dose dependent fashion except the hexane fraction (**Figure 4.3.2B**) and the acetone fraction showed a significantly higher (P<0.05) α-amylase inhibitory effects than other fractions. Based on the high activity of the acetone fraction in most of the assays described above, it was selected for further studies.

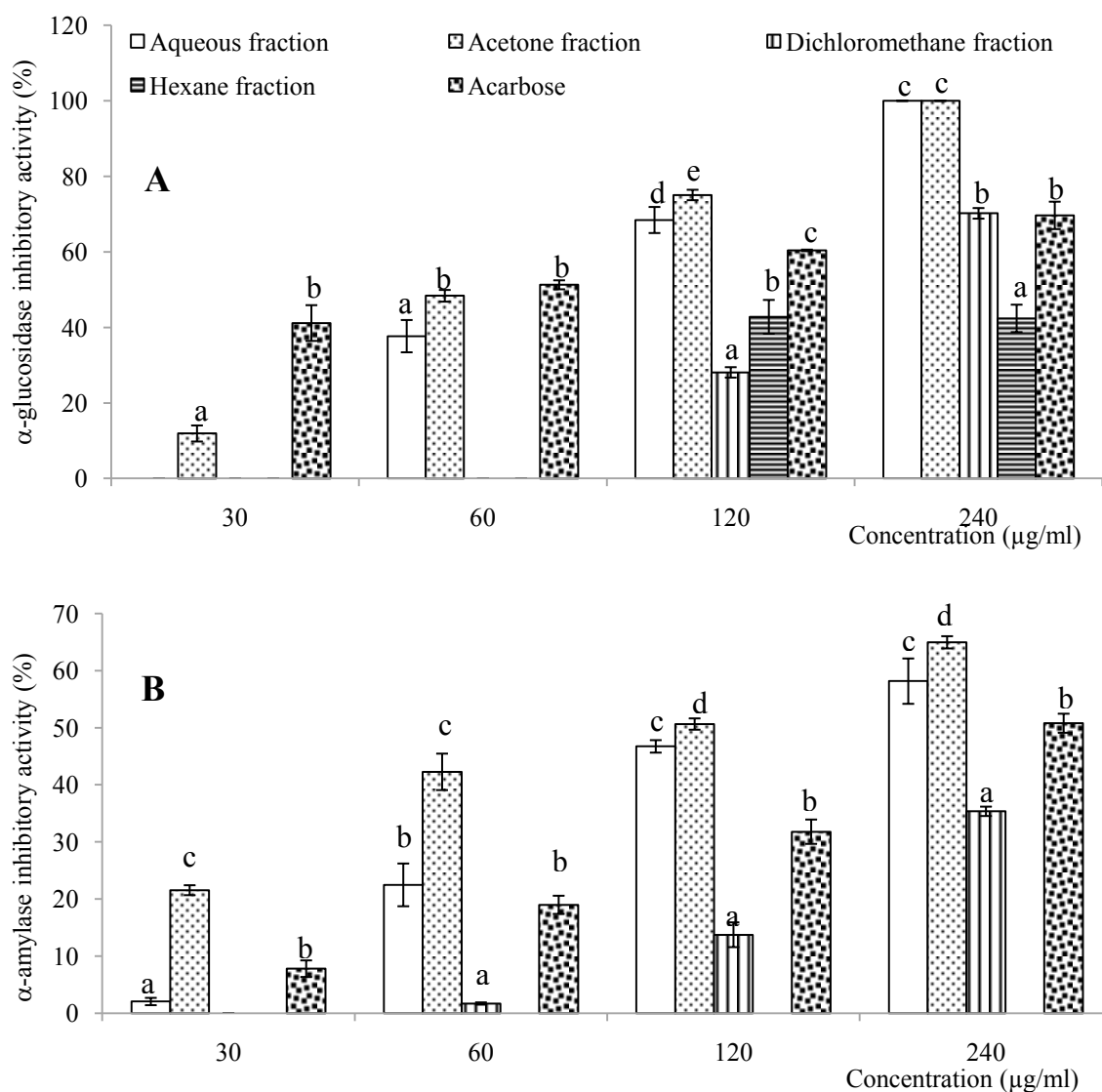


Figure 4.3.2: α -glucosidase (A) and α -amylase (B) inhibitory activities of different solvent fractions of the ethyl acetate extract of *C. singueana* stem bark. Data are presented as mean \pm SD of triplicate determinations. ^{a-c}Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

A steady state kinetic analysis of both α -glucosidase and α -amylase activities at varied substrate concentrations in the presence and absence of 60 $\mu\text{g/ml}$ of the acetone fraction (the most active fraction) was further conducted to determine the mechanism of inhibition. From the experiment, the acetone fraction was found to be a non-competitive inhibitor of α -glucosidase (**Figure 4.3.3A**) with V_{max} decreasing from 655.09 $\mu\text{mol/min}$ to 180.45 $\mu\text{mol/min}$. The K_M remained unchanged at 2.00 mM and the computed K_i value for inhibiting α -glucosidase was 22.81 $\mu\text{g/ml}$ (**Table 4.3.2**). Conversely, the mechanism of inhibition of α -amylase by the acetone fraction revealed a competitive inhibition pattern (**Figure 4.3.3B**) with a K_M value of 2.00 % and 0.25% in the presence and absence of the inhibitor

respectively. The V_{\max} of the α -amylase was not affected (33.70 $\mu\text{mol}/\text{min}$) and K_i for inhibiting α -amylase by the fraction was 8.57 $\mu\text{g}/\text{ml}$ (**Table 4.3.2**).

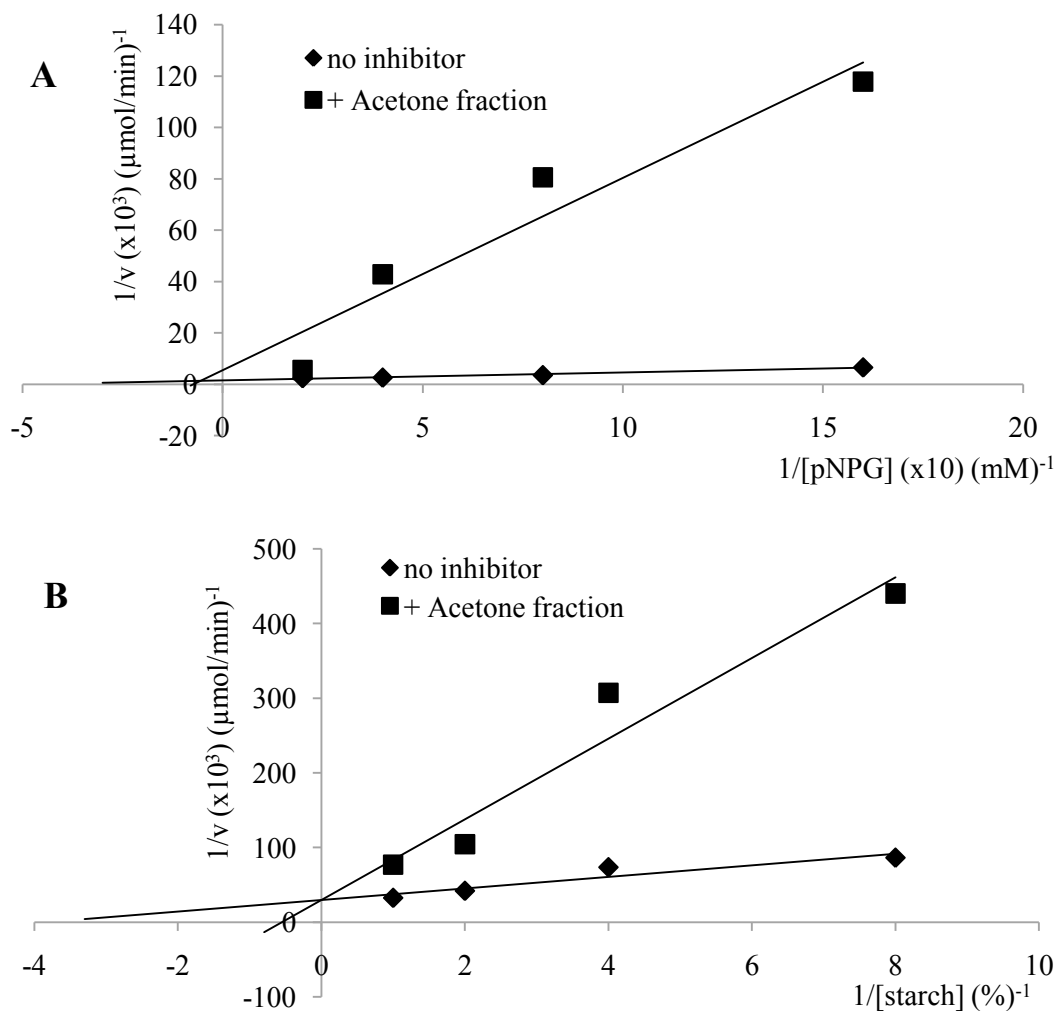


Figure 4.3.3: Lineweaver-Burke's plot of α -glucosidase (A) and α -amylase (B) catalyzed reactions in the presence and absence of the acetone fraction derived from the ethyl acetate extract of *C. singueana* stem bark

Table 4.3.2: Effects of the acetone fraction derived from the ethyl acetate extract of *C. singueana* stem bark (60 $\mu\text{g}/\text{ml}$) on some kinetic parameters of α -glucosidase and α -amylase

Kinetic parameters	α -glucosidase		α -amylase	
	control	+ acetone fraction	control	+ acetone fraction
K_M	2.00*	2.00*	0.25 [#]	2.00 [#]
V_{\max} ($\mu\text{mol}/\text{min}$)	655.09	180.45	33.70	33.70
K_i ($\mu\text{g}/\text{ml}$)	-	22.81	-	8.57

The units for K_M was mM (*) and % ([#])

The major peaks detected in the GC-MS chromatogram of the acetone fraction (Figure 4.3.4) were those of resorcinol, 2' 4'-dihydroxy chalcone and 2-(3-carbethoxyphenylamino)-4,6-diphenylpyrimidine identified by their fragmentation patterns and in conjunction with the NIST library (Table 4.3.3). The retention time and the molecular mass of these compounds are also provided in Table 4.3.3.

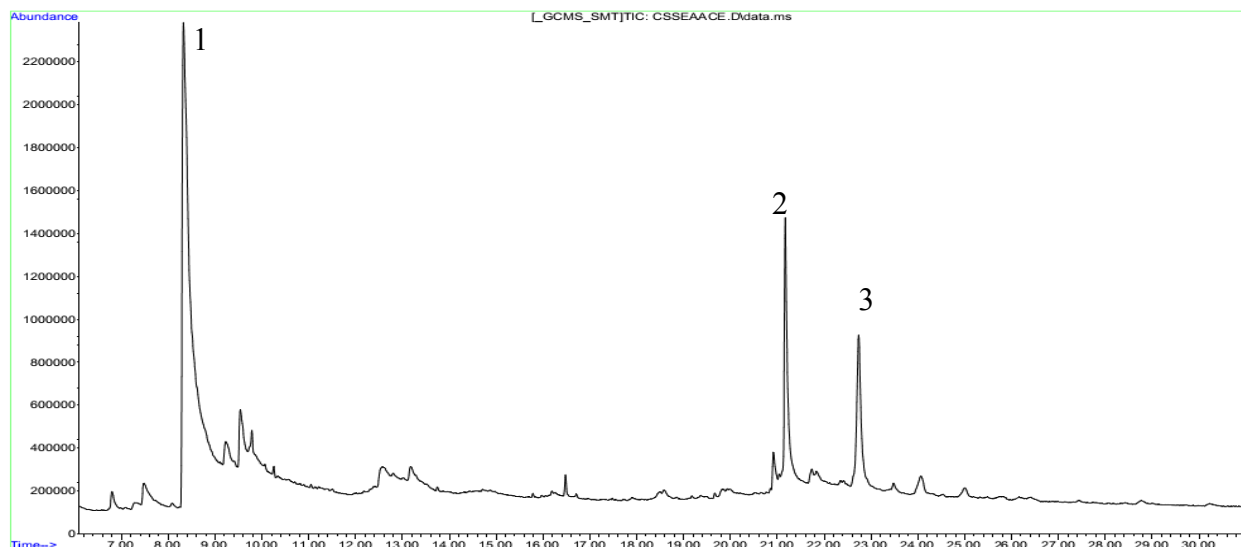


Figure 4.3.4: GC-MS chromatogram of the acetone fraction derived from the ethyl acetate extract of *C. singueana* stem bark.

Table 4.3.3: Identified components of the acetone fraction derived from the ethyl acetate extract of *C. singueana* stem bark by GC-MS

§	Compound	Retention time (min)	Molecular mass
1	Resorcinol	8.32	110.11
2	2' 4'-dihydroxy chalcone	21.13	240.25
3.	2-(3-Carbethoxyphenylamino)-4,6-diphenylpyrimidine	22.75	395.50

4.3.5 Discussion

Phytochemicals or other chemotherapeutic agents with potent anti-oxidative as well as α -glucosidase and α -amylase inhibitory effects are useful in the management of postprandial hyperglycemia, T2D and related chronic vascular complications, thereby providing a holistic therapeutic strategy. In the present study, acetone fraction fractionated from the crude ethyl acetate of *C. singueana* stem bark elicited remarkable anti-oxidative as well as α -glucosidase and α -amylase inhibitory activities.

Plants have wide array of phytochemicals ranging from both nonpolar to polar. Thus, we fractionated the crude ethyl acetate extract to further narrow down on the phyto-components responsible for the observed high activity [48] and to also determine their *in vitro* anti-diabetic potentials. For the anti-oxidative activity of the fractions, different models for *in vitro* anti-oxidative studies were used because a single method cannot give a full evaluation of the anti-oxidative capabilities due to the involvement of multiple mechanisms in the induction of oxidative stress. The total reducing power of phytochemicals is considered as a reliable marker of their antioxidant effects. The reductants terminate the free radical chain reaction by donating hydrogen atoms to the radical molecules. Free radicals are known to be a major factor in cellular damages in biological systems and DPPH method has been used to investigate the free radical scavenging activity of natural anti-oxidative agents. On the other hand, hydroxyl radicals are also extremely reactive species that could damage any biomolecule in living systems [49] and NO is an unstable species which reacts with oxygen to generate the reactive nitrite and peroxynitrite anions [50]. All these radicals are implicated in the pathogenesis of T2D and associated complications [32, 51]. From the present study, the acetone fraction contains better group(s) of phytochemical(s) which could reasonably scavenge all the various forms of radicals. This further suggests the presence of powerful phytochemical(s) that might have the ability to inhibit free radical upsurge as well as oxidative stress. These phytochemicals, either in pure form or in combination could be useful therapeutic agents for treating oxidative stress related metabolic disorders such as T2D.

Analysis of the α -glucosidase inhibitory activity of the fractions also demonstrated that the acetone fraction contains the most powerful inhibitors against this enzyme which could slow down the breakdown of disaccharides to liberate glucose; thereby reducing glucose absorption from the small intestine [52]. Consequently, this will decrease the flow of glucose into the bloodstream; thereby controlling postprandial hyperglycemia. The observation could be linked to the high phenolics content recorded in the fraction since phenolic fractions from plants have been reported to inhibit α -glucosidase activity and allow for a tight control of blood glucose levels [53]. Indeed, some isolated phenolics have been reported to be the main bioactive anti-diabetic agents of *Brickellia cavanillesii* [54] and *Garcinia mangostana* [55] and the activity was mediated through the inhibition of α -glucosidase. Furthermore, Mai et al. [56] demonstrated a strong positive correlation between polyphenolic content and α -glucosidase inhibitory effects of 28 extracts from Vietnamese edible plants which corroborates with this study. On the other hand, analysis of α -amylase inhibitory activity also revealed that the acetone fraction had the highest activity which suggests the presence of better group(s) of phytochemicals that could inhibit the breakdown of complex carbohydrates to oligosaccharides, thereby diminishing the effect of carbohydrate consumption on postprandial hyperglycemia.

Kinetic delineation of the α -glucosidase inhibitory effects of the acetone fraction indicated that the mechanism of inhibition is non-competitive suggesting that the bioactive phytochemicals binds the α -glucosidase at separate site(s) of the enzyme (rather than the active site) but caused conformational modification at the active site, thereby preventing effective binding of the substrate and consequently reduced the α -glucosidase activity [57]. On the contrary, the pattern of α -amylase inhibition by the fraction (competitive) suggests that the active site of the enzyme is directly involved in the inhibitory action and the fraction likely contains some phytochemical(s) that could serve as substrate analogues, thereby competing for the active site of the α -amylase [58].

Phytochemical analysis of the acetone fraction revealed that phenolics such as resorcinol and 2' 4'-dihydroxy chalcone are the main bioactive agents of the fraction. This observation corroborates with our earlier assertion on the involvement of phenolics in the observed inhibitory effects.

We concluded that the acetone fraction derived from the crude ethyl acetate extract of *C. singueana* stem bark contains powerful anti-oxidative agents and inhibitors of α -glucosidase and α -amylase which could be exploited for the development of holistic therapeutic strategy for the control of postprandial hyperglycemia, T2D and related complications while phenolics are the main bioactive agents responsible for the observed activities investigated in the study. We are currently working on a detailed study on the anti-diabetic activity of the acetone fraction in a T2D diabetes model of rats as well as isolating some pure compounds from it.

Postscript: From the above studies, the acetone fraction had the best α -glucosidase and α -amylase effects and therefore it was selected for detailed *in vivo* anti-diabetic study

4.4 Anti-diabetic activity of the acetone fraction of *Cassia singueana* stem bark in a type 2 diabetes model of rats

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Preface: This article reports the detailed *in vivo* anti-diabetic studies of the acetone fraction and it has been read and corrected by the supervisors. The article is currently under review in the “Journal of Ethnopharmacology”.

4.4.1 Abstract

The present study examined the anti-diabetic activity of the acetone fraction of *Cassia singueana* stem bark (CSAF) in a type 2 diabetes (T2D) model of rats and the enzyme inhibitory activity of 3 β -O-acetyl betulinic acid isolated from the fraction. Rats were randomly allocated into six groups: normal control (NC), diabetic control (DBC), diabetic rats treated with 150 mg/kg bw (DCL) and 300 mg/kg bw (DCH) of CSAF, diabetic rats treated with 300 mg/kg bw of metformin (DMF) and normal rats treated with 300 mg/kg bw of CSAF (NCT). T2D was induced by feeding the rats a 10% fructose solution for two weeks *ad libitum* followed by an intraperitoneal injection of streptozotocin (40 mg/kg b.w). After 4 weeks of intervention, non-fasting blood glucose levels were significantly ($P<0.05$) lowered and the glucose tolerance ability was significantly improved in the DCL and DCH groups compared to the DBC group. Furthermore, serum insulin concentrations, pancreatic β -cell function (HOMA- β) and liver glycogen level were significantly ($P<0.05$) increased while serum alanine transaminase, alkaline phosphatase and urea were significantly decreased in the CSAF treated diabetic rats compared to the DBC group. Other T2D-induced abnormalities ameliorated, though insignificantly ($P>0.05$), by the CSAF treatments in diabetic rats were feed and fluid intakes, body weight changes, serum lipids alterations, serum fructosamine level and peripheral insulin resistance (HOMA-IR) Analysis of 3 β -O-acetyl betulinic acid isolated from CSAF revealed that it is a potent non-competitive inhibitor of α -glucosidase and α -amylase activities. It was concluded that orally administered CSAF could ameliorate most of T2D-induced abnormalities and 3 β -O-acetyl betulinic acid is a possible anti-diabetic agent in the fraction.

4.4.2 Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia which results from anomalies in the secretion of insulin and/or its action [59]. According to a projection of the International Diabetes Federation (IDF), approximately 366 million people are living with diabetes and this figure is

projected to increase to 552 million by the year 2030 [38]. Among the two major types of diabetes, type 2 is more prevalent than type 1, with more than 90% of the total diabetic patients are suffering from it. Type 2 diabetes (T2D) is a heterogeneous disorder characterized by a gradual decline in insulin action (insulin resistance) and subsequent failure of pancreatic β -cells to compensate for the insulin resistance (β -cell dysfunction) which leads to hyperglycemia with oxidative stress being a major contributor to the β -cell damage [60, 61].

Hyperglycemia is a major factor responsible for the development of several chronic complications associated with T2D such as diabetic neuropathy, diabetic retinopathy, diabetic nephropathy, diabetic cardiomyopathy, and diabetic foot diseases [62]. Approximately, 50% of people with T2D are affected with one or more above-mentioned complications [63]. The control of hyperglycemia is therefore of prime importance to impede the disease progression. At present, the use of insulin secretagogues and sensitizers constitutes the predominant line of therapy, however, the use of inhibitors of carbohydrate digesting enzymes in order to reduce the intestinal absorption of sugar is also vital as they do not interfere with the carbohydrate metabolism and help to control hyperglycemia in a noninvasive manner [64]. Alpha glucosidase inhibitors are the current class of inhibitors of intestinal absorption which are shown to control postprandial hyperglycemia. However, the clinically available glucosidase inhibitors, acarbose and miglitol, are frequently found to cause diarrhea and other gastrointestinal side effects such as bloating, flatulence, cramping and abdominal pain [44]. Hence, research on diabetes therapy is focused on the search for alternative agents which could decrease postprandial hyperglycemia and other diabetic complications with fewer or no side effects.

Cassia singueana Delile, also known as golden shower, is a member of the family Caesalpiniaceae and is commonly used by traditional medical practitioners to treat diabetes mellitus [3] as well as bathe nursing mothers. In a previous study, the *in vitro* anti-oxidative potential of the methanolic extract from the leaves was reported [11]. However, in a most recent study, we subjected different solvent crude extracts of the stem bark, root and leaves of the plant to anti-oxidative activity assays using several models and reported that the crude stem bark ethyl acetate extract had the best anti-oxidative activity among all other extracts [48].

In the present study, the ethyl acetate extract was further partitioned across solvents of different polarity and the acetone fraction derived from it was found to have the highest α -glucosidase and α -amylase inhibitory activities (among other solvent fractions). Subsequently, the acetone fraction was subjected to a detailed anti-diabetic study in a T2D model of rats.

4.4.3 Materials and methods

Please see chapter two sub-sections 2.3-2.4 (pages 35-36); 2.6.1-2.6.3 pages 38-39; 2.7.1-2.7.7 pages 40-43; and 2.8.4 (page 46) for detailed material and methods that affect *C. singueana*. However, the group codes DFL, DFH and NFT used in the chapter two are replaced with DCL, DCH and NCT respectively. CSAF refers to the acetone fraction derived from the ethyl acetate extract of *C. singueana* stem bark

4.4.4 Results

Analysis of the α -glucosidase and α -amylase inhibitory effects of the fractions revealed that the more polar fractions demonstrated significantly ($P<0.05$) higher inhibitory effects than the less polar fractions. However, within the more polar fractions, the α -glucosidase and α -amylase inhibitory activities demonstrated by the acetone fraction was significantly ($P<0.05$) higher than the other solvent fractions (Table 4.4.1). Based on the high activity of the acetone fraction in most of the assays described above, it was selected for further *in vivo* studies.

Table 4.4.1: IC₅₀ values for the inhibition of α -glucosidase and α -amylase by various solvent fractions of the ethyl acetate extract of *C. singueana* stem bark

Fractions/standard	IC ₅₀ (μ g/ml)	
	α -glucosidase	α -amylase
Aqueous	80.99 \pm 0.97 ^c	157.24 \pm 4.96 ^b
Acetone	67.58 \pm 3.59 ^b	108.36 \pm 2.64 ^a
Dichloromethane	175.59 \pm 1.68 ^d	769.54 \pm 165.71 ^d
Hexane	278.66 \pm 48.64 ^e	NIL
Acarbose	55.59 \pm 5.22 ^a	256.66 \pm 20.52 ^c

The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Values with different alphabets along a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

The mean food and fluid consumption per day per rat over the seven week experimental period is presented in Figure 4.4.1. The food and fluid intakes of the DBC group was significantly higher ($P<0.05$) compared to NC and NCT groups; however the CSAF treated groups consumed an insignificantly ($P>0.05$) lower food and fluid than the DBC group. The fluid intake of DCH group was markedly lower compared to the DBC, DCL and DMF groups.

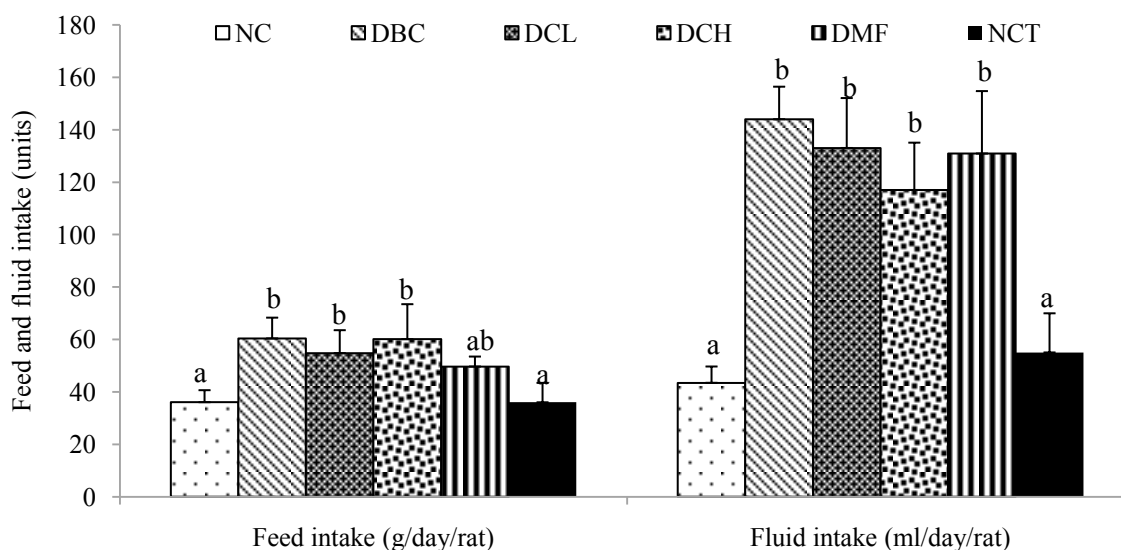


Figure 4.4.1: Food and fluid intake of the different groups during the experimental period. Data are presented as mean \pm SD of eight animals. ^{a-b}Values with different letters over the bars for a given parameter are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

No significant difference in the body weight among the different animal groups during the first 2 weeks of the experiment was observed. However, the body weight gains of the diabetic groups were significantly lower compared to NC and NCT groups during the entire experimental period (**Figure 4.4.2**).

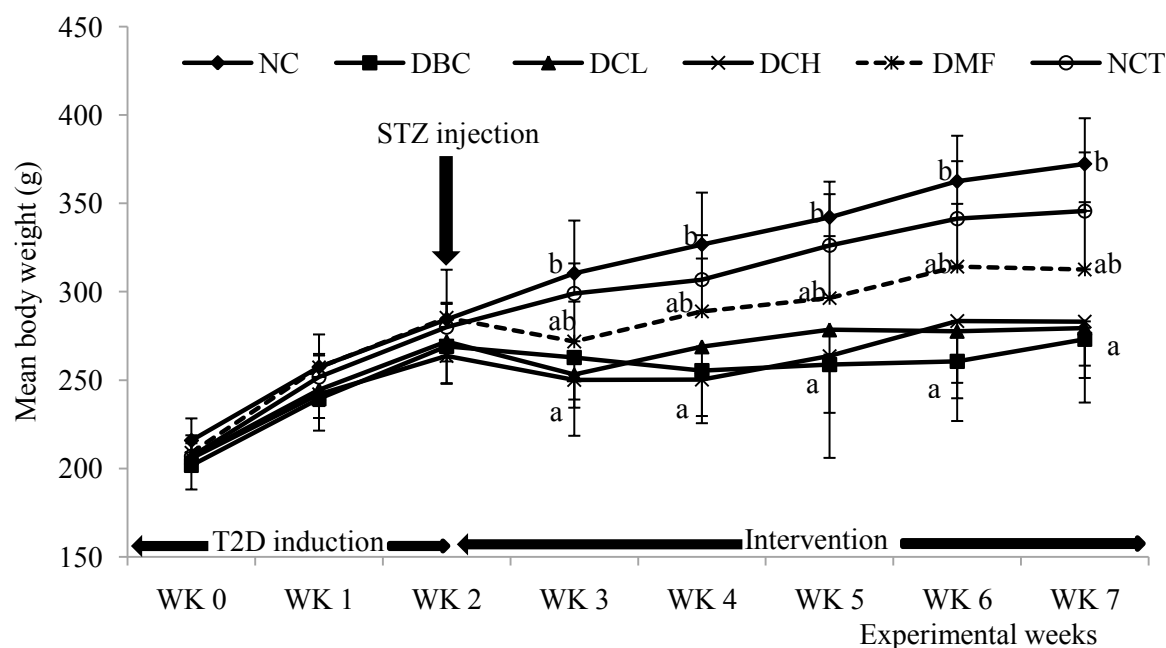


Figure 4.4.2: Mean body weight gain for all groups of experimental animals over the seven weeks experimental period. Data are presented as the mean \pm SD of eight animals. ^{a-b}Values with different letters for a given week are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The blood glucose concentrations of the diabetic groups were significantly higher than the NC and NCT groups at week 1, however, as soon as the intervention started, the DCL and DCH groups maintained a significantly lower ($P<0.05$) blood glucose levels than the DBC group throughout the experimental period (**Figure 4.4.3**). The blood glucose lowering effect of the fraction seems to be dose dependent because the DCH had a relatively lower blood glucose levels than DCL throughout the intervention period. These results also suggest the significant effects of CSAF on both non-fasting (week 1-4) and fasting blood glucose (week 5) levels compared to DBC group (**Figure 4.4.3**).

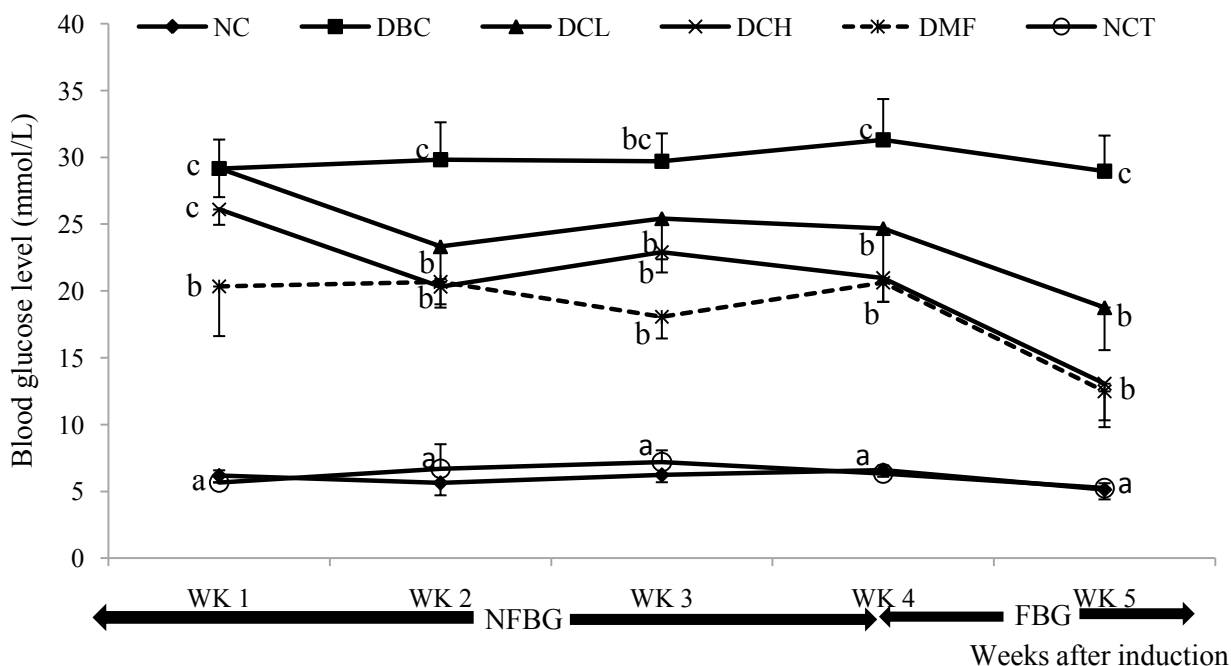


Figure 4.4.3: Weekly blood glucose concentrations (post induction) of different animal groups. Data are presented as the mean \pm SD of eight animals. ^{a-c}Values with different letters for a given week are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The data for OGTT are shown in **Figure 4.4.4**. The glucose tolerance abilities of DCL and DCH groups were significantly ($P<0.05$) better than the DBC group during the entire experimental period. Even better glucose tolerance was observed for DCH group compared to the metformin consuming DMF group at 120 min point of the glucose tolerance test. The glucose tolerance ability of DCH group was also significantly better than the DCL group at almost the entire period of the test.

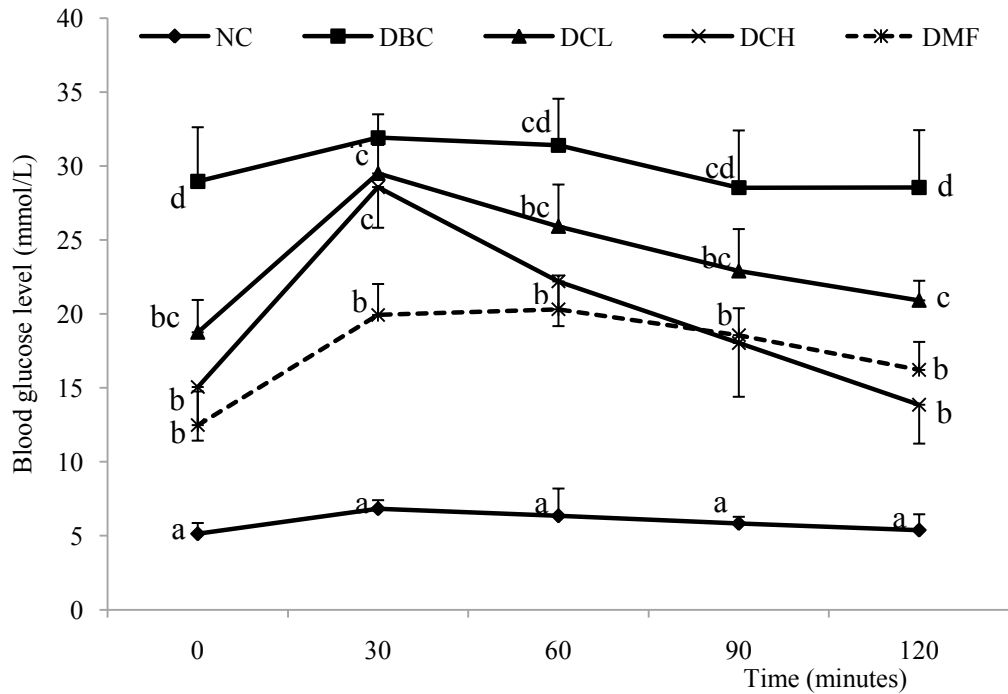


Figure 4.4.4: Oral glucose tolerance test (OGTT) for all groups of animals in the last week of the experimental period. Data are presented as the mean \pm SD of eight animals. ^{a-d}Values with different letters for a given time are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 4.4.2 presents the data for serum insulin and fructosamine concentrations as well as the computed HOMA-IR and HOMA- β scores. A significantly ($P < 0.05$) higher serum insulin concentration and better β -cell function (HOMA- β) were recorded in the DCL and DCH groups in comparison to DBC group. However, the serum fructosamine levels and insulin resistance (HOMA-IR) were markedly decreased in the DCH group in comparison to the DBC group (**Table 4.4.2**).

Table 4.4.2: Serum insulin and fructosamine concentrations as well as HOMA-IR and HOMA- β scores at the end of the experimental period

	NC	DBC	DCL	DCH	DMF	NCT
Serum insulin (pmol/L)	153.1 \pm 9.9 ^b	64.2 \pm 29.9 ^a	119.7 \pm 17.8 ^b	138.3 \pm 12.6 ^b	119.2 \pm 13.0 ^b	140.4 \pm 17.8 ^b
Serum fructosamine (μ mol/L)	196.3 \pm 5.6 ^a	258.4 \pm 10.9 ^b	268.4 \pm 14.1 ^b	214.4 \pm 28.9 ^{ab}	253.8 \pm 30.4 ^b	185.0 \pm 8.4 ^a
HOMA-IR	4.9 \pm 1.1 ^a	16.2 \pm 5.7 ^b	13.0 \pm 5.7 ^b	13.4 \pm 4.9 ^b	9.8 \pm 3.8 ^b	4.6 \pm 0.7 ^a
HOMA- β	304.1 \pm 53.7 ^d	9.68 \pm 5.2 ^a	20.3 \pm 4.7 ^b	32.8 \pm 16.7 ^{bc}	54.9 \pm 27.5 ^c	232.4 \pm 55.5 ^d

Data are presented as the mean \pm SD of eight animals. ^{a-d}Values with different letters along a row are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The results for liver weights and liver glycogen levels are presented in **Table 4.4.3** and there were no significant differences (P>0.05) in the liver weights of all groups of experimental rats but the relative liver weights of the diabetic groups were significantly higher than NC and NCT groups. The DCL, DCH and DMF groups had insignificantly (P>0.05) lower relative liver weights in comparison to the DBC group. A significantly higher liver glycogen contents were detected in the NC, DCL, DCH and DMF groups compared to DBC and NCT groups.

Table 4.4.3: Liver weights and liver glycogen concentrations in different animal groups at the end of the experimental period

	NC	DBC	DCL	DCH	DMF	NCT
Liver weight (g)	11.8 \pm 2.4	10.9 \pm 1.9	10.6 \pm 1.2	9.9 \pm 0.8	10.4 \pm 1.7	10.8 \pm 1.4
Relative liver weight (%)	3.3 \pm 0.3 ^a	3.9 \pm 0.2 ^b	3.8 \pm 0.1 ^b	3.8 \pm 0.2 ^b	3.7 \pm 0.1 ^b	3.1 \pm 0.1 ^a
Liver glycogen (mg/g tissue)	3.5 \pm 0.8 ^c	1.54 \pm 0.1 ^a	3.2 \pm 0.2 ^{bc}	2.7 \pm 0.2 ^b	2.7 \pm 0.4 ^b	1.5 \pm 0.6 ^a

Data are presented as the mean \pm SD of eight animals. ^{a-c}Values with different letters along a row are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The concentrations of the serum lipids are presented in **Figure 4.4.5**. Although there were no significant differences in the concentrations of total cholesterol and LDL cholesterol among the experimental groups, the DBC had relatively higher values which were reduced in the DCL and DCH groups. The DBC group also recorded a significantly lower level of HDL cholesterol compared to the DCL and DCH groups. Furthermore, T2D caused a significant increase in the serum triglycerides which was ameliorated in the DCL, DCH and DMF groups.

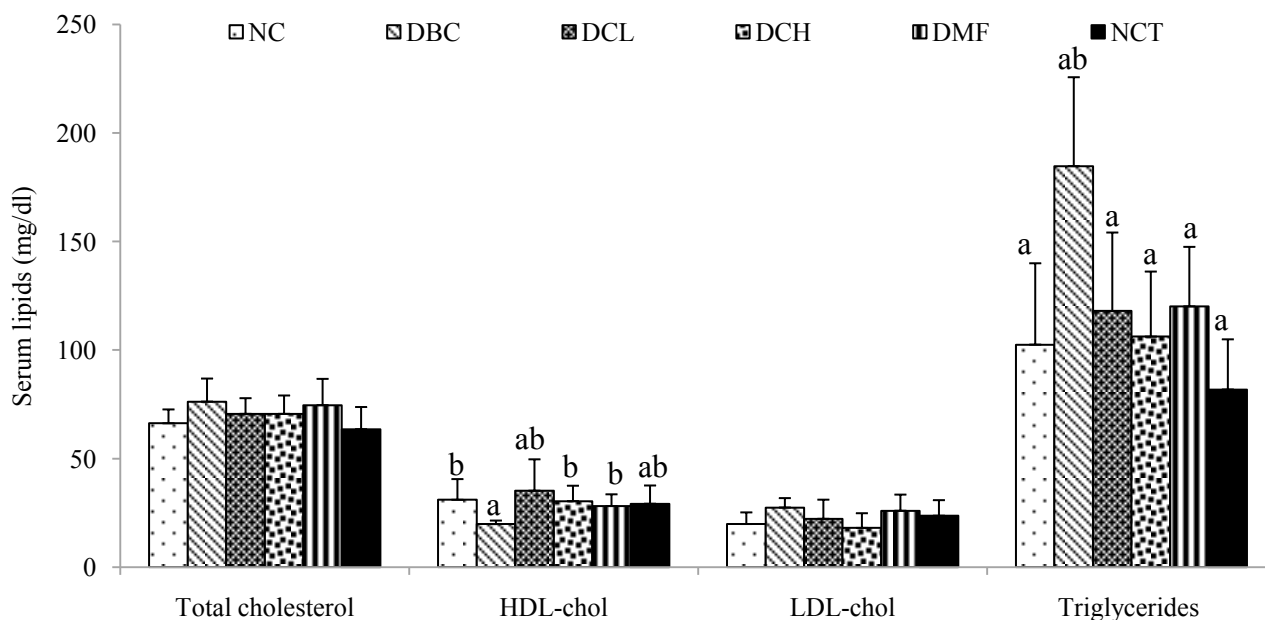


Figure 4.4.5: The serum lipid profile in different animal groups at the end of the experimental period. Data are presented as the mean \pm SD of eight animals. ^{a-b}Values with different letters for a given parameter are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The data for serum AST, ALT, ALP, urea and creatinine concentrations are presented in **Table 4.4.4**. The serum levels of ALT, ALP and urea were significantly elevated in the DBC group compared to NC group but the DCL, DCH and DMF groups recorded significantly lower values of these biochemical parameters when compared to the DBC group (**Table 4.4.4**). Conversely, relatively higher serum levels of creatinine was observed in the DMF group compared to the DBC group whereas no significant difference was observed for serum AST levels among all experimental groups.

Table 4.4.4: Serum biochemical parameters for all groups of animals at the end of experimental period

	NC	DBC	DCL	DCH	DMF	NCT
AST (U/L)	88.0±14.9	73.6±7.6	78.3±4.9	76.2±3.6	88.7±17.9	86.0±13.7
ALT (U/L)	56.3±12.6 ^a	77.8±4.6 ^b	64.6±9.2 ^a	62.3±9.3 ^a	63.3±18.2 ^a	48.5±15.4 ^a
ALP (U/L)	188.8±19.9 ^b	898.6±174.5 ^d	320.4±25.8 ^c	344.6±45.4 ^c	472.0±74.9 ^c	117.5±12.5 ^a
Urea (mg/dl)	48.0±6.2 ^b	93.0±16.4 ^c	69.2±10.7 ^{bc}	78.0±10.9 ^{bc}	42.0±11.8 ^{ab}	30.2±4.5 ^a
Creatinine (µg/dl)	582.0±70.5 ^{ab}	485.0±40.4 ^{ab}	570.0±104.6 ^{ab}	545.0±84.1 ^{ab}	675.0±95.2 ^b	470.0±67.8 ^a

Data are presented as the mean ± SD of eight animals. ^{a-d}Values with different letters along a row are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

In the histopathological examination of the pancreas, the DBC group had a reduced number of β -cells which were highly dispersed compared with the NC group. Higher number of β -cells was observed in the DCL and DMF groups compared to the DBC group although the size of the islets were morphologically smaller in size compared to the NC group (**Figure 4.4.6**).

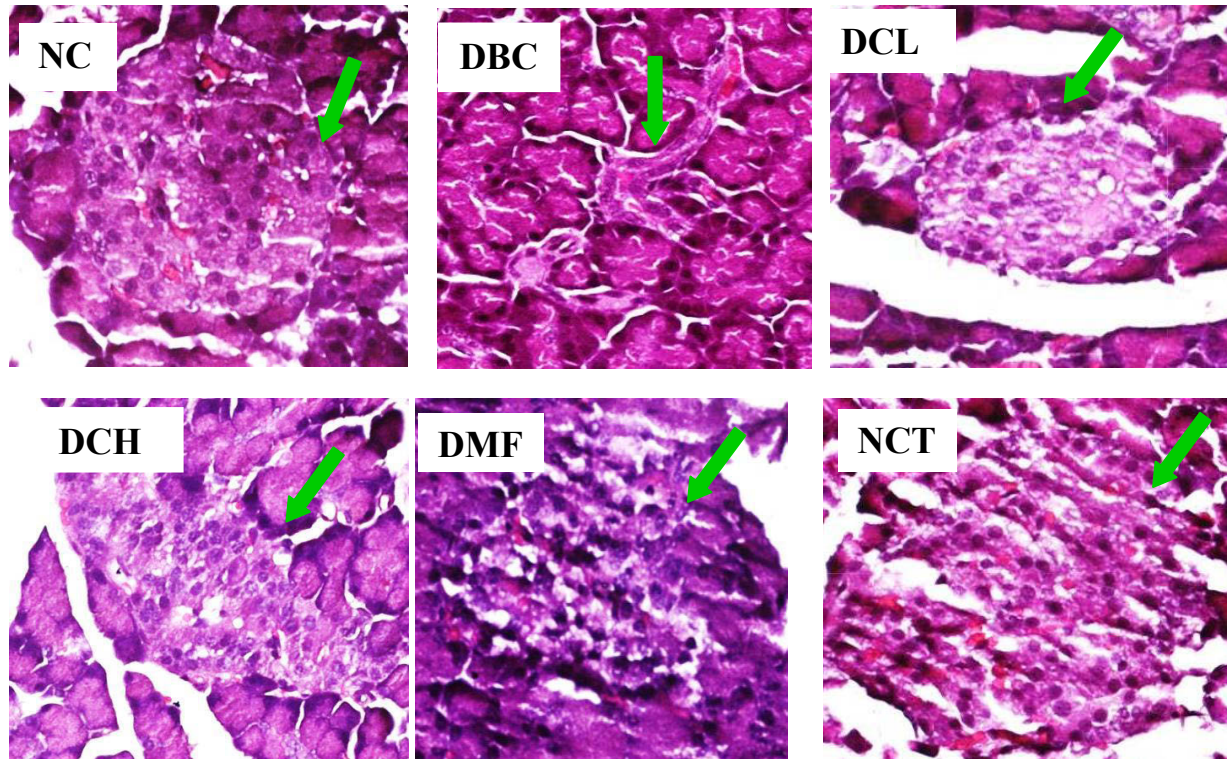


Figure 4.4.6: Representative histopathological examinations of the pancreas of different experimental groups at the end of the experiment. The NC had high number of β -cells while the DBC had highly dispersed and morphologically deformed β -cells. The DCL, DCH and DMF groups had higher number of β -cells (compared to DBC) but morphologically smaller whereas relatively reduced number of β -cells (compared to NC) but not morphologically deformed.

The structure of the active compound was determined as 3 β -O-acetyl betulinic acid (**Figure 4.4.7**) from its ^1H and ^{13}C NMR data (Appendix 1 and 2) and by comparison of the data with the data reported in the literature [65]. Analysis of α -glucosidase and α -amylase inhibitory activity of 3 β -O-acetyl betulinic acid revealed that the compound is a potent inhibitor of α -glucosidase and α -amylase activity with IC_{50} values of 45.12 ± 3.47 and 98.52 ± 2.816 $\mu\text{g/ml}$ respectively. Furthermore, enzyme kinetics studies revealed that 3 β -O-acetyl betulinic acid exerts a non-competitive inhibition pattern on both enzymes thereby decreasing the V_{max} of the enzymes while the K_{M} remained unchanged (**Figure 4.4.8**). The computed inhibition binding constants (K_i) were 5.56 and 3.76 $\mu\text{g/ml}$ for α -glucosidase and α -amylase inhibitions respectively.

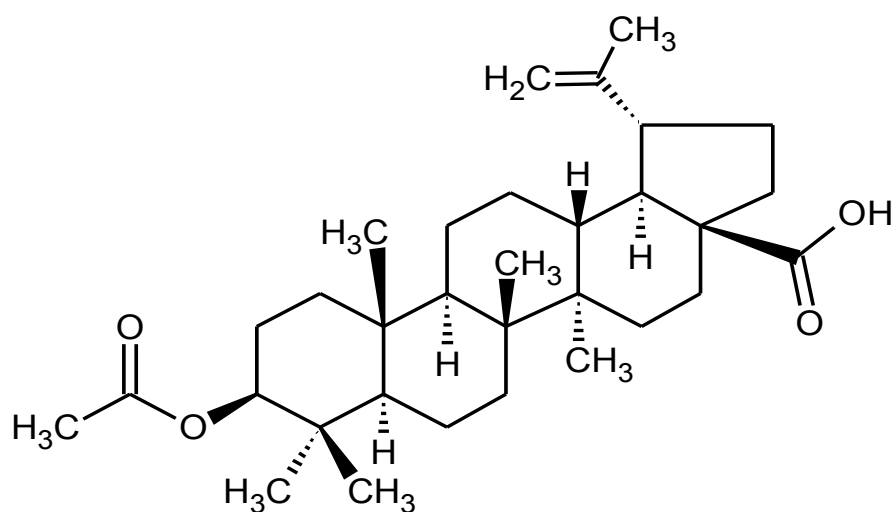


Figure 4.4.7: The structure of 3 β -O-acetyl betulinic acid isolated from the acetone fraction of *Cassia singueana* stem bark

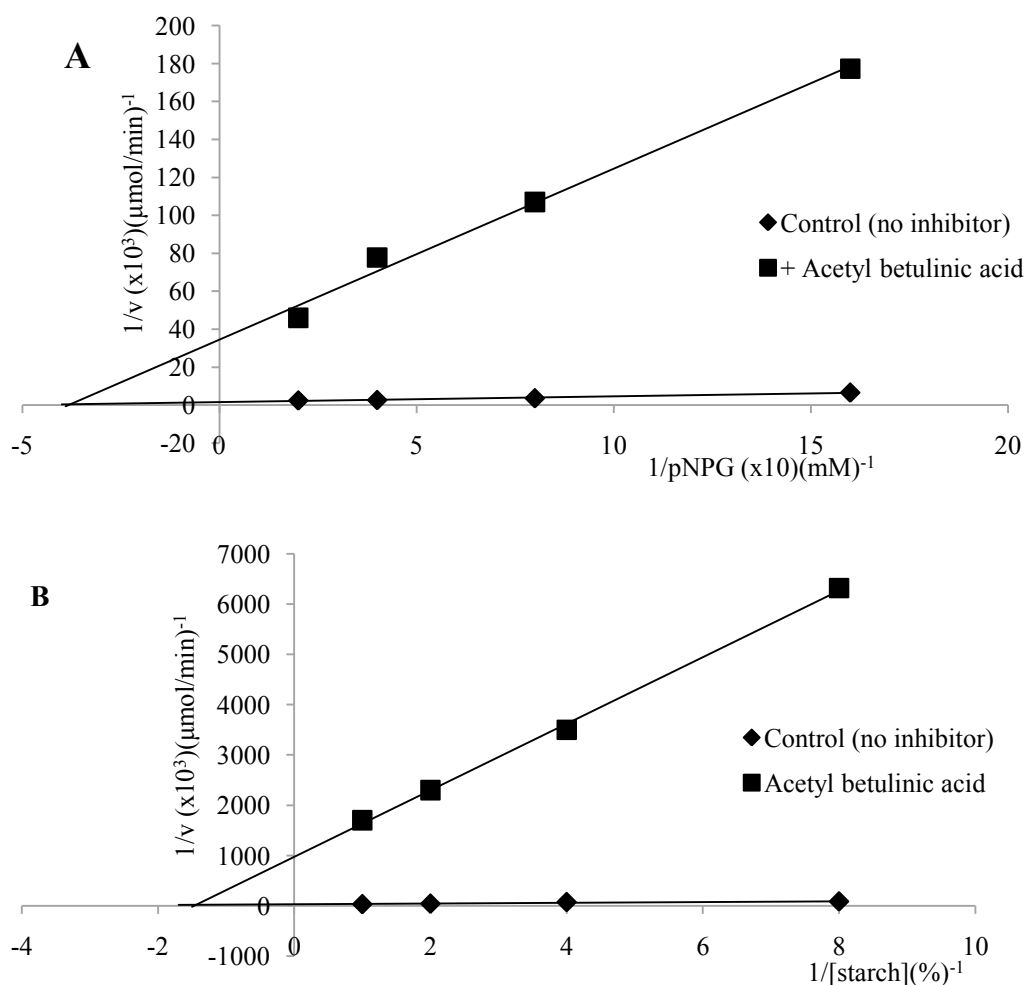


Figure 4.4.8: Lineweaver-Burke's plot for α -glucosidase (A) and α -amylase (B) catalyzed reactions in the presence and absence of 3 β -O-acetyl betulinic acid as an inhibitor

4.4.5 Discussion

The development of anti-diabetic agents that are devoid of adverse effects is still a challenge to the health care systems globally. Thus, medicinal plants are constantly being explored with the hope of developing a relatively safe antidiabetic plant-based product alone or in combination with other agents [66]. In the present study, we reported the anti-diabetic activity of an acetone fraction of the stem bark of *Cassia singueana* in a newly developed model of T2D. *Cassia singueana* is used for the traditional remedy of diabetes mellitus and the acetone fraction was selected for the study based on the higher α -glucosidase and α -amylase inhibitory activity showed by the fraction as compared to other solvent fractions.

Polyphagia and polydipsia with concomitant reduction of body weight are major symptoms of diabetes mellitus [67] which were also evidently observed in the diabetic groups of our experiment. These

parameters are usually dependent on energy expenditure, urinary excretion and catabolic processes among others. Treatment with the fraction did not completely reverse the T2D-induced polyphagia, polydipsia and weight loss (**Figures 4.4.1 and 4.4.2**) possibly because the experimental period was short for the reversal of these parameters due to their dependency on other metabolic parameters.

Fasting or postprandial hyperglycemia is a common pathogenesis of T2D which is induced by insulin resistance and partial pancreatic β cell destruction [68]. Effective control of the blood glucose concentration is a vital step in ameliorating diabetic complications and enhancing the quality of life in type 2 diabetic patients [69]. In the present study, oral treatment of diabetic animals with the CSAF resulted in a significant, consistent and dose dependent decrease in blood glucose levels throughout the experimental period, indicating its potent anti-diabetic activity. Furthermore, in the OGTT analysis, better glucose tolerance abilities were observed in the CSAF treated groups than the DBC group. These results could be linked to the potent α -glucosidase and α -amylase inhibitory activity exhibited by the fraction which could cause a decrease in the digestion of carbohydrates and intestinal absorption of sugar [70]. On the other hand, the CSAF stimulated insulin secretions and improved pancreatic β -cell function whereas insulin resistance was not affected. Based on the foregoing observations, it is logical to suggest that the mechanism of anti-T2D action of the CSAF is elicited through delaying glucose absorption, improving β cell function and stimulating insulin secretions rather than by increasing insulin sensitivity. Interestingly, the above hypothesis was further supported by the histopathological examinations of the pancreatic islets where CSAF groups had more as well as healthier pancreatic islets than the DBC group. The CSAF was found to have high *in vitro* anti-oxidative activity (data not shown) and thus, we speculated that the protection of the pancreatic islets could be mediated through an anti-oxidative dependent mechanism because oxidative stress is an important biological phenomenon in the process of pancreatic β -cell damage in T2D [71]. The serum fructosamine level is a measure of early protein glycation which is formed by the covalent binding of glucose to serum proteins via a non-enzymatic glycation reaction [72, 73]. It predicts the overall metabolic control of diabetes over the preceding 2–3 weeks. Although, the result was not significant, CSAF treatments tended to ameliorate the T2D-induced elevation in serum fructosamine level especially in the DCH group.

Previous studies reported contradictory findings on the effect of diabetes on liver weights. Some workers have shown an increase in hepatic weight in animals as well as humans while others have reported no change [74]. Our findings revealed that relative but not absolute liver weights were elevated by the disease and was not significantly reversed by the treatments. Hepatic glycogenesis is associated with extracellular glucose concentration and insulin availability. *In vivo* glycogen metabolism is regulated by a multifunctional enzyme glycogen synthase and glycogen phosphorylase [75]. Reduced hepatic glycogen reserve in diabetic animals has been attributed to reduced glycogen synthase activity and increased

glycogen phosphorylase activity. Thus, the ability of the CSAF to restore the depleted glycogen reserves in diabetic animals could indicate that the fraction also decreases the activity of glycogen phosphorylase and/or increases glycogen synthase activity. This could further suggest that the fraction not only acts as insulin secretagogue but also by inhibiting hepatic glycogenolysis thereby reducing an upsurge in blood glucose concentrations.

Profound changes in the serum lipid and lipoprotein profile with an increased risk in cardiovascular diseases is associated with T2D. Hyperlipidemia is a well-known complication in diabetic patients characterized by increased cholesterol and triglycerides levels as well as changes in lipoprotein composition [76]. This hyperlipidemia is mainly as a result of uncontrolled actions of lipolytic hormones on the fat depots that arise from the impairment of insulin secretions in the diabetic state. In our study, serum total and LDL-cholesterols were relatively elevated but triglycerides were significantly increased in the untreated diabetic rats. Interestingly, the CSAF treated diabetic groups had relatively lower serum lipid concentrations than the untreated control for these lipid parameters. This observation is possibly linked to the stimulation of insulin secretion by the fraction which could modulate the actions of the lipolytic hormones in fat reserves.

The serum levels of ALT and ALP but not AST were significantly increased in the untreated diabetic animals indicating impaired liver function, which is obviously due to hepatocellular necrosis. Diabetic complications such as increased ketogenesis and gluconeogenesis may be due to elevated aminotransferase activities [77]. Therefore, restoration of these biomarker enzymes towards normal level indicates decreased diabetic complications in the CSAF treated groups. Furthermore, the fraction reversed the T2D-induced increase in serum urea level which could suggest decreased renal impairments associated with diabetic complications. Apart from assessing renal damage, creatinine was reported to be a predictor for T2D and insulin resistance [78]. Creatinine is a product of creatine metabolism which mostly occur in skeletal muscle, the major center for the action of insulin and subsequent glucose disposal. Muscle mass has been proposed to inversely correlate with insulin resistance but directly correlate to serum creatinine. Thus, the serum creatinine could be a reliable marker to assess insulin resistance [78]. Thus, previous studies have demonstrated lower serum creatinine level in T2D human subjects compared to normal individuals [78, 79] which corroborates with our findings. It is thus possible to suggest that the insignificant decrease in the insulin resistance (HOMA-IR) of DCL and DCH groups compared to the DBC group also led to an insignificant elevation in the serum creatinine levels in these groups.

In order to investigate the possible bioactive compounds that might play a role in eliciting the above-mentioned anti-diabetic activity of CSAF, we isolated 3 β -O-acetyl betulinic acid and subjected it to *in vitro* α -glucosidase and α -amylase inhibitory assays. Results from the studies indicated that 3 β -O-acetyl

betulinic acid isolated from CSAF is a potent inhibitor of α -glucosidase and α -amylase which is mediated through non-competitive patterns for both enzymes. This indicates that 3 β -O-acetyl betulinic acid binds α -glucosidase and α -amylase at separate site(s) of the enzyme (rather than the active sites) but caused conformational modification at the active sites thereby preventing effective binding of the substrates. This type of inhibition could further suggest that the compound could interact with the enzyme-substrate complexes [57].

In summary, data from the study suggest that the acetone fraction of the stem bark of *C. singueana* has a strong potent anti-T2D activity which could also ameliorate other diabetes-related complications. The mechanism of action is mediated through inhibition of carbohydrate-hydrolysing enzymes, modulation of β -cell function and stimulation of insulin secretions while 3 β -O-acetyl betulinic acid is a possible bioactive anti-T2D agent in the fraction.

Postscript: In order to further explore the possible mechanism of anti-diabetic action of the fraction, the serum, liver, kidney, heart and pancreas were investigated for *in vivo* anti-oxidative parameters

4.5 Modulation of *in vivo* anti-oxidative status by the acetone fraction of *Cassia singueana* stem bark in a type 2 diabetes model of rats

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Preface: This article describes the *in vivo* anti-oxidative status of the serum and organs collected from the rats in the above experiment.

4.5.1. Abstract

The effects of oral administration of the acetone fraction derived from the ethyl acetate extract of *Cassia singueana* stem bark (CSAF) on the oxidative stress parameters of the serum and organs of type 2 diabetic rats were investigated. Type 2 diabetes was found to induce oxidative stress in the serum, liver, kidney, heart and pancreas as evidenced by the significant ($P < 0.05$) increase in the levels of thiobarbituric acid reactive substances (TBARS) and significant ($P < 0.05$) decreases in the levels of glutathione, superoxide dismutase (SOD) and catalase. However, treatment with CSAF ameliorated T2D-changes in all these parameters for the serum and organs under investigation. The effects of the CSAF on this parameters was dose dependent as treatment with a high dose of 300 mg/kg bw recorded a significant ($P < 0.05$) effect for all the parameters except the TBARS level of the heart whereas low dose (150 mg/kg bw) treatment achieved less potent effects. It was concluded that the CSAF contains phytochemicals that could modulate oxidative stress in type 2 diabetic rats.

4.5.2. Introduction

Reactive oxygen species are products of aerobic metabolism which are continuously produced under normal metabolic conditions but become deleterious when overproduced or the enzymatic and non-enzymatic anti-oxidative defense system is attenuated leading to a process called oxidative stress [80]. Chronic hyperglycemia is a hallmark in the pathophysiology of diabetes mellitus and is implicated as a vital contributory factor in the initiation and progression of most of the secondary complications associated with the disease [62]. In type 2 diabetes (T2D), hyperglycemia induces oxidative stress via multiple pathways which include glucose autooxidation, increased metabolic flux of the polyol (sorbitol) pathway, activation of protein kinase C, increased generation of advanced glycation end products, activation of protein kinase C and increased hexosamine pathway flux [40, 81]. The oxidative stress is thus considered as an important unifying mechanism that plays major roles in all aspects of T2D pathology that include pancreatic β cell dysfunction, microvascular and macrovascular complications [39,

40, 82]. Thus, anti-oxidative agents, which are able to modulate the hyperglycemia-induced oxidative stress, are beneficial, and anti-diabetic drugs with anti-oxidative potential would certainly have a higher therapeutic value.

Cassia singueana Delile, also known as golden shower, is a member of the family Caesalpiniaceae and is commonly used by African traditional medical practitioners to treat several diseases [1]. In northern Nigeria, the plant is commonly exploited for the traditional management of diabetes mellitus [3] as well as bathes nursing mothers. In a previous study, we subjected different solvent crude extracts of the stem bark, root and leaves of the plant to anti-oxidative activity assays using several models and reported that the ethyl acetate extract of the stem bark had the highest anti-oxidative activity among all other extracts [48]. Subsequently, we subjected the ethyl acetate extract of the stem bark to solvent-solvent fractionation and found the acetone fraction (among other solvent fractions) had the best *in vitro* α -glucosidase and α -amylase inhibitory activities as well as significant anti-hyperglycemic and insulinotropic effects in a T2D model of rats (see above article 4.4.). Furthermore, the fraction was found to ameliorate most of the T2D-induced alterations in metabolic parameters including some indices of T2D complications. It is thus possible that the fraction exerts its actions, wholly or in part, via an anti-oxidative dependent mechanism. Therefore, in the present study, the effects of the fraction on the anti-oxidative status of the serum, liver, kidney, heart and pancreas of type 2 diabetic rats were investigated considering the crucial role of oxidative stress in the progression and complications of T2D.

4.5.3 Materials and methods

See Chapter two sub sections 2.3-2.4 pages (34-36); 2.7.1-2.7.5 (pages 41-42); and 2.7.8 (2.7.8.1-2.7.8.4) pages 44-45 for details that affect *C. singueana*.

4.5.4 Results

The T2D was found to significantly ($P<0.05$) increase the TBARS levels and significantly decrease the glutathione, SOD and catalase levels in the liver, kidney, heart, pancreas and serum. However, the CSAF treatment ameliorated these T2D-induced changes in the liver. The ameliorative effects seem to be dose-dependent because all the changes were significant ($P<0.05$) in the DCH group (**Table 4.5.1**). Also, the CSAF treatment significantly ($P<0.05$) boosted the kidney level of glutathione and catalase but insignificantly ($P>0.05$) reduced the disease-induced increase in kidney TBARS level. The SOD level was also boosted in the kidney of the CSAF-treated diabetic rats but the effect was only significant in the DCH group (**Table 4.5.2**).

Table 4.5.1: Effects of CSAF on hepatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DCL	DCH	DMF	NCT
TBARS (μM)	10.6 \pm 3.0 ^a	33.9 \pm 4.9 ^b	31.7 \pm 7.7 ^b	14.8 \pm 4.1 ^a	35.9 \pm 9.5 ^b	16.9 \pm 3.3 ^a
Glutathione (μM)	120.8 \pm 6.0 ^c	29.9 \pm 9.3 ^a	82.6 \pm 17.6 ^b	86.7 \pm 29.9 ^b	73.6 \pm 10.2 ^b	103.1 \pm 26.6 ^{bc}
SOD (nmol/min)	25.5 \pm 5.3 ^b	12.4 \pm 1.6 ^a	14.9 \pm 4.6 ^{ab}	20.5 \pm 3.9 ^b	17.5 \pm 5.2 ^{ab}	23.2 \pm 7.0 ^b
Catalase($\mu\text{mol/min}$)	16.5 \pm 0.9 ^c	1.5 \pm 0.3 ^a	6.0 \pm 1.8 ^b	9.3 \pm 1.9 ^b	1.7 \pm 0.6 ^a	7.4 \pm 5.9 ^{ab}

The results are expressed as the mean \pm SD of five animals. ^{a-c}Data with different alphabets along a row are significantly different (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 4.5.2: Effects of CSAF on kidney lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DCL	DCH	DMF	NCT
TBARS (μM)	14.1 \pm 5.8 ^a	40.8 \pm 12.5 ^b	27.1 \pm 9.9 ^{ab}	21.5 \pm 6.0 ^{ab}	33.4 \pm 10.0 ^b	18.6 \pm 6.2 ^{ab}
Glutathione (μM)	68.2 \pm 20.8 ^{ab}	46.0 \pm 8.6 ^a	92.7 \pm 23.1 ^b	69.9 \pm 9.3 ^b	70.8 \pm 10.4 ^b	52.9 \pm 3.9 ^a
SOD (nmol/min)	16.1 \pm 3.4 ^b	6.8 \pm 1.5 ^a	9.2 \pm 2.7 ^{ab}	11.2 \pm 2.2 ^b	11.5 \pm 4.4 ^{ab}	16.7 \pm 5.8 ^b
Catalase ($\mu\text{mol/min}$)	1.7 \pm 0.3 ^b	0.9 \pm 0.2 ^a	1.8 \pm 0.6 ^b	2.2 \pm 0.5 ^b	1.6 \pm 0.4 ^b	1.4 \pm 0.8 ^{ab}

The results are expressed as the mean \pm SD of five animals. ^{a-c}Data with different alphabets along a row are significantly different (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The data for the effects of the CSAF treatment on the heart oxidative stress parameters of type 2 diabetic rats also indicated that the treatment significantly (P<0.05) ameliorated the T2D-induced changes in heart TBARS level as well as glutathione and SOD levels. The catalase level of the heart was boosted compared to the DBC group but the effect was only significant (P<0.05) in the DCH group (**Table 4.5.3**).

Table 4.5.3: Effects of CSAF on lipid peroxidation and anti-oxidant status in the heart of type 2 diabetic rats

	NC	DBC	DCL	DCH	DMF	NCT
TBARS (μM)	9.4 \pm 2.6 ^a	24.6 \pm 8.8 ^b	24.2 \pm 7.7 ^b	20.7 \pm 7.4 ^b	33.9 \pm 5.9 ^b	9.1 \pm 4.6 ^{ab}
Glutathione (μM)	122.2 \pm 22.0 ^c	48.6 \pm 14.6 ^a	77.3 \pm 13.4 ^b	70.1 \pm 2.8 ^b	79.2 \pm 18.3 ^{ab}	109.9 \pm 5.9 ^c
SOD (nmol/min)	15.2 \pm 4.0 ^b	5.8 \pm 1.2 ^a	10.2 \pm 2.6 ^b	10.5 \pm 2.0 ^b	12.8 \pm 2.0 ^b	13.5 \pm 3.6 ^b
Catalase($\mu\text{mol/min}$)	2.1 \pm 0.5 ^b	0.9 \pm 0.2 ^a	1.4 \pm 0.6 ^{ab}	1.8 \pm 0.4 ^b	1.4 \pm 0.4 ^{ab}	1.9 \pm 0.5 ^b

The results are expressed as the mean \pm SD of five animals. ^{a-c}Data with different alphabets along a row are significantly different (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The pancreatic levels of SOD and catalase were also significantly (P<0.05) higher in the DCL and DCH groups than the DBC group but the pancreatic glutathione level was significantly (P<0.05) higher in the DCH group and relatively higher in the DCL group in comparison to the DBC group (**Table 4.5.4**).

Table 4.5.4: Effects of CSAF on pancreatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DCL	DCH	DMF	NCT
TBARS (μM)	6.0 \pm 1.2 ^a	35.7 \pm 9.1 ^c	24.3 \pm 6.3 ^{bc}	16.2 \pm 3.8 ^b	18.4 \pm 4.4 ^b	10.1 \pm 4.2 ^{ab}
Glutathione (μM)	80.0 \pm 8.1 ^d	6.2 \pm 3.5 ^a	7.5 \pm 1.2 ^a	22.9 \pm 7.9 ^b	37.3 \pm 8.6 ^{bc}	43.9 \pm 11.2 ^c
SOD(nmol/min)	2.6 \pm 0.5 ^c	0.4 \pm 0.2 ^a	0.8 \pm 0.2 ^b	1.2 \pm 0.4 ^{bc}	1.0 \pm 0.5 ^{bc}	2.0 \pm 0.7 ^c
Catalase ($\mu\text{mol/min}$)	0.7 \pm 0.2 ^b	0.35 \pm 0.04 ^a	0.81 \pm 0.29 ^b	0.9 \pm 0.2 ^b	1.11 \pm 0.2 ^b	0.9 \pm 0.3 ^b

The results are expressed as the mean \pm SD of five animals. ^{a-c}Data with different alphabets along a row are significantly different (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 4.5.5 presents the modulatory effects of the CSAF treatment on the serum oxidative stress parameters of type 2 diabetic rats. Although the CSAF treatment was able to prevent the T2D-induced changes in all the parameters but significant results were only observed for glutathione and SOD levels in both DCL and DCH groups. The T2D-induced changes in TBARS and catalase levels were significantly ($P < 0.05$) reversed in the DCH group only.

Table 4.5.5: Effects of CSAF on serum lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DCL	DCH	DMF	NCT
TBARS (μM)	63.0 \pm 15.2 ^a	201.2 \pm 19.4 ^c	138.1 \pm 48.2 ^{bc}	134.4 \pm 26.4 ^b	142.0 \pm 32.0 ^b	66.3 \pm 22.7 ^a
Glutathione (μM)	84.5 \pm 7.7 ^e	19.9 \pm 2.5 ^a	30.7 \pm 7.2 ^b	42.3 \pm 10.7 ^{bc}	65.2 \pm 6.8 ^d	54.1 \pm 3.8 ^c
SOD (nmol/min)	18.1 \pm 2.4 ^d	0.9 \pm 0.2 ^a	2.1 \pm 0.7 ^b	4.3 \pm 0.5 ^c	3.2 \pm 0.7 ^{bc}	12.1 \pm 3.4 ^d
Catalase ($\mu\text{mol/min}$)	3.6 \pm 0.8 ^c	1.5 \pm 0.4 ^a	1.9 \pm 0.4 ^{ab}	2.9 \pm 0.8 ^b	2.4 \pm 0.5 ^b	3.1 \pm 0.6 ^{bc}

The results are expressed as the mean \pm SD of five animals. ^{a-d}Data with different alphabets along a row are significantly different (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

4.5.5 Discussion

The anti-oxidative status of the serum and the various organs of the CSAF treated diabetic rats were investigated to determine, whether or not, the anti-diabetic effects of the fraction in various organs was elicited through an anti-oxidative-related phenomenon.

Insulin resistance and hepatic and renal damages are established features of T2D and were linked to the induction of oxidative stress during the disease [41, 83]. The CSAF treatment was able to reduce the degree of insulin resistance and the organs damages (see manuscript 4.4 above) as well as alleviate the T2D-induced oxidative stress in these organs. Based on the above statements, it is logical to deduce, although without a correlation analysis, that the effects of the CSAF on the insulin resistance and liver and kidney damages is related to the modulatory effects of the fraction on the anti-oxidant status.

Cardiomyopathy and other cardiovascular complications associated with T2D are common secondary diabetic complications that are linked to oxidative stress in numerous studies [84, 85]. Therefore, agents that could impede the oxidative stress process in the heart during diabetes would ultimately be beneficial to the patients. Interestingly, the CSAF treatment was able to boost the anti-oxidative status in the heart, suggesting its beneficial effects. This could, perhaps, explain our observation on the ability of the CSAF to maintain the weight of the heart to near-normal (data not shown) despite the decrease in the heart weight caused by the diabetes (data not shown).

The observed effects of CSAF on the pancreatic anti-oxidative parameters suggest that the fraction protected the pancreas from oxidative attack which consequently leads to an improvement in the function of the pancreas and the β -cells. This is evident by the higher serum insulin concentrations, improved β cell function and higher number of β -cells recorded in the CSAF treated diabetic rats in comparison to the diabetic control. Thus, considering the crucial role of oxidative stress in the pathogenesis of pancreatic β cell dysfunction, it is possible to surmise that CSAF acts via an anti-oxidative dependent mechanism to prevent pancreatic β cell dysfunction and consequently improved the diabetic condition.

Taken the findings of this study as a whole, it is evident that CSAF could modulate the anti-oxidative status of type 2 diabetic rats and this phenomenon seems to play a role in the anti-diabetic effects of the fraction.

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CHAPTER 5

5.0 THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITIES OF *Parkia biglobosa* IN VITRO AND IN VIVO

5.1.1 *Parkia biglobosa* A. Jacq (Mimosaceae)



Figure 5.1: *Parkia biglobosa* A. Jacq (Mimosaceae); common names: African locust bean tree (English); Dorawa (Hausa, Nigeria); Caoubier African (French); Narehi (Fulani, Nigeria); Mkunde (Swahili, Tanzania). Photo: Mohammed Auwal Ibrahim (2013), Zaria, Nigeria

5.1.2 Background

It is a medium sized tree that make up to 20-30 m height with spreading branches that are usually gnarled. It has thick bark which is usually charred. The leaves are bi-pinnate (up to 36 cm long) with 6-11 pairs of pinnae. The plant produces flowers of about 17 mm long that are well anchored to stamens as well as a carbohydrate-rich yellowish fruit which is commonly consumed. The seeds are embedded in yellowish endocarp and when fermented, they are extensively used as cooking condiment in West Africa under various names such as *dawa dawa* in Nigeria and *afitin* in Benin republic. The habitat of this species lies between 5°N and 15°N from Senegal (west Africa) to southern Sudan (North Africa) and Uganda (East Africa).

5.1.3 Ethnomedicinal uses

Documented ethnobotanical survey reveals that the plant is used to treat various types of wounds, pain and fungal infections in Mali [1]. Also, various parts of the plant are used in the treatment of a number of ailments in Nigeria such as bronchitis, pneumonia, diarrhea, sores and ulcers [2]. Reports from Ghana

reveal that the medicinal uses of the plant include toothache, rheumatism, schistosomiasis, leprosy, fever, burns and skin infections [3]. In an investigation from Burkina Faso, the stem bark is successfully used in the treatment of jaundice, amoebiasis, cough, conjunctivitis and dermatosis in addition to most of the other ailments listed in other reports [4]. Moreover, rural villagers from the Oio region of Guinea Bissau reported that the smoke of the seed capsules of the plant is effective as mosquito repellent [5]. More relevant to this dissertation, ethnopharmacological surveys have indicated that the plant is exploited in the treatment of diabetes mellitus in northern Nigeria [6] and central region of Togo [7].

5.1.4 Biological activities

The most highly investigated biological activity of different parts of *P. biglobosa* is antibacterial activity using different bacteria species. Millogo-Kone et al. [4] studied the activity of extracts from the stem bark of the plant against three species of Shigellae isolated from hospitals in Burkina Faso and found that the hydroalcoholic extract was more active than aqueous extract particularly against *S. dysenteriae*. In a subsequent comparative study, the same research group further reported that the hydroalcoholic extract of the stem bark is more effective than extracts from the leaf against clinical isolates of enterobacteria [8]. In another study, aqueous extract of the root was reported to have a broader spectrum of antibacterial activity than corresponding extracts from other parts [2]. In a more recent study from South Africa, solvent fractions prepared from the crude methanolic extract of the stem bark was reported to possess antimicrobial properties which favourably compared with those of streptomycin [9]. The antivenom activity of the hydroalcoholic extract of the stem bark has also been investigated where it was found to inhibit the pathological effects of *N. nigricolis* venom as well as protected mice from death caused by *E. ocellatus* venom [10]. Preliminary analgesic and anti-inflammatory activities of stem bark extracts was investigated where the hexane extract was found to possess marked analgesic activity in an abdominal writhing test in mice [11]. In an effort to validate the anti-diarrhoeal activity of some plants, Agunu et al. [12] reported 100% protection by water-methanol extract of the plant against castor-oil induced diarrhoea. Conversely, semi ethanolic extracts from different parts of *P. biglobosa* did not induce any changes in corticosteroid secretion in rat [13]. The wound healing potentials of the plant was also validated [14] where the stem bark extracts of the plant were found to promote *in vitro* wound healing via stimulation of fibroblasts. Despite the extensive use of the leaves of this plant in traditional medicine, the only comprehensive investigation performed on this part is its antihypertensive properties of the procyanidin-rich fraction which was reported to cause redox-sensitive endothelium-dependent relaxation involving NO and EDHF in porcine coronary artery [15]. The fermented seed extracts were also investigated for pharmacological activities. For example, Odetola et al. [16] demonstrated the preliminary hypoglycemic effects of the fermented seed extract in an alloxan-induced type 1 diabetes model of rats.

5.1.5 Phytochemistry

Most reports on the phytochemistry of *P. biglobosa* involves preliminary phytochemical screening where alkaloids, tannins, flavonoids, saponins, steroids and glycosides were reported in different extracts derived from the plant [2, 9, 17]. The only detailed phytochemistry study performed on the plant reported the isolation of lupeol, 4-O-methyl-epi-gallocatechin, epi-gallocatechin, epi-catechin 3-O-gallate, long chain ester of trans-ferulic acid as well as an unsaparable mixture of long chain *cis*-ferulates [18].

This study is aimed to comprehensively investigate the anti-diabetic activity of this plant using *in vitro* and *in vivo* models. To accomplish this, various solvent crude extracts of the stem bark, root and leaves of the plant were subjected to detailed *in vitro* anti-oxidative studies (using four models) from where the ethanolic extract of the leaves was found to have the best activity. Subsequently, the ethanolic extract of the leaves was fractionated across various solvents and the fractions were subjected to the anti-oxidative as well as α -glucosidase and α -amylase inhibitory studies where the butanol fraction was found to have the best anti-oxidative and enzymes inhibitory activities. Thus, the butanol fraction was investigated for *in vivo* anti-diabetic activity in type 2 diabetes rats model as well as the possible mechanism of action. A pure bioactive anti-diabetic compound was also isolated from the butanol fraction derived from the ethanolic extract.

5.2 *In vitro* anti-oxidative activities of the various parts of *Parkia biglobosa* and GC-MS analysis of extracts with high activity

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Preface: As the first preliminary study, the *in vitro* anti-oxidative activities of the crude solvents extracts from the stem bark, root and leaves of the plant were investigated in this article. The article has been published in the journal ‘African Journal of Traditional, Complementary and Alternative Medicine’ 2013, Vol. 10 issue 5, page 283-291.

5.2.1 Abstract

The anti-oxidative activities of sequentially extracted solvent fractions of different parts of *P. biglobosa* were investigated using four complementary *in vitro* assays. Our findings indicated that all extracts had electron donating and free radical scavenging activities but the ethanol (EtOH) extracts from all the parts demonstrated more promising anti-oxidative effects in these experimental models. Apart from the aqueous extracts of the stem bark and leaves, all other extracts exhibited hydroxyl radical scavenging (HRS) activity but the stem bark ethyl acetate (EtOAc) extract and EtOH extracts of the root and leaves possessed more powerful HRS activity than other corresponding extracts in the parts. Further, nitric oxide (NO) inhibition activities were observed in all the extracts except the EtOAc extract of the stem bark which showed pro-oxidative activity. However, the EtOH extract of the stem bark and root as well as the EtOAc extract of the leaves displayed more potent anti-NO activity than other extracts in the parts. The GC-MS analysis of the EtOH extracts revealed that the most abundant phytochemicals are pyrogallol derivatives. Data from this study suggest that the EtOH extracts from different parts of *P. biglobosa* contained potent anti-oxidative agents and pyrogallol could be the main bioactive constituent.

5.2.2 Introduction

Reactive oxygen species (ROS) and free radicals are generated via normal biochemical and physiological processes in living system and are quenched by a cascade of endogenous antioxidant systems in the body [19]. Excess generation of such free radicals and/or inactivation of the endogenous anti-oxidative systems usually shift the ROS/antioxidants balance in favor of stress, a phenomenon called oxidative stress (OS) [20]. The OS is implicated as a crucial factor in the pathogenesis of a number of diseases which include hypertension, cardiovascular diseases, diabetes mellitus and other metabolic syndromes [21].

The application of medicinal plant resources for the management of various diseases continues to be an important component of the health care delivery system, especially in Africa where more than 5400

medicinal plants were reported to have over 16,300 medicinal uses [22]. The role of these medicinal plants on drug discovery is impressive because most of the currently available clinically active drugs are either directly discovered from natural products or have a natural product pharmacophore [23].

Parkia biglobosa (Jacq.) Benth. (Mimosaceae), commonly known as the African locust bean tree, is native to Nigeria alongside other West African countries. The fermented seeds are well appreciated as condiments in cooking under various names such as ‘*dawadawa*’ in Nigeria. All of the different parts of this plant are used by traditional healers to cure several metabolic or non-metabolic disorders like hypertension, haemorrhages and dermatosis [2,15]. A recent ethnopharmacological survey in northern Nigeria also revealed that the stem bark part of the plant was among the most commonly used plants for the traditional healing of diabetes mellitus [6]. The analgesic and anti-inflammatory [11], antivenom [10], antidiarrhoeal [12], antibacterial [8], vasorelaxant [15], and wound healing [14] activities of extracts from different parts of this plant have been demonstrated. Furthermore, the fermented seed extract was also reported to possess hypoglycemic activity in a type 1 diabetes model of rats [16]. Although several studies were investigated to examine the above-mentioned effects of this plant, however the anti-oxidative effects have not been examined either *in vitro* or *in vivo* despite its extensive use for the treatment of diabetes mellitus, an OS associated metabolic disorder.

Hence, our present study was firstly designed to conduct a comprehensive investigation on the anti-oxidative effects of the various solvent extracts of the stem bark, root and leaves of this plant with a view to find compound(s) that could be used to ameliorate the OS mediated metabolic disorders. Based on the results of anti-oxidative activities, the most promising anti-oxidative extracts were subjected to GC-MS analysis in order to identify the phytochemicals contained therein.

5.2.3 Materials and methods

Please refer to the chapter 2 sub sections 2.3 – 2.5 page 35-38 for detailed material and method that affect *P. biglobosa*

5.2.4 Results

Higher yields of EtOAc and EtOH extracts were obtained from the leaves in comparison to the stem bark and roots, however the aqueous extract from the stem bark had the highest yield among the different plant parts. The EtOH extracts contained a significantly ($P<0.05$) higher amount of total polyphenols than other solvent extracts within the parts of the plant (**Table 5.2.1**).

Table 5.2.1: Percentage yield and total polyphenol content of various solvent extracts of *P. biglobosa* parts

Samples	% Yield	Total polyphenol (mg/g GAE)
Stem bark		
EtOAc	0.74	57.76 ± 0.88 ^e
EtOH	4.80	114.77 ± 0.99 ^g
Aqueous	3.98	50.96 ± 0.37 ^d
Root		
EtOAc	0.17	0.47 ± 0.04 ^a
EtOH	0.15	168.98 ± 11.32 ^h
Aqueous	1.55	8.95 ± 0.78 ^b
Leaves		
EtOAc	1.54	32.41 ± 0.17 ^c
EtOH	4.95	84.57 ± 1.06 ^f
Aqueous	3.32	31.37 ± 0.65 ^c

The results are expressed as mean ± SD of triplicate determinations. ^{a-h}Different superscript alphabets within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The EtOH extracts of the stem bark and leaves had significantly higher (P<0.05) total reducing power (GAE) than trolox and other solvents extracts in these parts at all concentrations. The EtOH extracts of the stem bark and leaves also had significantly higher (P<0.05) total reducing power than ascorbic acid at most concentrations tested. Extracts obtained from the root demonstrated weaker reducing power that were significantly lower (P<0.05) than those of ascorbic acid and trolox (**Table 5.2.2**).

Table 5.2.2: Total reducing power (GAE) of solvent extracts from various parts of *P. biglobosa*

Extracts	Concentration (µg/ml)				
	15	30	60	120	240
Stem bark					
EtOAc	2.89 ± 0.95 ^b	30.98 ± 1.11 ^e	22.44 ± 1.14 ^c	40.66 ± 1.33 ^f	70.42 ± 4.68 ^f
EtOH	44.11 ± 1.30 ^g	45.60 ± 2.45 ^f	39.65 ± 1.33 ^g	61.71 ± 1.14 ^h	108.76 ± 9.07 ⁱ
Aqueous	27.69 ± 3.39 ^f	23.93 ± 4.54 ^d	25.88 ± 0.88 ^{ef}	51.63 ± 5.69 ^g	77.11 ± 1.00 ^g
Root					
EtOAc	0.00 ± 0.00 ^a	0.11 ± 0.01 ^a	0.70 ± 0.24 ^a	1.64 ± 0.17 ^a	2.99 ± 0.46 ^a
EtOH	5.55 ± 1.12 ^c	4.35 ± 2.66 ^b	4.92 ± 0.45 ^b	11.87 ± 2.39 ^c	16.27 ± 1.05 ^b
Aqueous	5.15 ± 2.02 ^c	7.38 ± 0.20 ^c	3.94 ± 0.63 ^b	5.98 ± 1.80 ^b	15.79 ± 1.10 ^b
Leaves					
EtOAc	3.30 ± 1.12 ^{bc}	5.60 ± 1.50 ^{bc}	7.73 ± 0.55 ^c	15.42 ± 0.65 ^d	25.01 ± 0.38 ^c
EtOH	29.59 ± 6.45 ^f	44.50 ± 6.38 ^f	36.79 ± 2.82 ^g	68.25 ± 3.39 ⁱ	94.82 ± 0.85 ^h
Aqueous	11.74 ± 3.01 ^d	15.79 ± 1.01 ^d	15.59 ± 1.31 ^d	25.70 ± 3.02 ^e	48.36 ± 3.29 ^d
Ascorbic acid	31.50 ± 4.92 ^f	32.66 ± 3.00 ^e	35.62 ± 5.51 ^g	52.56 ± 5.43 ^g	89.95 ± 6.21 ^h
Trolox	17.84 ± 1.86 ^e	20.88 ± 7.33 ^d	24.42 ± 3.95 ^{ef}	29.61 ± 1.96 ^e	52.77 ± 0.90 ^e

The results are expressed as mean ± SD values of triplicate determinations. ^{a-i}Different superscript alphabets within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The scavenging effects of the different solvent extracts of *P. biglobosa* parts on DPPH radical are presented in **Figure 5.2.1**. While all the extracts demonstrated the ability to scavenge DPPH radicals as manifested by the concentration-dependent increase in the percentage inhibitions, the EtOH extracts of the stem bark and root displayed significantly higher (P<0.05) radical scavenging activity than other extracts within these parts at higher concentrations (60-240 µg/ml) and the difference insignificant (P>0.05) compared to the standard antioxidants in some cases. Interestingly, the EtOH extract of the leaves extract maintained >90% free radical scavenging activity at 30-240 µg/ml. It is important to note that the IC₅₀ values of the aqueous extracts of the root and leaves and the EtOH extract of the stem bark were lower than the corresponding values for other extracts in these parts (**Table 5.2.5**).

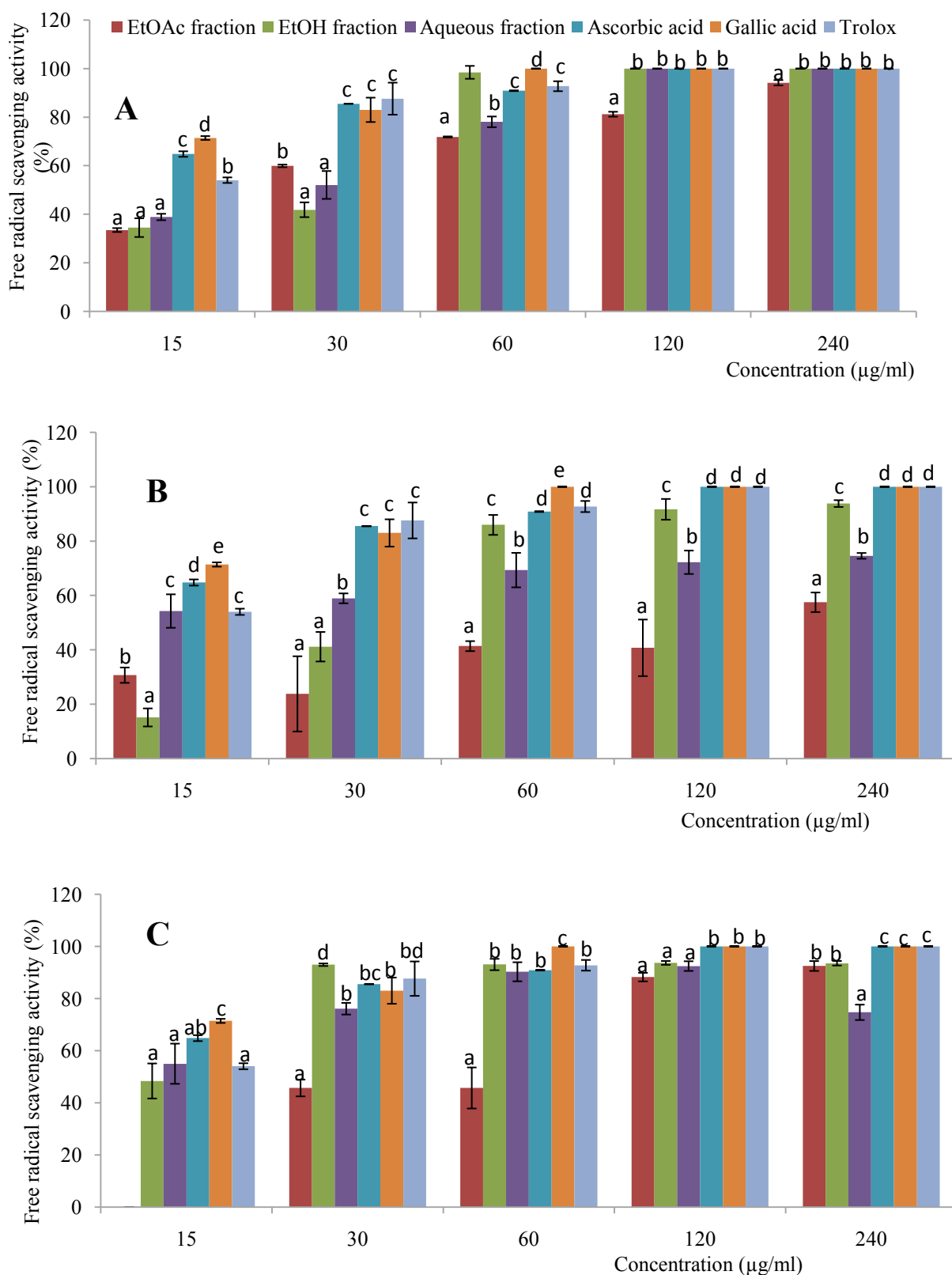


Figure 5.2.1: DPPH radical scavenging activity of stem bark (A), root (B) and leaves (C) extracts of *P. biglobosa*. The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different alphabets over the bars on a given concentration of each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The results of the HRS assay indicated that all extracts could scavenge hydroxyl radicals generated by Fenton's reaction except the aqueous extracts of the stem bark and leaves which displayed pro-oxidative tendencies at all concentrations tested (**Table 5.2.3**). Although all organic extracts obtained from the stem bark and root exhibited a non-concentration dependent pattern of HRS activity, the EtOAc and EtOH extracts of the stem bark displayed significantly higher ($P<0.05$) activity at 15-60 $\mu\text{g/ml}$ and 120-240 $\mu\text{g/ml}$ respectively, than other extracts. It is also noteworthy that the EtOH extract of the root consistently demonstrated a significantly higher ($P<0.05$) HRS activity than other extracts in this part. Contrary to this, the EtOAc extract of the leaves displayed a significantly higher ($P<0.05$) hydroxyl radical inhibition than other extracts in this part at all concentrations and an activity that inversely correlates with concentration was observed with the EtOH extract of the leaves.

Table 5.2.3: Percentage HRS activity of extracts from various parts of *P. biglobosa*

Extracts	Concentration ($\mu\text{g/ml}$)				
	15	30	60	120	240
Stem bark					
EtOAc	20.93 ± 0.85^f	39.10 ± 0.68^e	19.30 ± 1.48^d	8.71 ± 0.40^c	-5.57 ± 0.37^b
EtOH	0.00 ± 0.00^c	10.31 ± 5.49^c	17.38 ± 3.00^d	33.79 ± 7.69^e	28.10 ± 11.80^e
Aqueous	-33.31 ± 0.97^b	-51.73 ± 3.25^b	-69.62 ± 2.00^a	-100.00 ± 0.00^a	-100.00 ± 0.00^a
Root					
EtOAc	0.80 ± 0.07^d	12.39 ± 3.07^c	9.76 ± 1.07^c	$24.61 \pm 1.37^{d,e}$	$22.39 \pm 1.96^{d,e}$
EtOH	46.00 ± 2.86^h	48.82 ± 2.73^f	51.41 ± 1.01^f	44.79 ± 0.78^f	$38.63 \pm 0.39^{e,f}$
Aqueous	19.07 ± 0.28^e	27.87 ± 0.74^d	26.67 ± 0.96^e	30.75 ± 1.34^e	28.40 ± 2.90^e
Leaves					
EtOAc	64.69 ± 1.57^j	68.62 ± 14.25^g	78.81 ± 0.81^g	85.02 ± 0.27^h	88.63 ± 0.70^g
EtOH	29.00 ± 1.32^g	15.32 ± 0.31^c	7.98 ± 0.57^b	1.88 ± 0.23^b	1.06 ± 0.23^c
Aqueous	-41.26 ± 1.25^a	-60.90 ± 3.39^a	-81.80 ± 13.10^a	-100.00 ± 0.00^a	-100.00 ± 0.00^a
Trolox	57.32 ± 2.95^i	73.11 ± 1.44^g	76.04 ± 2.05^g	80.09 ± 3.93^g	79.82 ± 3.50^h

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-i}Different superscript alphabets for values within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 5.2.4 presents the NO inhibition activities of solvents extracts of *P. biglobosa* parts. Apart from the EtOAc extract of the stem bark, all other extracts were found to exhibit NO inhibition activity. The aqueous and EtOH extracts of the stem bark and root as well as the EtOAc and aqueous extracts of the leaves demonstrated a non-concentration dependent NO inhibition effects while the EtOH extract of the leaves showed an NO inhibition activity which inversely correlates with concentration. Furthermore, the EtOH extracts of the stem bark and root as well as the EtOAc extract of the leaves possessed lower IC_{50} values than the corresponding extracts in these plant parts (**Table 5.2.5**).

Table 5.2.4: Percentage NO scavenging activities of extracts from various parts *P. biglobosa*

Extracts	Concentration ($\mu\text{g/ml}$)				
	15	30	60	120	240
Stem bark					
EtOAc	-24.74 ± 2.31^a	-47.96 ± 1.53^a	-47.96 ± 16.07^a	-100.00 ± 0.00^a	-100.00 ± 0.00^a
EtOH	55.47 ± 7.54^g	50.78 ± 3.67^f	31.60 ± 9.42^e	36.91 ± 8.00^c	43.45 ± 1.82^e
Aqueous	10.73 ± 0.72^d	18.53 ± 0.69^d	33.40 ± 1.33^e	32.22 ± 5.05^c	22.51 ± 1.14^b
Root					
EtOAc	0.00 ± 0.00^b	0.00 ± 0.00^b	0.00 ± 0.00^b	14.57 ± 7.66^b	$24.71 \pm 2.93^{b,c}$
EtOH	34.39 ± 2.45^f	49.67 ± 0.85^f	$48.73 \pm 0.88^{e,f}$	$39.55 \pm 1.62^{c,d}$	29.08 ± 0.73^d
Aqueous	28.92 ± 1.78^e	32.36 ± 0.65^e	14.61 ± 2.20^c	20.20 ± 6.75^b	38.49 ± 6.05^e
Leaves					
EtOAc	$61.06 \pm 4.96^{g,h}$	63.54 ± 6.89^g	$62.74 \pm 14.33^{f,g,h}$	$42.53 \pm 9.34^{c,d,f}$	60.51 ± 2.86^g
EtOH	73.45 ± 1.98^i	64.88 ± 7.31^g	65.19 ± 3.97^h	15.03 ± 6.28^b	18.02 ± 3.29^b
Aqueous	15.19 ± 7.08^d	32.45 ± 1.23^c	41.30 ± 7.71^e	51.67 ± 3.82^f	39.36 ± 4.67^e
Ascorbic acid	47.81 ± 0.51^g	50.97 ± 1.35^f	52.82 ± 1.35^g	$56.72 \pm 3.69^{f,g}$	64.08 ± 4.25^g
Gallic acid	4.43 ± 2.69^c	7.52 ± 1.11^c	$25.74 \pm 0.44^{d,e}$	$27.66 \pm 1.91^{b,c}$	31.70 ± 2.51^d
Trolox	66.30 ± 1.23^h	65.51 ± 1.27^g	63.39 ± 1.84^h	59.53 ± 1.81^g	52.21 ± 2.47^f

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-i}Different superscript alphabets for values within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 5.2.5: IC₅₀ values of various solvent extracts of *P. biglobosa* parts in different anti-oxidative models

Samples	IC ₅₀ ($\mu\text{g/ml}$)		
	DPPH	HRS	NO
Stem bark			
EtOAc	24.43	3.63	P
EtOH	1.37	979.22	18.29
Aqueous	22.34	P	8.69*
Root			
EtOAc	177.39	52.79	5.71*
EtOH	34.78	13.21	2.36
Aqueous	7.60	118.16*	>1**
Leaves			
EtOAc	51.69	3.13	4.64
EtOH	4.26	1.18	52.54
Aqueous	1.83	P	252.43
Ascorbic acid	2.56	ND	26.28
Gallic acid	1.27	ND	1.03
Trolox	5.04	2.61	599.21

ND means not determined and P means the extract showed pro-oxidative properties in the experimental model. *The units of these values are mg/ml. ** The unit of this value is g/ml.

Based on the anti-oxidative activities, the EtOH extracts were selected for GC-MS analysis (**Figure 5.2.2**). The major peak detected in the chromatogram of the EtOH extracts of the stem bark and root were due to those of a 1,2,3-trioxygenated benzene compound (pyrogallol) with a hydroxy, methoxy and acetoxy group at each of the oxygenated positions, while an additional phloroglucinol derivative with the same substituents as well as an alloside of the pyrogallol derivative and two sugars, 3-O-methyl-D-fructose and 4-O-methyl-mannose were detected in the EtOH extract of the leaves (**Table 5.2.6**). The exact pyrogallol and phloroglucinol derivatives were not detected by the library of the instrument. Nevertheless, a parent ion at M^+ 182 is evident in the mass spectrum as well as fragments at m/z 167 and 125 for the loss of the methyl group and the loss of the methyl and acyl groups respectively. The resultant fragmentation pattern at m/z 125 and below is consistent with that of pyrogallol and therefore the oxygenation pattern is probably consistent with that of pyrogallol.

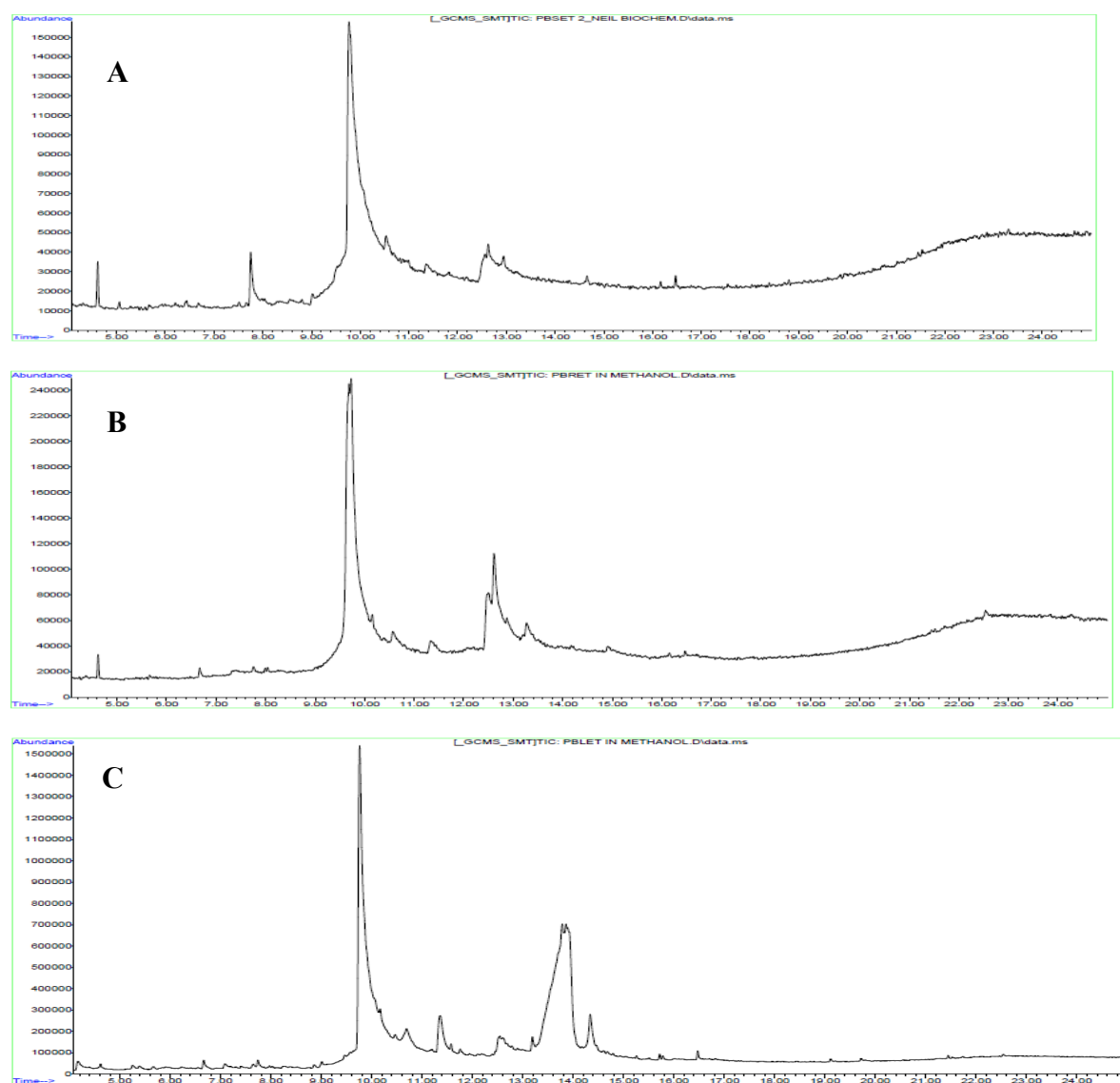


Figure 5.2.2: GC-MS chromatogram of EtOH extracts of stem bark (A), root (B) and leaves (C) of *P. biglobosa*

Table 5.2.6: Identified compounds of EtOH extracts of different parts of *P. biglobosa* by GC-MS

Compounds	Retention time (min)	Molecular mass	Relative abundance (%)
Stem bark			
Pyrogallol derivative (acetoxyl, hydroxyl and methoxy substituents)	9.76	182	100
Root			
Pyrogallol derivative (acetoxyl, hydroxyl and methoxy substituents)	9.73	182	100
Leaves			
Pyrogallol derivative (acetoxyl, hydroxyl and methoxy substituents)	9.76	182	52.27
*Pyrogallol derivative - alloside	11.36	ND	3.19
Phloroglucinol derivative	12.54	182	0.46
3-O-methyl-D-fructose	13.78	194.18	25.33
4-O-methyl mannose	13.92	194.18	6.92

* The molecular ion was not detected but the fragmentation pattern is indicative of the compound.

5.2.5 Discussion

The genus *Parkia* comprises of over 70 species across the world but only a preliminary report on the antioxidant activity of a single plant (*P. speciosa*) from the genus appears in the literature [24]. Ethnobotanical surveys have indicated that different parts of *P. biglobosa* are used in the traditional treatment of diabetes mellitus and other diseases whose pathogenesises are, in part, linked to OS. This study investigated the anti-oxidative activities of various solvent extracts from different parts of *P. biglobosa* as a prelude to finding agent(s) that could be used to ameliorate OS mediated metabolic disorders.

In the present study, four experimental models for *in vitro* anti-oxidative studies were used because a single model cannot give a full evaluation of the anti-oxidative capabilities of the different extracts tested due to the multiple mechanisms via which antioxidant compounds act. The total reducing ability of the extracts was measured as Fe^{3+} – Fe^{2+} transformation using the procedure of Oyaizu [25]. Thus, the reducing power of an extract or compound could be a vital marker of its potential anti-oxidative activity and therefore the FRAP method is a viable procedure to investigate the anti-oxidative activity of different extracts and/or compounds. The strong reducing capacity of the stem bark and leaves EtOH extracts

indicate that the phytochemical components of this plant with high redox potential, at least in these parts, are EtOH extractable.

The DPPH radical is a nitrogen-containing free radical which undergoes reduction through electron or hydrogen donation and changes color from violet to yellow. Agents which can undergo this reaction are considered as free radical scavengers and antioxidants [26]. The high DPPH radical scavenging effects of the different solvent extracts, especially the EtOH extracts that were comparable to standard antioxidants used in most cases, suggest that these extracts possess high proton donating ability which could make them free radical inhibitors. However, the EtOH extracts of the stem bark and leaves as well as the aqueous extracts of the leaves exhibited stronger DPPH radicals quenching ability with lower IC₅₀ values than trolox. The computed IC₅₀ values for the EtOH extract of the stem bark (1.37 µg/ml) and aqueous extract of the leaves (1.83 µg/ml) is especially remarkable because only a few authors [27] reported such low IC₅₀ values as found with these extracts in spite of hundreds of reports on the DPPH radical scavenging effects of extracts from plants in various continents of the world. This could further indicate that these extracts contain powerful free radical scavenging phytochemicals that might have the ability to inhibit free radical upsurge as well as OS and therefore might be valuable as curative or preventing agents for the treatment diseases associated with radicals.

The hydroxyl radicals are highly potent reactive free radicals generated in living organisms and have been implicated as detrimental species that can cause damage to all life essential biomolecules [28]. Thus, agents with excellent scavenging effects toward hydroxyl radicals would be important in treating diseases where OS is important for the disease initiation and/or progression. From the present study, all organic extracts from the various parts of *P. biglobosa* contain phytochemicals with hydroxyl radical inhibition activity but the activity is more pronounced in the EtOH extracts except in that of the stem bark. The non-concentration dependent pattern of HRS activity displayed by the organic extracts of the stem bark and root as well as the reciprocal activity by the EtOH extract of the leaves could be attributed to a hormesis phenomenon by these extracts in this experimental model. Hormesis is a dose-response relationship for a single endpoint that is associated with changes of response between high and low dosages of biological agents, chemicals or any other agent that could initiate a response [29] and its occurrence has been documented in numerous biological, toxicological and pharmacological investigations [30]. The foregoing therefore indicates that maximum scavenging activity towards hydroxyl radicals by these extracts occur at optimal points. On the other hand, the pro-oxidative activities of the aqueous extract of the stem bark and leaves in the hydroxyl radical based anti-oxidative model further indicated the need to use a multi method approach before a definite statement can be made on the anti-oxidative effects of a plant extract, extract or pure compound.

Nitric oxide is a free radical produced by macrophages, endothelial cells or neurons and has been associated with the regulating a number of physiological processes [31]. High concentration of NO is implicated in the pathology of several diseases including T2D [32]. Excess NO reacts oxygen to produce peroxy nitrite anions and nitrite, which serve as free radicals [33]. In the present study, the incubation of sodium nitroprusside leads to a time-dependent nitrite generation [34], which was scavenged by all the tested extracts except the EtoAc extract of the stem bark. This could be linked to the anti-NO phytochemicals (mostly phenolics) in the extracts which could affect the NO-oxygen reaction, thereby diminishing the nitrite generation. Further, the hormetic responses shown by some extracts toward scavenging NO indicate that the anti-NO principles have some optimal points of effective inhibition and/or antagonism occurs with other phytochemicals at certain concentrations.

Polyphenols are a well-known class of secondary metabolites with anti-oxidative activities. This is mostly linked to their high redox abilities that play crucial role in absorbing and scavenging free radicals, inhibiting triplet and singlet oxygen as well as decomposing peroxides [35]. Although correlation analysis was not performed, our results tend to suggest strong positive correlation between the polyphenolic content and anti-oxidative activity because a broad view of the results indicates that the EtOH extracts with highest polyphenolic content within the plants demonstrated a higher anti-oxidative activity with no pro-oxidative activity in any of the experimental models used. Thus, these extracts were further subjected to direct GC-MS analysis. It is evident from the results that a pyrogallol derivative is the only phytochemical detected in the stem bark and root sample of the plant and could therefore be the main bioactive anti-oxidative agent. However, the presence of the pyrogallol derivative peaks coupled with other phenolics in the leaf sample could account for the observed higher anti-oxidative activities in this part of the plant. On a general note, the observed quantitative difference in the anti-oxidative activities among the various parts of this plant could be attributed to the variations in concentrations and compositions of the anti-oxidative principles in the different parts.

From the results of this study, we can conclude that the EtOH extracts of the various parts of *P. biglobosa* contain more potent anti-oxidative agents than other extracts and that a pyrogallol derivative could be the main bioactive agent. Future work would entail isolating the pyrogallol derivative and other phenolics as well as evaluating the anti-oxidative activity of the pure isolates and mixtures of isolates.

Postscript: From the above experiment, the crude ethanol extract from the leaves was found to have relatively higher anti-oxidative activity that cuts across various types of radicals than other extracts. It was therefore selected for further investigations.

5.3 Butanol fraction of *Parkia biglobosa* leaves ethanol extract contains anti-oxidative agents and potent inhibitors of α -glucosidase and α -amylase

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Preface: In this sub-chapter, solvent-solvent fractionation of the crude ethanol extract from the leaves was carried out and the solvent fractions were investigated for anti-oxidative and α -glucosidase and α -amylase inhibitory activities. The inhibitory mechanism of the most active (butanol) fraction and the bioactive phytochemicals were also investigated. This article is yet to be submitted to the supervisors for proof reading and eventual submission for publication.

5.3.1 Abstract

The *in vitro* anti-oxidative as well as the α -glucosidase and α -amylase inhibitory effects of various solvent fractions derived from the crude ethanol extract of *Parkia biglobosa* leaves were investigated. Crude ethanol extract of the leaves of *P. biglobosa* was subjected to solvent-solvent partitioning which yielded a butanol fraction with significantly ($P < 0.05$) more potent anti-oxidative as well as α -glucosidase and α -amylase activities than other solvent (aqueous, ethyl acetate, dichloromethane and hexane) fractions. Further kinetic studies on the inhibitory mechanism of the butanol fraction on α -glucosidase revealed a mixed inhibition pattern with inhibition binding constants (K_i) of 9.47 and 18.98 $\mu\text{g/ml}$ for competitive and non-competitive inhibitions, respectively. However, the mechanism of α -amylase inhibition was non-competitive with a K_i of 5.32 $\mu\text{g/ml}$. GC-MS analysis of the butanol fraction revealed that the main bioactive components are phenolics such as pyrogallol and methoxyeugenol. It was concluded that the butanol fraction contains potent anti-oxidative agents and inhibitors of α -glucosidase and α -amylase activities and could thus be useful therapeutic agents against diabetes mellitus.

5.3.2 Introduction

Diabetes mellitus could be defined as a heterogenous group of metabolic disorder associated with persistent hyperglycemia and derangement in the metabolism of major macromolecules resulting from anomalies in the secretion of insulin or its action [36]. At present, different pharmacological strategies are used in the treatment of the disease in addition to life style modification. These include stimulation of insulin, inhibition of gluconeogenesis, increasing peripheral insulin sensitivity and delaying glucose absorption from the intestine. One of the beneficial therapeutic approaches to delay glucose absorption is by decreasing the activity of carbohydrate-hydrolyzing enzymes such as α -amylase and α -glucosidase [37] which would consequently retard the release of glucose from complex dietary carbohydrates to the bloodstream, thereby reducing the postprandial hyperglycemia.

Available scientific evidences have shown that hyperglycemia induces oxidative stress and this phenomenon is regarded as the common biological event that plays vital roles in all aspects of the diabetes pathogenesis such as insulin resistance, pancreatic β cell dysfunction as well as macrovascular and microvascular complications [38-40]. Based on the foregoing, research on diabetes therapy has focused attention on the search for novel agents with potent anti-oxidative activities that could also decrease postprandial hyperglycemia via α -amylase and α -glucosidase inhibition, thereby providing a holistic therapeutic approach to control hyperglycemia and other diabetic complications resulting from oxidative stress.

Traditional medicinal plants play an essential role in the healthcare delivery systems of many parts of the world as well as in drug discovery [23]. According to World Health Organization (WHO), approximately 60% of the total population of the world depends, directly or indirectly, on traditional medicinal plants [41]. A survey of plant-derived pure compounds used as drugs in countries hosting WHO-Traditional Medicine Centers indicated that, out of the 122 compounds so far identified, 80% were used for the same or related ethnomedical purposes and were derived from only 94 plant species. Furthermore, 60% of the anticancer drugs and 75% of the anti-infectious disease drugs approved from 1981-2002, were originally derived from natural sources [42] whereas 61% of all new chemical entities introduced worldwide as drugs during the same period could be traced to natural products [43]. Indeed, the widely used anti-diabetic drug metformin was originally developed from a medicinal plant [44]. Thus, investigating medicinal plants for anti-oxidative agents with potent α -amylase and α -glucosidase inhibitory activities is an appealing alternative.

Parkia biglobosa (Jacq.) Benth. (Mimosaceae) is known as the African locust bean tree and native to West African countries. It is highly reputed for numerous medicinal values [1, 8] and all of the different parts of the plant are used by traditional healers to cure several metabolic or non-metabolic disorders such as hypertension, haemorrhages diarrhea, ulcers and dermatosis [2, 15]. Also, the fermented seeds of the plant are also frequently used as natural nutritional condiment with hypoglycemic activity [16]. Recent ethnopharmacological surveys also revealed that the plant is among the most commonly utilized plants in the traditional remedy of diabetes mellitus in northern Nigeria [6] and the central region of Togo [7]. In order to systematically study the anti-diabetic potentials of the plant, we previously subjected different solvent crude extracts of the stem bark, root and leaf samples of the plant to anti-oxidative activity assays using several models and found that the ethanol crude extract of the leaves had the most potent anti-oxidative activity among all other extracts [45]. In the present study, we further fractionated the crude ethanol extract of the leaves by solvent-solvent partitioning and then conducted a comprehensive study on the *in vitro* anti-oxidative as well as the α -glucosidase and α -amylase inhibitory effects of the fractions.

Additionally, we subjected the most bioactive fraction to GC-MS analysis in order to identify the phytochemicals contained therein.

5.3.3 Materials and methods

Please refer to the chapter 2 sub-sections 2.6.1-2.6.6 pages 38-40 for detailed material and methods that affect *P. biglobosa*

5.3.4 Results

The total phenolics were significantly higher ($P<0.05$) in the butanol fraction than other solvent fractions. The butanol fraction also recorded a significantly ($P<0.05$) higher DPPH and NO radicals scavenging activities than other solvent fractions (**Table 5.3.1**). However, the aqueous fraction was found to have significantly ($P<0.05$) higher total reducing capacity (**Figure 5.3.1**) and hydroxyl radical scavenging activity than other fractions (**Table 5.3.1**).

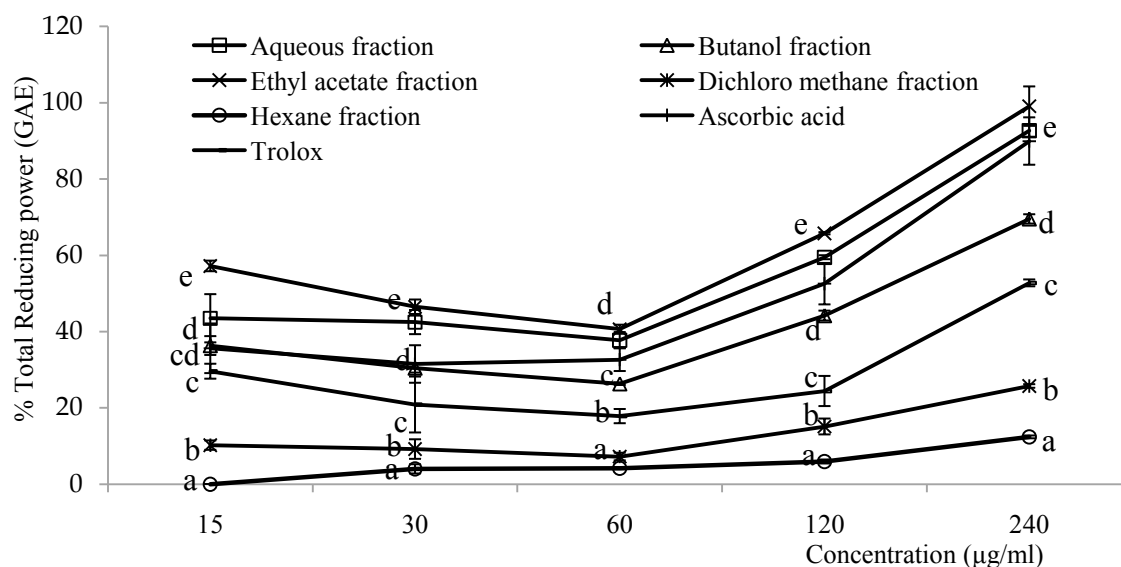


Figure 5.3.1: Total reducing power (gallic acid equivalent) of different solvent fractions of the ethanol extract of *P. biglobosa* leaves. The results are expressed as mean \pm SD of triplicate determinations. ^{a-} Different alphabets presented for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 5.3.1: IC₅₀ values of various solvent fractions of ethanol extract of *P. biglobosa* leaves in different anti-oxidative models

Fractions	Total phenolics (mg/g GAE)	IC ₅₀ (µg/ml)		
		DPPH	Hydroxyl radical	NO
Aqueous	68.75 ± 0.75 ^d	20.30 ± 1.46 ^e	13.97 ± 3.16 ^b	1734.38 ± 58.69 ^f
Butanol	82.66 ± 5.51 ^e	16.05 ± 0.74 ^d	44.05 ± 3.12 ^c	227.63 ± 34.96 ^b
Ethyl acetate	32.73 ± 0.59 ^c	43.15 ± 3.26 ^f	160.42 ± 25.93 ^e	3285.96 ± 1085.47 ^g
Dichloromethane	11.51 ± 0.52 ^b	78.64 ± 0.82 ^g	76.46 ± 5.17 ^d	302.75 ± 21.58 ^c
Hexane	2.54 ± 0.37 ^a	348.35 ± 48.40 ^h	195.51 ± 10.08 ^f	3697.62 ± 267.73 ^g
Ascorbic acid	-	2.56 ± 0.26 ^b	ND	26.40 ± 6.46 ^a
Gallic acid	-	1.40 ± 0.43 ^a	ND	1053.48 ± 239.92 ^e
Trolox	-	8.47 ± 2.88 ^c	3.23 ± 0.49 ^a	629.76 ± 63.98 ^d

The results are expressed as mean ± SD values of triplicate determinations. ^{a-h}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, p<0.05).

Data from the α -glucosidase and α -amylase inhibitory actions revealed that all the fractions could inhibit the carbohydrate digesting enzymes in a dose dependent pattern (**Figure 5.3.2**) but the butanol fraction was also found to possess significantly (P<0.05) more potent α -glucosidase and α -amylase inhibitory phytochemicals as evidenced by the significantly (P<0.05) lower IC₅₀ values compared to other fractions (**Table 5.3.2**). Based on the high activity of the butanol fraction in most of the assays described above, it was selected for further studies.

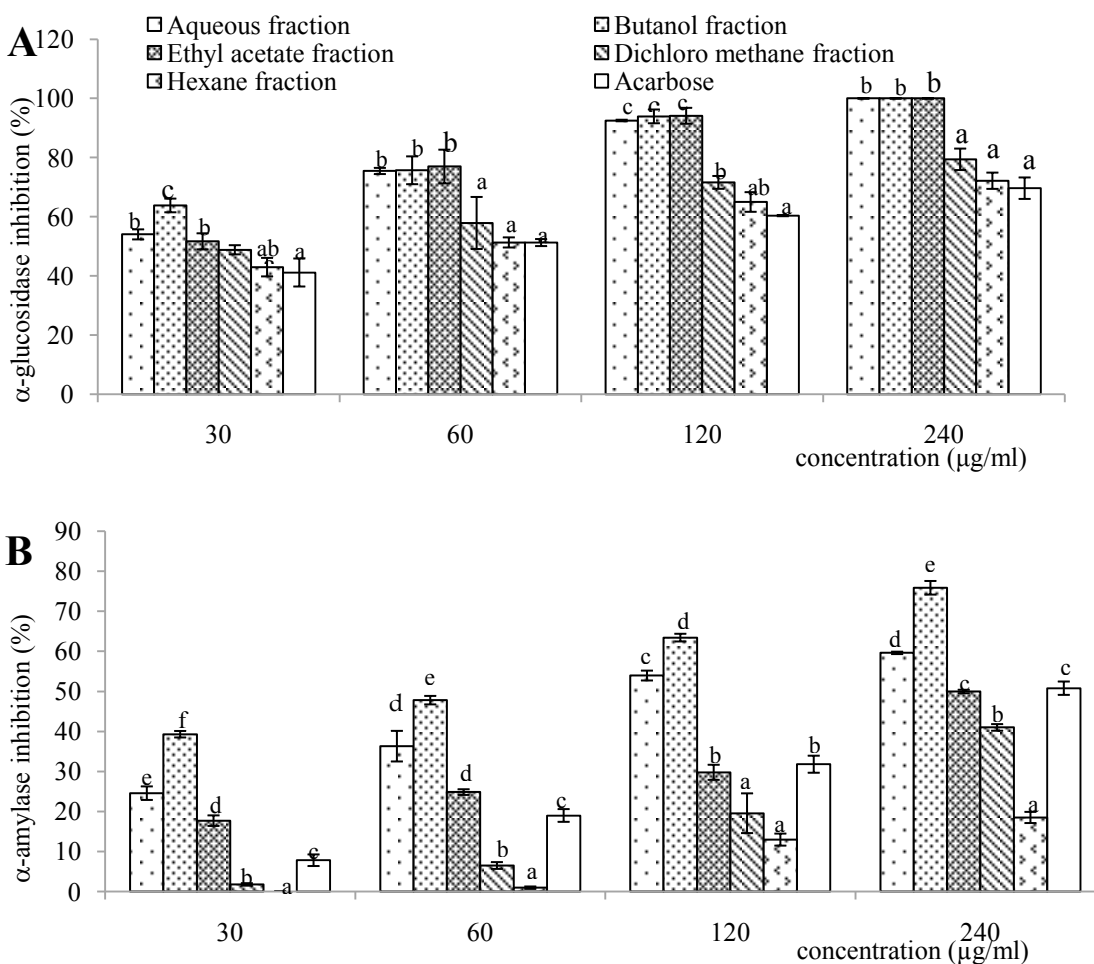


Figure 5.3.2: α -glucosidase (A) and α -amylase (B) inhibitory actions of different solvent fractions of the ethanol extract of *P. biglobosa* leaves. The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different alphabets over the bars for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 5.3.2: IC_{50} values for the inhibition of α -glucosidase and α -amylase by various solvent fractions of the ethanol extract of *P. biglobosa* leaves

Fractions/standard	IC_{50} (µg/ml)	
	α -glucosidase	α -amylase
Aqueous	21.08 ± 3.12^b	120.23 ± 4.58^b
Butanol	13.28 ± 1.33^a	58.00 ± 0.80^a
Ethyl acetate	22.31 ± 3.12^b	318.90 ± 32.41^d
Dichloromethane	32.43 ± 8.40^c	488.80 ± 88.37^e
Hexane	48.80 ± 5.96^d	6615.49 ± 768.31^f
Acarbose	55.59 ± 5.22^d	256.66 ± 20.52^c

The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$).

Steady state kinetic analysis was used to delineate the inhibitory mechanism of the fraction on both enzymes and the findings indicated that the α -glucosidase was inhibited by the fraction using a mixed inhibition pattern (**Figure 5.3.3A**) with inhibition binding constants (K_i) of 9.47 and 18.98 $\mu\text{g/ml}$ for competitive and non-competitive inhibitions respectively (**Table 5.3.3**). On the other hand, the α -amylase was non-competitively inhibited by the fraction (**Figure 5.3.3B**) with a K_i of 5.32 $\mu\text{g/ml}$.

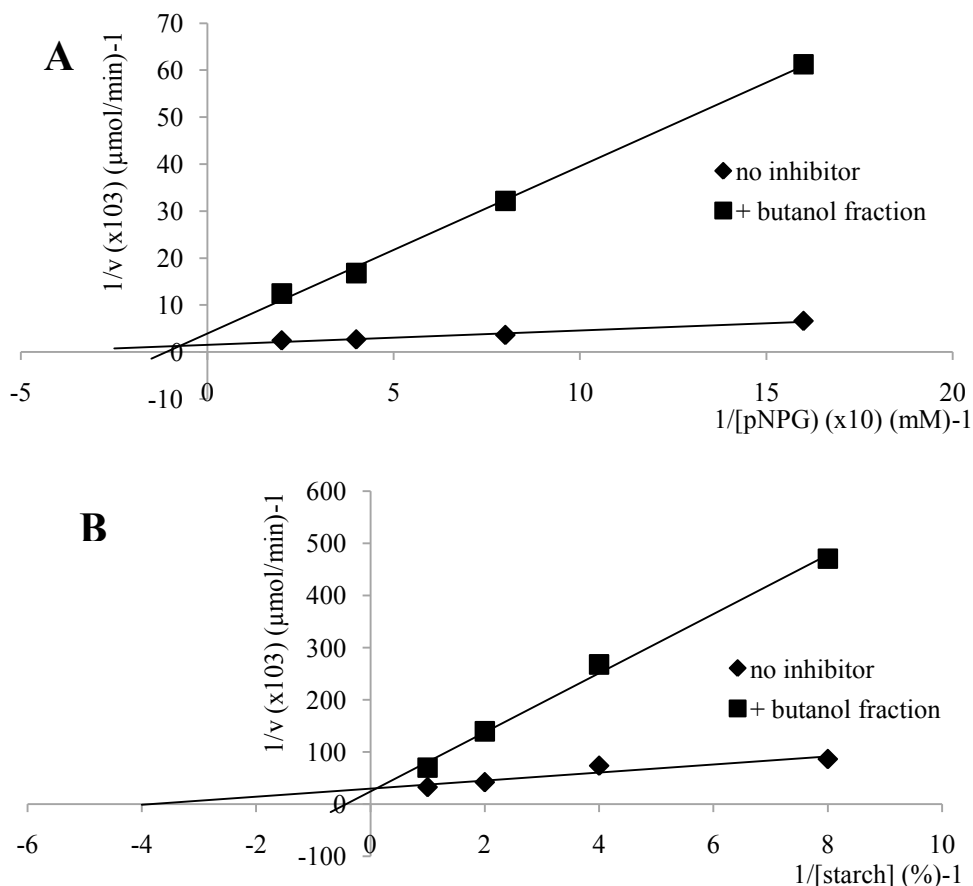


Figure 5.3.3: Lineweaver-Burke's plot of α -glucosidase (A) and α -amylase (B) catalysed reactions in the presence and absence of the butanol fraction obtained from the ethanol extract of *P. biglobosa* leaves.

Table 5.3.3: Effects of the butanol fraction derived from the ethanol extract of *P. biglobosa* leaves (30 $\mu\text{g/ml}$) on some kinetic parameters of α -glucosidase and α -amylase

Kinetic parameters	α -glucosidase		α -amylase	
	control	+ butanol fraction	control	+ butanol fraction
K_M	2.00*	8.33*	0.25 [#]	1.66 [#]
V_{max} ($\mu\text{mol/min}$)	655.09	253.55	33.70	33.70
K_i ($\mu\text{g/ml}$)	-	9.47 (competitive) 18.98 (non-competitive)	-	5.32

The units for K_M was mM (*) and % ([#])

In order to identify the possible bioactive agents of this potent fraction, GC-MS analysis was conducted and the chromatogram is shown in **Figure 5.3.4**. The major peaks detected were those of pyrogallol, methoxyeugenol, 3-(1-isopropyl-but-3-enyloxy)-butyric acid and sugars (**Table 5.3.4**) which were identified by their fragmentation pattern in conjunction with the NIST library.

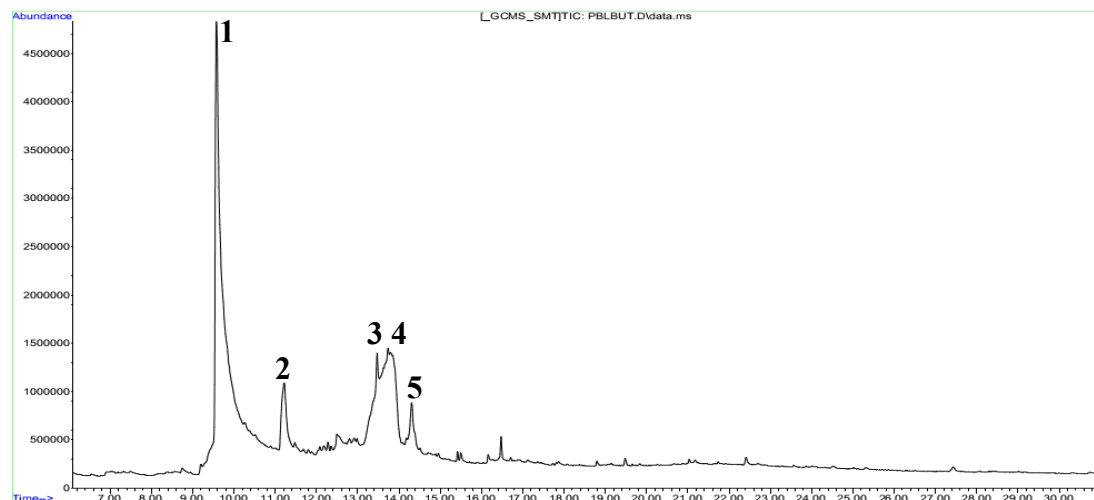


Figure 5.3.4: GC-MS chromatogram of the butanol fraction derived from the ethanol extract of *P. biglobosa* leaves

Table 5.3.4: Identified components of the butanol fraction derived from the ethanol extract of *P. biglobosa* leaves by GC-MS

§	Compound	Retention time (min)	Molecular mass
1	Pyrogallol	9.57	126.10
2	D-Allose	11.21	180.15
3	Methoxyeugenol	13.46	194.10
4	3-O-methyl-fructose	13.62	194.18
5	3-(1-isopropyl-but-3-enyloxy)-butyric acid	14.30	167.10

§ means annotated peak number

5.3.5 Discussion

The traditional use of *P. biglobosa* in the treatment of diabetes mellitus [6,7] prompted us to conduct a systematic study to investigate the anti-diabetic potentials of the plant. In a previous study, it was found that the leaves ethanolic extract had the best anti-oxidative activity among other solvent crude extracts from different parts of the plant [45]. In the present study, butanol fraction obtained from the crude

ethanol extract of the leaves elicited remarkable anti-oxidative as well as α -glucosidase and α -amylase inhibitory effects.

Sequential partitioning of the crude ethanol extract of the leaves across solvents of varying polarity was aimed at separating the polar phytochemicals from the non-polar ones for detailed investigation. Thus, the recovered fractions were subjected to *in vitro* anti-oxidative activity assays using different models because a single method cannot give a full evaluation of the anti-oxidative capabilities due to the involvement of multiple mechanisms in the induction of oxidative stress. The total reducing power of phytochemicals is considered as a reliable marker of their anti-oxidative potential. The reductants inhibit the chain reaction of free radicals by donating hydrogen atoms to the radical molecules. Free radicals are known to be a major factor in cellular damages in biological systems and DPPH method has been used to investigate the free radical scavenging effects of natural anti-oxidative agents. On the other hand, hydroxyl radicals are also extremely reactive species that can damage any biological molecule available in living cells [28] and NO is an unstable species which reacts with oxygen to generate the reactive nitrite and peroxynitrite anions [33]. All these radicals are implicated in the development of diabetes and associated complications [32]. From the present study, the aqueous fraction had the best total reducing power and hydroxyl radical scavenging activity while the butanol fraction had the best DPPH and NO radicals scavenging activities and this observation could suggest preferential difference in the response of the phytochemicals to the different anti-oxidative models. Furthermore, the findings suggests that the more polar (butanol and aqueous) fractions contain strong phytochemicals that could inhibit free radical upsurge and oxidative stress in diseases where the phenomenon is important mechanism of pathogenesis such as diabetes.

All the solvent fractions inhibited α -glucosidase and α -amylase dose dependently which suggest that these fractions could slow down the initial hydrolysis of starch and other complex polysaccharides to oligosaccharides as well as the subsequent hydrolysis of the oligosaccharides to glucose for intestinal absorption and blood circulation [37]. This would obviously decrease the liberation of glucose from complex dietary carbohydrates to the bloodstream, thereby impeding postprandial hyperglycemia. However, the butanol fraction demonstrated the best α -glucosidase and α -amylase inhibitory effects which were mediated through mixed and non-competitive inhibition patterns respectively. The inhibitory pattern exerted on α -glucosidase indicates that the butanol fraction contain powerful inhibitors that were able to bind both at the active site and at an allosteric site of the enzyme [46] both the computed inhibition binding constants for competitive and non-competitive inhibitions suggest that the inhibitory constituents are more oriented to active site binding. On the other hand, the observed inhibitory pattern on α -amylase suggests binding at an allosteric site by the inhibitory phytochemicals which could consequently cause

conformational modification at the active site, thereby preventing the effective binding of the starch and resulting in decreased α -amylase activity [47].

Phenolics are the major plants constituents responsible for anti-oxidative activity and are also known to inhibit some enzymatic activity such as α -glucosidase and α -amylase [48]. Interestingly, the butanol fraction had the highest phenolics content as well as the most potent α -glucosidase and α -amylase inhibitory activities among other fractions. It could thus imply, although without a correlation analysis, that phenolics play a vital role in the enzymes inhibitory activities of this fraction. This is supported by previous studies which reported that the α -glucosidase and α -amylase inhibitory effects of *Ocimum basilicum*, *Citrus maxima*, cinnamon and tea extracts are mediated by phenolics [48-51]. Furthermore, GC-MS analysis also revealed that the butanol fraction contained some phenolics which include pyrogallol and methoxyeugenol. Indeed, a phenolic peak is the most prominent peak (**1**) in the GC-MS chromatogram. Thus, while not discounting the possible contribution of other phytocomponents, it is reasonable to suggest that phenolics act individually or synergistically to bring about the observed inhibitory effects.

In conclusion, our data suggests that the butanol fraction derived from the crude ethanol extract of the leaves of *P. biglobosa* contains powerful anti-oxidative agents and inhibitors of α -glucosidase and α -amylase which could be exploited for the development of holistic therapeutic strategy for the control of postprandial hyperglycemia and other diabetic complications while phenolics could be the main bioactive agents. We are currently conducting a comprehensive investigation on the anti-diabetic activity of the butanol fraction in type 2 diabetic rats as well as isolating some pure compounds from it.

Postscript: From the above studies, the butanol fraction had the best α -glucosidase and α -amylase effects and therefore it was selected for detailed *in vivo* anti-diabetic study and isolation of the possible bioactive compound.

5.4 Butanol fraction of *Parkia biglobosa* leaves modulates β cell functions, stimulates insulin secretion and ameliorates diabetic complications in a type 2 diabetes model of rats

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Preface: This article reports the detailed *in vivo* anti-diabetic studies of the butanol fraction and the isolation of the possible bioactive anti-diabetic compound. It is yet to be submitted for publication.

5.4.1 Abstract

This study investigated the anti-diabetic effects and the mechanism of action of the butanol fraction of *Parkia biglobosa* leaves (PBBF) in a type 2 diabetes (T2D) rats model as well as the isolation of possible bioactive compound from the fraction. T2D was induced in rats by feeding with a 10% fructose solution *ad libitum* for two weeks followed by intraperitoneal injection of 40 mg/kg bw streptozotocin and the animals were treated with 150 and 300 mg/kg bw of the fraction for four weeks. The PBBF treatments significantly ($P < 0.05$) decreased the blood glucose concentrations and enhances the glucose tolerance capacity compared to untreated diabetic rats. Furthermore, the treatments were found to improve β -cell function, stimulate insulin secretions, decrease insulin resistance, restore liver glycogen, ameliorate serum lipids alterations and prevent hepatic and renal damages compared to untreated diabetic rats. Phytochemical analysis of the fraction led to the isolation to lupeol which inhibited α -glucosidase and α -amylase with IC_{50} values of 45.12 ± 3.47 and 256.47 ± 16.47 μ g/ml. Kinetic studies revealed that lupeol is a non-competitive inhibitor of α -glucosidase and an uncompetitive inhibitor of α -amylase. The findings of this study suggest that PBBF possessed remarkable anti-T2D activity which is mediated through modulation of β -cell function and stimulation of insulin secretion while lupeol is an important bioactive anti-T2D agent in the fraction.

5.4.2 Introduction

Type 2 diabetes (T2D) is a complex, heterogenous and polygenic disorder associated with insulin resistance and pancreatic β -cell dysfunction [52]. Impaired postprandial insulin secretion due to functional defects and the loss of surviving pancreatic β -cells leads to hyperglycemia and a subsequent decline in insulin sensitivity [53]. At present, the use of insulin secretagogues and sensitizers as well as of inhibitors of carbohydrate-hydrolyzing enzymes such as α -glucosidase and α -amylase are the commonly exploited approach for the treatment of the disease [37]. Although a number of antidiabetic drugs are currently used for T2D treatment, adverse reactions and side effects are serious impediments to their use [54]. Recently, many researchers are searching medicinal plants or dietary interventions to

prevent or treat T2D. In this context, African medicinal plants may provide the much needed alternative therapies because of their multiple health benefits [22].

Parkia biglobosa (Jacq.) Benth. Is also called the African locust bean tree and belongs to the family Mimosaceae which is native to Nigeria and other West African countries. It is highly reputed for numerous medicinal values [1,8] and all of the different plant's part are exploited in traditional medicine to cure several metabolic or non-metabolic disorders such as hypertension, haemorrhages and dermatitis [2, 15]. Also, the fermented seeds of the plant are also frequently used as natural nutritional condiment with different local names such as 'afitin' in Benin, 'nététou' in Senegal, and dawadawa in Nigeria [15]. Scientific reports have validated the analgesic and anti-inflammatory [11], antivenom [10], antidiarrhoeal [12], antibacterial [8], vasorelaxant [15], wound healing [14] and anti-oxidative [45] activities of extracts from various parts of the plant.

On the other hand, ethnopharmacological surveys revealed that *P. biglobosa* is commonly used in the traditional remedy of diabetes mellitus in northern Nigeria [6] and the central region of Togo [7]. In a previous study, the fermented seed extract was also reported to possess hypoglycemic activity in an alloxan-induced type 1 diabetes model of rats [16]. However, the anti-diabetic effects of the parts of the plant which are actually used for the traditional management of diabetes mellitus have not yet been examined either in humans or experimental animal models of T2D.

Hence, the present study was conducted to comprehensively evaluate the *in vivo* anti-diabetic activity of the butanol fraction of *P. Biglobosa* leaves in a T2D model of rats as well as to isolate a pure α -glucosidase and α -amylase inhibitory compound from the fraction. Additionally, the mechanism of α -glucosidase and α -amylase inhibition by the active compound was determined using the enzyme kinetics approach.

5.4.3 Materials and methods

Please refer to the chapter 2 sub-sections 2.3-2.4 (pages 35-36); 2.6.1-2.6.4 (pages 38-40); 2.7.1-2.7.7 pages (40-43); 2.8.2 page 46 for detailed materials and methods that affect *P. biglobosa*. However, the group codes DFL, DFH and NFT used in the chapter two are replaced with DPL, DPH and NPT respectively. PBBF refers to the butanol fraction derived from the crude ethanol extract of *P. biglobosa* leaves.

5.4.4 Results

The DBC group had significantly higher mean feed and fluid intake than the NC group while the PBBF-treated groups had relatively lower feed and fluid intakes compared to the DBC group. The PBBF treatment did not significantly ($P < 0.05$) affect the feed and fluid intakes of normal rats (**Figure 5.4.1**). Body weight gain was significantly retarded in the DBC group but the PBBF treatment caused an

insignificant ($P>0.05$) increase in the body weight gain especially in the last three weeks of the experiment (**Figure 5.4.2**).

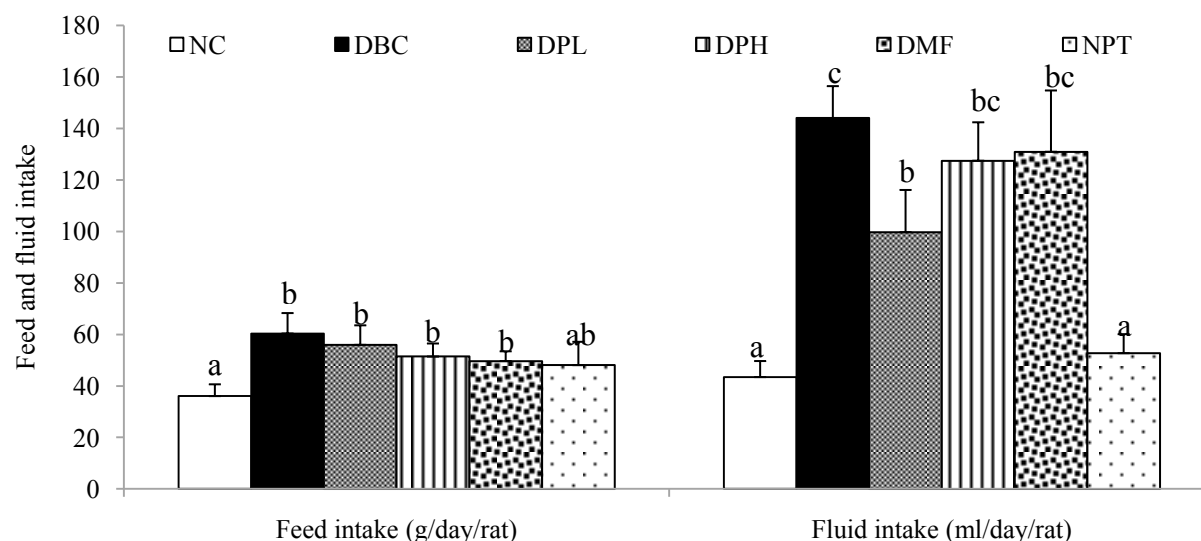


Figure 5.4.1: Feed and fluid intakes of different groups of during the experimental period. The results are expressed as the mean \pm SD of eight animals. ^{a-c}Different alphabets over the bars for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

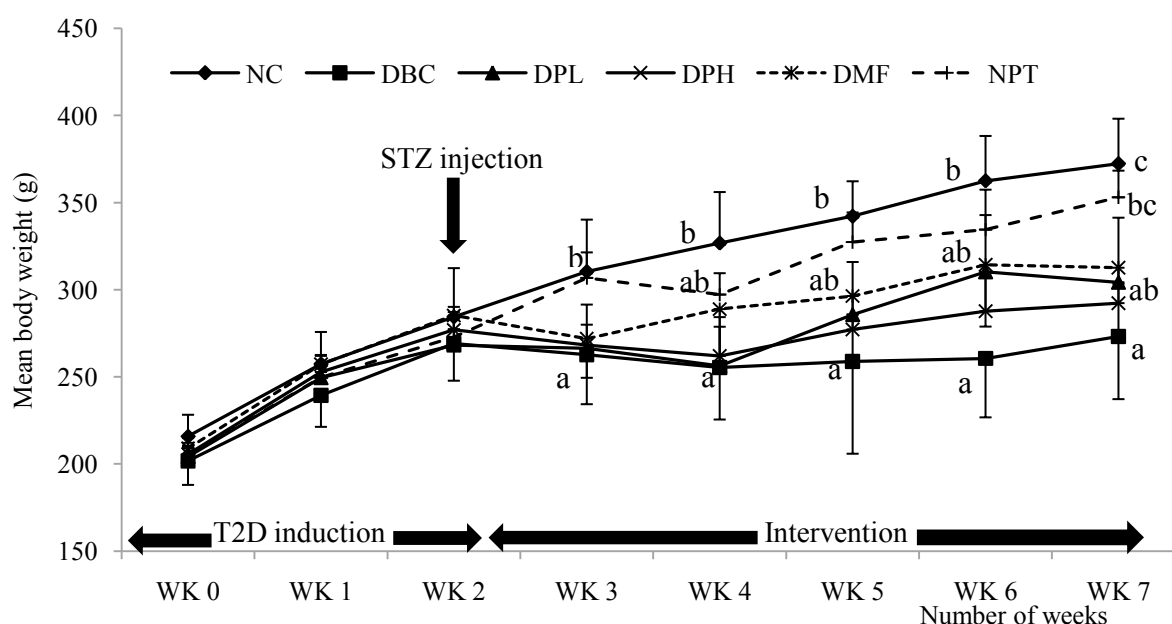


Figure 5.4.2: Mean body weight gain for all groups of experimental animals over the seven weeks experimental period. The results are expressed as the mean \pm SD of eight animals. ^{a-c}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

The profile of the weekly NFBG levels indicated that all diabetic groups had an elevated blood glucose level at the first week after induction (**Figure 5.4.3**). However, the PBBF treatment significantly ($P<0.05$) decreased the T2D-induced elevation in NFBG in the first week after treatment and the pattern was consistent throughout the experimental period. It is noteworthy that the NFBG of the DPL group was

brought to near normal and was significantly ($P<0.05$) lowered compared to the DPH group. In the OGTT, a rise in blood glucose levels was observed in all animal groups after 30 min of glucose administration but thereafter, the PBBF-treated groups recorded a significantly lowered blood glucose levels compared to the DBC group which was maintained throughout the 120 min experiment (**Figure 5.4.4**). Also, the DPL group recorded significantly ($P<0.05$) better glucose tolerance ability compared to the DPH group. The serum insulin concentrations and β -cell function (HOMA- β) of the DBC group were significantly ($P<0.05$) lowered compared to the NC group but the PBBF-treated groups recorded significantly ($P<0.05$) higher insulin levels and β -cell function than the DBC group (**Table 5.4.1**). Indeed, the DPL group had a significantly higher ($P<0.05$) insulin concentration than normal rats. Conversely, serum fructosamine concentration and insulin resistance (HOMA-IR) were significantly ($P<0.05$) elevated in the DBC group but they were insignificantly ($P>0.05$) ameliorated in the PBBF-treated groups.

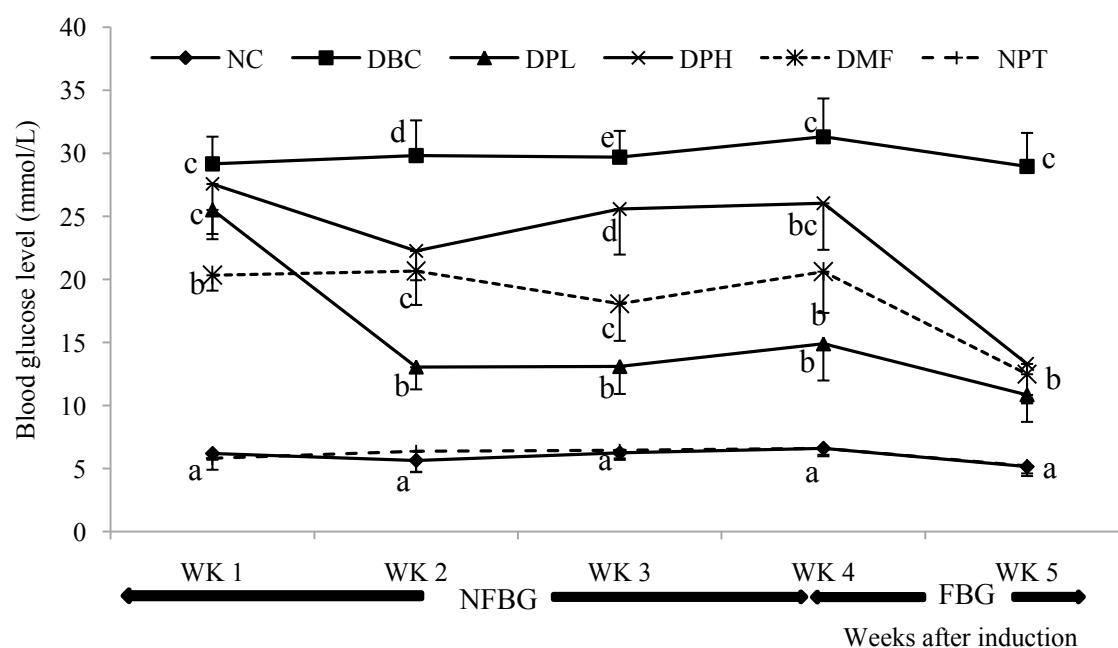


Figure 5.4.3: The effects of different doses of PBBF on weekly blood glucose levels (post induction). Results are expressed as the mean \pm SD of eight animals ^{a-e}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

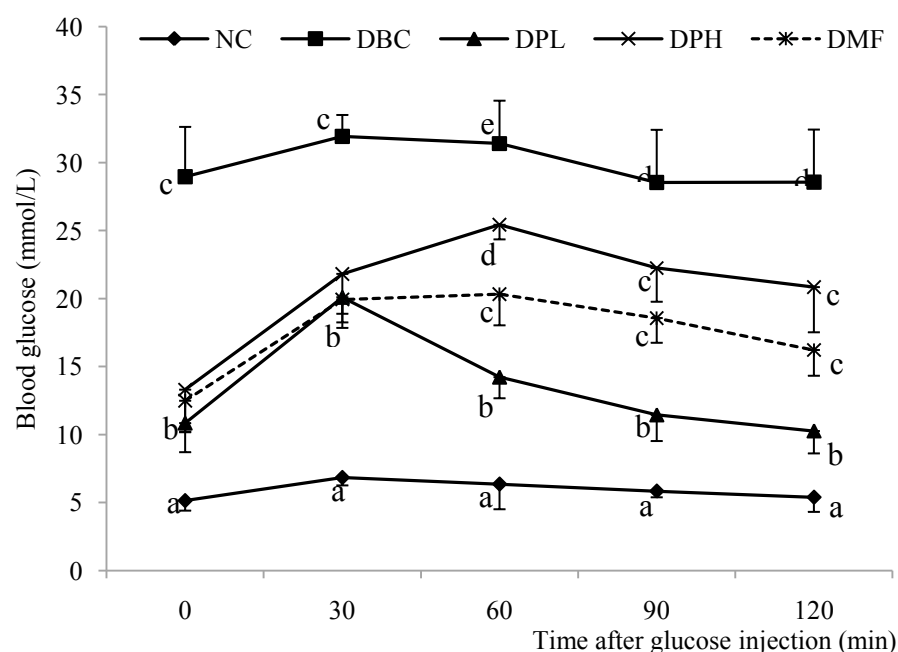


Figure 5.4.4: Oral glucose tolerance test (OGTT) for all groups of animals in the last week of the experiment. Results are expressed as the mean \pm SD (n=8). ^{a-d}Different alphabets for a given time indicate significant difference from each other (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 5.4.1: Serum insulin and fructosamine concentrations, indices of hepatic and renal damages as well as computed HOMA-IR and HOMA- β scores for the animal groups at the end of the experiment

	NC	DBC	DPL	DPH	DMF	NPT
Serum insulin (pmol/L)	153.1 \pm 9.9 ^{bc}	64.2 \pm 29.9 ^a	192.6 \pm 21.5 ^c	128.7 \pm 20.9 ^b	119.1 \pm 13.0 ^b	159.4 \pm 3.7 ^c
Serum fructosamine (μ mol/L)	196.3 \pm 5.5 ^a	258.4 \pm 10.9 ^b	240.2 \pm 26.5 ^b	243.0 \pm 21.5 ^b	253.8 \pm 30.3 ^b	195.1 \pm 18.6 ^a
HOMA-IR	4.9 \pm 1.1 ^a	16.2 \pm 5.6 ^b	8.1 \pm 3.8 ^b	9.6 \pm 2.51 ^b	9.8 \pm 3.7 ^b	5.1 \pm 0.7 ^a
HOMA- β	304.1 \pm 53.7 ^d	9.6 \pm 5.2 ^a	106.8 \pm 46.4 ^c	28.6 \pm 12.2 ^b	54.8 \pm 27.5 ^{bc}	299.9 \pm 83.3 ^d
AST (U/L)	88.0 \pm 14.8	73.6 \pm 7.6	79.6 \pm 12.5	73.7 \pm 8.8	88.6 \pm 17.8	80.1 \pm 7.9
ALT (U/L)	56.3 \pm 12.6 ^a	77.7 \pm 4.5 ^b	75.8 \pm 22.1 ^{ab}	71.0 \pm 12.9 ^{ab}	63.3 \pm 18.1 ^{ab}	48.85 \pm 8.1 ^a
ALP (U/L)	188.8 \pm 19.8 ^a	898.6 \pm 174.5 ^c	333.8 \pm 26.1 ^b	426.6 \pm 87.6 ^b	472.0 \pm 74.9 ^b	155.4 \pm 48.6 ^a
Urea (mg/dl)	48.0 \pm 6.2 ^a	93.0 \pm 16.4 ^b	53.5 \pm 18.7 ^a	63.4 \pm 20.4 ^{ab}	42.0 \pm 11.7 ^a	41.63 \pm 9.37 ^a
Creatinine (μ g/dl)	582.0 \pm 70.5 ^{ab}	485.0 \pm 40.4 ^a	548.3 \pm 36.0 ^{ab}	555.0 \pm 52.3 ^{ab}	675.0 \pm 95.2 ^b	471.2 \pm 42.9 ^a

The results are expressed as the mean \pm SD (n=8). ^{a-d}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The data for the liver weights and glycogen concentration of the experimental animals is presented in **Table 5.4.2**. No significant differences in the absolute liver weights of all groups of animals were observed, however, the relative liver weights of the diabetic groups were significantly higher than NC and NPT groups. The PBBF-treated groups recorded a significantly lower ($P<0.05$) relative liver weight in comparison to DBC group. The liver glycogen store was significantly ($P<0.05$) depleted in the DBC group compared to the NC group but the PBBF treatment significantly ($P<0.05$) boosted the liver glycogen reserves in the diabetic animals.

Table 5.4.2: The effects of different doses of PBBF on liver weights and liver glycogen concentrations of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
Liver weight (g)	11.8±2.4	10.9± 1.9	11.2± 0.9	9.9±0.4	10.4±1.7	10.7±1.4
Relative liver weight (%)	3.3±0.3 ^a	3.9± 0.2 ^c	3.6± 0.2 ^b	3.6±0.2 ^b	3.7±0.1 ^b	3.1±0.2 ^a
Liver glycogen (mg/g tissue)	3.5±0.8 ^{bc}	1.5±0.1 ^a	3.7± 0.6 ^c	2.6±0.3 ^b	2.6±0.4 ^b	2.6±0.4 ^b

Results are expressed as mean ± SD (n=8). ^{a-c}Different alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 5.4.3 presents the serum lipid profile of all groups of experimental animals. Both PBBF and T2D did not significantly affect total and LDL cholesterol except in the DPH group which had significantly ($P<0.05$) higher total cholesterol in comparison to NC group. On the contrary, DBC group had a significantly ($P<0.05$) lower concentration of HDL-cholesterol which was boosted in the PBBF treated diabetic groups. Furthermore, T2D caused a significant ($P<0.05$) elevation in the level of serum triglycerides which was ameliorated in the PBBF treated diabetic groups. Results of other indices of diabetic complications also indicated that the T2D-induced increase in ALT, ALP and urea were ameliorated by the PBBF treatments (**Table 5.4.1**). Moreover, relatively lower serum levels of AST and creatinine were observed in the DBC group which were boosted in the DPL and DPH groups.

Table 5.4.3: The effects of different doses of PBBF on serum lipid profiles of type 2 diabetic rats

Serum lipids	NC	DBC	DPL	DPH	DMF	NPT
			(mg/dl)			
Total cholesterol	66.3±6.4 ^a	76.3±10.6 ^{ab}	73.5±5.8 ^{ab}	81.4±5.9 ^b	74.6±12.1 ^{ab}	69.1±16.4 ^{ab}
HDL cholesterol	31.0±9.6 ^b	19.8±1.6 ^a	26.3±4.3 ^b	25.8±3.5 ^b	28.1±5.4 ^b	27.2±8.8 ^{ab}
LDL cholesterol	19.9±5.4 ^a	27.5±4.3 ^a	22.2±4.9 ^a	30.3±9.4 ^a	25.9±7.4 ^a	17.1±5.1 ^a
Triglycerides	102.5±27.5 ^a	184.8±40.9 ^b	113.2±39.1 ^{ab}	118.2±34.2 ^{ab}	120.1±27.4 ^{ab}	111.3±28.2 ^a

Results are expressed as mean ± SD (n=8). ^{a-b}Different alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Histopathological examination of the pancreas indicated massive destruction of pancreatic islets with small number of β cells in the DBC group compared to NC. However, the PBBF treatments protected the T2D-associated destruction of β cells because larger islets with higher number of β cell were detected in the DPL and DPH groups. It is also noteworthy that the DPL group had more β cells than DPH group but they appear morphologically healthier in the DPH group than the DPL (Figure 5.4.5).

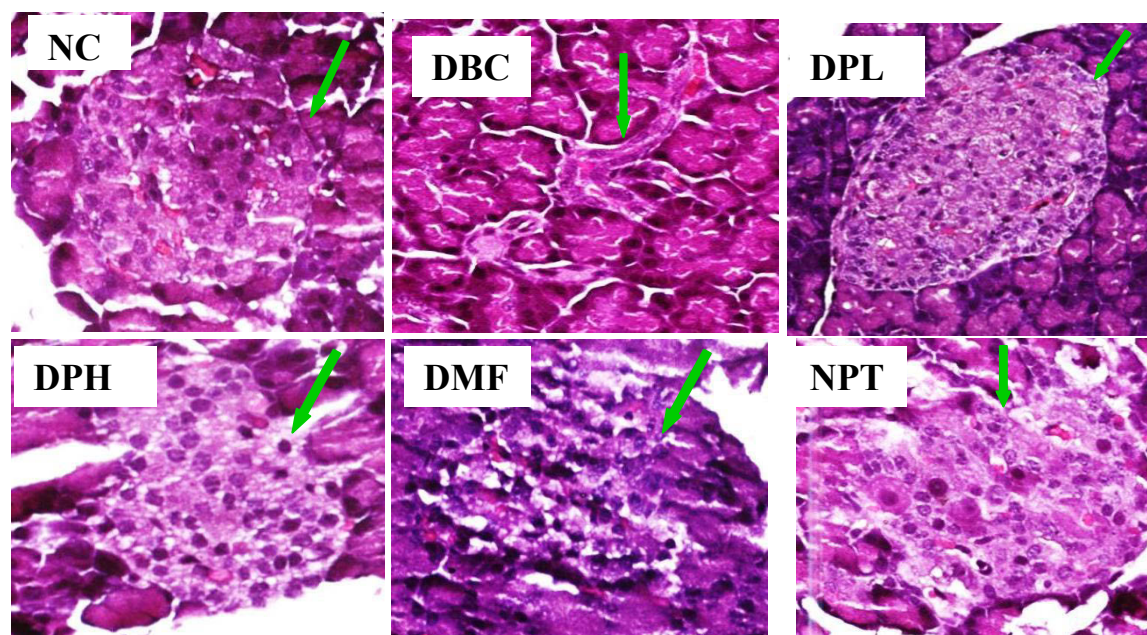


Figure 5.4.5: Histopathological pictures of the pancreas of different experimental groups at the end of the experiment. The NC had high number of β -cell while the DBC had highly dispersed and morphologically deformed β -cells. The DPL, DPH and DMF groups had higher number of β -cells compared to DBC. Higher dose of the PBBF causes slight damage to pancreatic islets as well as the β -cells as evidenced in the NPT group.

The structure of the active compound was identified as lupeol (**Figure 5.4.6**) through detailed spectroscopic analysis and confirmed by comparison with data from the literature [55, 56]. The ^1H NMR spectrum showed two doublet resonances at δ 4.66 and 4.54 corresponding to the germinal olefinic protons (H-29a and H-29b) of the ring D isopropenyl side chain (Appendix 3). The H-3 oxygenated proton was seen at δ 3.20 and the methyl resonances between δ 0.70 and δ 1.70. The ^{13}C NMR resonances at δ 150.94 and δ 109.32, attributed to the olefinic carbon atoms and the oxygenated carbon resonance at δ 79.02 further support the structure of lupeol (Appendix 4).

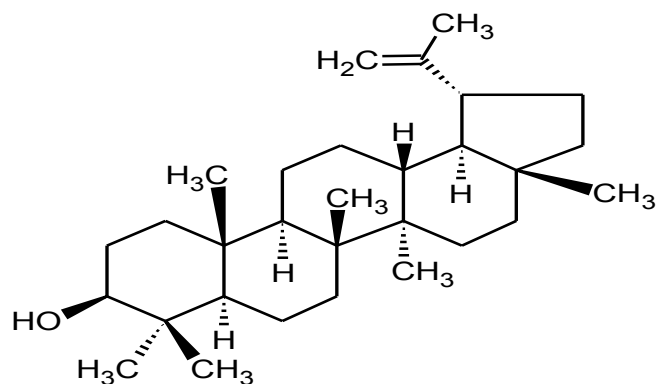


Figure 5.4.6: Structure of the lupeol isolated from the butanol fraction of *P.biglobosa* leaves

Results from the inhibitory effects of lupeol on α -glucosidase and α -amylase catalyzed reactions revealed that the compound inhibited the enzymes with IC_{50} values of 45.12 ± 3.47 and 256.47 ± 16.47 $\mu\text{g/ml}$ for α -glucosidase and α -amylase inhibitions respectively. Using the enzyme kinetic approach, lupeol was found to be a non-competitive inhibitor of α -glucosidase with V_{max} decreasing from 655.09 $\mu\text{mol/min}$ to 56.84 $\mu\text{mol/min}$ while the K_M remained unchanged at 2 mM (**Figure 5.4.7A**). On the other hand, lupeol displayed an uncompetitive inhibition pattern for α -amylase inhibition thereby decreasing both the V_{max} and K_M of the enzyme (**Figure 5.4.7B**). The V_{max} decreased from 33.70 $\mu\text{mol/min}$ to 0.75 $\mu\text{mol/min}$ while the K_M decreased from 0.25% to 0.14%.

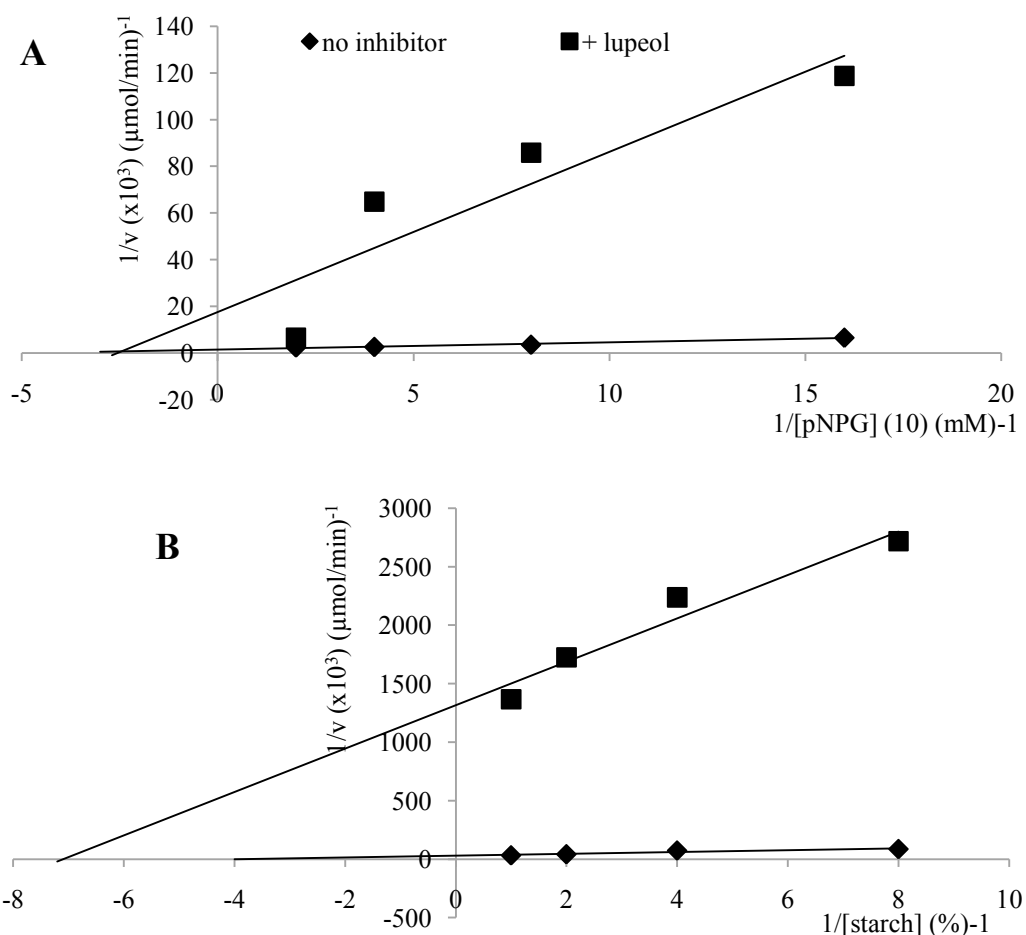


Figure 5.4.7: Lineweaver-Burke's plot for α -glucosidase (A) and α -amylase (B) catalysed reactions in the presence and absence of lupeol as an inhibitor

5.4.5 Discussion

A preliminary study on the hypoglycemic and antihyperlipidemic activities of the fermented seeds of *P. biglobosa* has been reported [16]. So far, information on the anti-T2D activity and the mechanism of action from traditionally used parts of *P. biglobosa* does not appear in the literature. The present study demonstrated that the butanol fraction of *P. biglobosa* stem bark possesses exceptionally high anti-T2D effect which is mediated through stimulation of β -cell function and insulin secretion.

The observed effects of PBBF on polyphagia, polydipsia, and body weight loss caused by T2D suggest that the fraction has a tendency to reverse these alterations caused by the disease. Shorter experimental period may be the reason for the insignificant amelioration of T2D-induced polyphagia and polydipsia as well as body weight gain.

Fasting or postprandial hyperglycemia is a common pathogenesis of T2D which is induced by insulin resistance and partial pancreatic β -cell destruction [57]. Effective control of the blood glucose level holds

a vital position in preventing diabetic complications as well as enhancing the living standard of type 2 diabetic patients [58]. The ability of PBBF to effectively and consistently control the hyperglycemia in diabetic animals could be attributed to anti-hyperglycemic, rather than hypoglycemic, activity because the PBBF treatment did not lower the blood glucose level of normal rats. In addition to the remarkable anti-hyperglycemic effects, the PBBF treatment also resulted in an improvement of glucose tolerance which could be associated with the increased insulin secretions [59] and/or relatively lower insulin resistance observed in the PBBF-treated animals and this is known to promote more glucose uptake by cells.

The effects of PBBF on serum insulin levels, HOMA-IR and HOMA- β scores as well as pancreatic histopathology were determined partly to give an insight into the mechanism of action. The elevated serum insulin levels in diabetic animals observed in the PBBF-treated diabetic animals suggest that the fraction is an insulin secretagogue whereas the relatively lower HOMA-IR in PBBF treated groups indicates that the fraction affects the peripheral insulin resistance. However, HOMA- β scores and pancreatic histopathology indicated that the fraction prevents T2D-induced pancreatic β -cell destruction and improves their functions. It seems therefore that, the anti-hyperglycemic activity of the fraction is related to both pancreatic (modulation of β -cells to increase the secretion of insulin) and extra pancreatic (glucose utilization at the peripheral tissues) mechanisms and/or the observed potent α -glucosidase and α -amylase inhibitory activities shown by the fraction (see manuscript 5.3 above). The anti-diabetic activity of a number of plant materials were reported to be through interference with gastrointestinal glucose absorption [60], insulinotropic action [61] or by promoting the regeneration of pancreatic β -cells [62]. Fructosamine is a product of protein glycation at an early stage which undergoes oxidative cleavage resulting in the formation of advanced glycation end products causing diabetic complications [63]. It is a useful indicator in monitoring middle term changes after alterations in the management of diabetes. Our study indicated that the PBBF relatively lowered the serum fructosamine which could suggest that the fraction interrupts the glycation cascade, thereby reducing the risk of diabetic complications.

The most important form of glucose storage is in the form of glycogen and its levels in different tissues especially the skeletal muscle provides a clear reflection of the activity of insulin. Insulin promotes intracellular glycogen deposition by stimulating glycogen synthase and inhibiting glycogen phosphorylase [64]. Thus, the reduced glycogen store in the DBC group may be attributed to the reduced insulin secretion with corresponding decrease in glycogen synthase activity and increase in glycogen phosphorylase activity. Based on the above, we speculated that the restoration of liver glycogen level in the PBBF treated animals might be linked to the insulinotropic activity of the fraction which could increase glycogen synthase activity and inhibit glycogen phosphorylase activity.

Although the results were not significant, an increasing tendency in the serum total and LDL cholesterol was observed in the DBC group. On the other hand, significant hypertriglyceridemia was also observed in the DBC compared to the NC group. The observed increase in serum lipids in diabetic animals is linked to the sensitivity of fatty acid mobilization (from adipose tissue) to the action of insulin. One of the most powerful actions of insulin is to reduce the process of lipolysis in the tissues [65]. Indeed, an increase in serum insulin level by only 5 IU/ml could inhibit lipolysis by 50%, while a decrease in insulin levels could profoundly accelerate tissue lipolysis [66]. Therefore, we also linked the observed restoration of these serum lipids in the PBBF treated groups to the insulintropic action of the fraction which may result in the inhibition of lipolysis with a corresponding decrease in the serum lipids. Moreover, the result suggests that the PBBF has a tendency to reduce T2D-associated complications such as coronary heart disease.

The serum levels of ALT and ALP but not AST were significantly increased in the untreated diabetic animals indicating impaired liver function, which is due to hepatocellular necrosis. Diabetic complications such as increased gluconeogenesis and ketogenesis is usually associated with elevated transaminase activities [67]. Therefore, restoration of these biomarker enzymes towards normal level indicates decreased diabetic complications in PBBF treated groups. Furthermore, the fraction reversed the T2D-induced increase in serum urea level which could suggest decreased renal impairments associated with diabetic complications. Apart from assessing renal damage, creatinine was reported to be a predictor for T2D and insulin resistance [68]. Creatinine is a product of creatine metabolism which mostly occur in skeletal muscle, the major center for the action of insulin and subsequent glucose disposal. Muscle mass has been proposed to inversely correlate with insulin resistance but directly correlate with serum creatinine. Thus, the serum creatinine could be a reliable marker to assess insulin resistance [68]. Indeed, previous studies have demonstrated lower serum creatinine level in T2D human subjects compared to normal individuals [68] which corroborates with our findings. It is thus plausible to suggest that the relatively lower insulin resistance (HOMA-IR) of the PBBF treated diabetic rats compared to the DBC group translated to a relatively elevated serum creatinine levels in these groups.

The anti-diabetic effects of DPL group were better than DPH group in most of the parameters measured in this study. One of the possible reasons for this observation might be the toxicological effects of the PBBF at higher dose (300 mg/kg bw) which was reflected in the histopathological results of the NPT group where pancreatic islets were slightly damage. This could eventually lead to a reduced function of the pancreas and ultimately decrease the overall anti-diabetic effects of the fraction at the high dose. Another possible explanation for the observation is that the fraction underwent hormesis phenomenon. Hormesis is a dose-response relationship for a single endpoint that is associated with changes of responses between high and low dosages of biological molecules, chemicals, or any other agent that can

initiate a response [29] and its occurrence has been documented in numerous biological, toxicological and pharmacological investigations [30].

After the anti-T2D of the PBBF was confirmed by *in vivo* studies, we attempted to investigate the potential bioactive anti-T2D agents in the fraction. A pentacyclic triterpene, lupeol was isolated from the PBBF and was subjected to *in vitro* α -glucosidase and α -amylase inhibitory assays. Although previous studies have demonstrated the α -glucosidase and α -amylase inhibitory activity of lupeol isolated from other plants [69, 70], we further investigated the mechanism of the inhibitions using the enzyme kinetics approach, which to our knowledge had not been done previously. Results from the enzyme kinetics studies indicated that lupeol mediates α -glucosidase and α -amylase inhibitory action through non-competitive and uncompetitive inhibition patterns respectively. These suggest that lupeol binds both to the free α -glucosidase protein or the α -glucosidase-pNPG complex and consequently reduces the rate of product formation. Conversely, lupeol decreased the rate of starch hydrolysis only through binding to the α -amylase-starch complex.

In summary, results from this study suggest that treatment of diabetic animals with the butanol fraction of *P. biglobosa* leaves leads to the stimulation of β -cell function and insulin secretion with a corresponding decrease in postprandial blood glucose levels and other T2D-associated complications whilst a dose of 150 mg/kg bw was more effective than 300 mg/kg bw possibly due to toxicological effects of the fraction at a higher dose. Furthermore, lupeol could be involved in decreasing postprandial hyperglycemia through α -glucosidase and α -amylase inhibitions.

Postscript: In order to further explore the possible mechanism of anti-diabetic action of the butanol fraction, the serum, liver, kidney, heart and pancreas were investigated for *in vivo* anti-oxidative parameters

5.5 The anti-diabetic effects of the butanol fraction of *Parkia biglobosa* leaves could be mediated via modulation of *in vivo* antioxidant status

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Preface: This article describes the *in vivo* anti-oxidative status of the serum and organs collected from the rats in the above experiment to determine whether an anti-oxidative dependent mechanism is involved in the anti-diabetic effects of the fraction.

5.5.1 Abstract

The effects of a butanol fraction of *Parkia biglobosa* leaves (PBBF) on the antioxidant status of the serum, liver, kidney, heart and pancreas of type 2 diabetic rats were investigated. Type 2 diabetes (T2D) was found to induce oxidative stress as manifested by a significant ($P < 0.05$) increase in the thiobarbituric acid reactive substances (TBARS) and significant ($P < 0.05$) decrease in the glutathione, superoxide dismutase (SOD) and catalase in the serum and organs under study. However, the PBBF treatment significantly ($P < 0.05$) ameliorated the T2D-induced changes in the TBARS levels glutathione, SOD and catalase in the serum, liver and pancreas of the type 2 diabetic rats. In the kidney, the T2D-decrease in glutathione, SOD and catalase was significantly ameliorated by the PBBF treatment but the reduction in TBARS level was not significant ($P > 0.05$). In the heart, the T2D-induced changes in TBARS level, glutathione and SOD were significantly ($P < 0.05$) ameliorated but the increase in catalase level was not significant ($P > 0.05$). It was concluded that PBBF contains therapeutically active phytochemicals that could modulate oxidative stress associated with T2D.

5.5.2 Introduction

Hyperglycemia is a hallmark of diabetes mellitus and has been implicated in the pathogenesis of macro and microvascular complications associated with the disease [36]. This is achieved via auto-oxidation of glucose and non-enzymatic protein glycation leading to the generation of advanced glycation end products as well as activation of protein kinase C which consequently results in excess free radicals in the body [20]. These highly reactive species attack membrane phospholipids and proteins leading to lipid peroxidation and a decrease in the levels of endogenous enzymatic (glutathione) and non-enzymatic (catalase, superoxide dismutase) antioxidants, a phenomenon called oxidative stress [20]. Thus, oxidative stress holds a central position in the secondary complications associated with diabetes mellitus and indeed all other aspects of the disease pathogenesis such as insulin resistance and pancreatic β cell dysfunction [38-40]. For these reasons, it is regarded as an important mechanism of diabetic pathogenesis.

Parkia biglobosa (Jacq.) Benth (Mimosaceae) is a traditional medicinal plant commonly exploited in the treatment of diabetes mellitus in Nigeria and Togo [6, 7]. In order to validate the claim, we conducted preliminary *in vitro* anti-oxidative studies on various crude extracts of the stem bark, root and leaf samples of the plant using complementary assays [45] and observed that the crude ethanol extract of the leaves had the best anti-oxidative activity. In a follow up study, butanol fraction obtained from the crude ethanol extract of the leaves, was subjected to an *in vivo* anti-diabetic study in a T2D rats model where it demonstrated potent anti-hyperglycemic activity and improved β -cell function, stimulated insulin secretions, decreased peripheral insulin resistance, ameliorated serum lipids alterations as well as prevented liver and kidney damages associated with T2D (see 5.4 above). In order to further probe, whether or not, the observed remarkable anti-diabetic activity was mediated through an anti-oxidative dependent mechanism, in the present study, the *in vivo* anti-oxidative status of the serum, liver, kidney, heart and pancreas of type 2 diabetic animals treated with the fraction was investigated.

5.5.3 Materials and methods

Please refer to the chapter two sub sections 2.3-2.4 (pages 35-36); 2.7.1-2.7.5 page 41-42; and 2.7.8 (2.7.8.1-2.7.8.4) page 44-45 for detailed materials and methods that affect *P. biglobosa*

5.5.4 Results

The TBARS level in the liver and kidney of the DBC group were significantly ($P<0.05$) higher than NC group but the DPH group recorded lower levels of TBARS than the DBC group and the difference was significant ($P<0.05$) in the liver (**Table 5.5.1**) and not the kidney (**Table 5.5.2**). The glutathione, SOD and catalase levels in the liver and kidney were significantly ($P<0.05$) lowered in the DBC group compared to the NC group but the DPL and DPH groups had significantly ($P<0.05$) higher levels of glutathione, SOD and catalase in both liver (**Table 5.5.1**) and kidney (**Table 5.5.2**) compared to the DBC group.

Table 5.5.1: Effects of PBBF on hepatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
TBARS (μM)	10.61 \pm 3.02 ^a	33.8 \pm 4.96 ^c	31.78 \pm 5.65 ^c	23.89 \pm 6.59 ^{bc}	35.94 \pm 9.46 ^c	17.82 \pm 2.23 ^b
Glutathione (μM)	120.82 \pm 6.01 ^c	29.99 \pm 9.34 ^a	137.45 \pm 15.06 ^c	75.28 \pm 10.28 ^b	73.62 \pm 10.22 ^b	81.64 \pm 12.90 ^b
Superoxide dismutase (nmol/min)	25.54 \pm 5.28 ^b	12.37 \pm 1.62 ^a	21.49 \pm 4.62 ^b	21.81 \pm 6.06 ^b	17.53 \pm 5.15 ^{ab}	19.14 \pm 5.45 ^b
Catalase ($\mu\text{mol/min}$)	16.5 \pm 0.92 ^c	1.54 \pm 0.27 ^a	8.57 \pm 2.12 ^b	13.14 \pm 3.39 ^b	1.67 \pm 0.57 ^a	20.05 \pm 4.37 ^c

Results are expressed as mean \pm SD (n=5). ^{a-c}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 5.5.2: Effects of PBBF on kidney lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
TBARS (μM)	14.1 \pm 5.8 ^a	40.8 \pm 12.5 ^b	48.2 \pm 13.5 ^b	33.7 \pm 9.9 ^b	33.4 \pm 10.0 ^b	10.0 \pm 3.9 ^a
Glutathione (μM)	68.2 \pm 20.8 ^b	46.0 \pm 8.6 ^a	89.8 \pm 19.5 ^b	113.9 \pm 8.7 ^c	70.8 \pm 10.4 ^b	121.6 \pm 10.1 ^c
Superoxide dismutase (nmol/min)	16.1 \pm 3.4 ^c	6.8 \pm 1.5 ^a	7.6 \pm 1.8 ^b	13.0 \pm 2.6 ^c	11.5 \pm 4.4 ^{bc}	9.9 \pm 2.8 ^{bc}
Catalase ($\mu\text{mol/min}$)	1.7 \pm 0.3 ^b	0.9 \pm 0.2 ^a	3.2 \pm 0.9 ^c	2.6 \pm 0.2 ^c	1.6 \pm 0.4 ^b	1.9 \pm 0.3 ^b

Results are expressed as mean \pm SD (n=5). ^{a-c}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

In the serum, heart and pancreas, the DBC group also had significantly (P<0.05) higher levels of TBARS as well as significantly (P<0.05) lower levels of glutathione, SOD and catalase than the NC group (**Table 5.5.3-5.5.5**). However, the DPL and DPH groups recorded significantly (P<0.05) higher levels of TBARS as well as significantly (P<0.05) lower levels of glutathione and SOD in the serum (**Table 5.5.3**), heart (Table 5.5.4) and pancreas (**Table 5.5.5**) compared to the DBC group. Catalase was significantly (P<0.05) elevated in the pancreas and serum of the DPL and DPH groups (**Table 5.5.4 & 5.5.5**) but was significantly (P<0.05) elevated in the heart of the DPH group only (**Table 5.5.3**).

Table 5.5.3: Effects of PBBF on lipid peroxidation and anti-oxidant status in the heart of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
TBARS (μM)	9.4 \pm 2.6 ^{ab}	24.6 \pm 8.8 ^c	9.1 \pm 1.7 ^b	11.7 \pm 3.6 ^b	33.9 \pm 5.9 ^c	6.0 \pm 1.2 ^a
Glutathione (μM)	122.2 \pm 22.0 ^c	48.6 \pm 14.6 ^a	92.7 \pm 10.6 ^b	117.3 \pm 9.4 ^c	79.2 \pm 18.3 ^{ab}	107.8 \pm 5.9 ^{bc}
Superoxide dismutase (nmol/min)	15.2 \pm 4.0 ^c	5.8 \pm 1.2 ^a	9.3 \pm 1.2 ^b	14.8 \pm 2.9 ^c	12.8 \pm 2.0 ^c	10.9 \pm 1.8 ^{bc}
Catalase ($\mu\text{mol/min}$)	2.1 \pm 0.5 ^b	0.9 \pm 0.2 ^a	1.0 \pm 0.1 ^a	1.2 \pm 0.6 ^{ab}	1.4 \pm 0.4 ^{ab}	1.9 \pm 0.4 ^b

Results are expressed as mean \pm SD (n=5). ^{a-c}Different alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 5.5.4: Effects of PBBF on pancreatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
TBARS (μM)	6.0 \pm 1.2 ^a	35.7 \pm 9.1 ^d	12.2 \pm 2.6 ^c	9.0 \pm 1.8 ^{bc}	18.4 \pm 4.4 ^c	8.5 \pm 0.5 ^b
Glutathione (μM)	80.0 \pm 8.1 ^d	6.2 \pm 3.5 ^a	22.3 \pm 3.5 ^b	27.4 \pm 5.4 ^{bc}	37.3 \pm 8.6 ^c	63.5 \pm 16.1 ^d
Superoxide dismutase (nmol/min)	2.6 \pm 0.5 ^d	0.4 \pm 0.2 ^a	1.6 \pm 0.06 ^c	0.7 \pm 0.2 ^b	1.0 \pm 0.5 ^{bc}	2.1 \pm 0.5 ^{cd}
Catalase ($\mu\text{mol/min}$)	0.7 \pm 0.2 ^b	0.35 \pm 0.04 ^a	1.4 \pm 0.3 ^c	1.3 \pm 0.2 ^c	1.11 \pm 0.2 ^c	0.7 \pm 0.08 ^b

Results are expressed as mean \pm SD (n=5). ^{a-d}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Table 5.5.5: Effects of PBBF on serum lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DPL	DPH	DMF	NPT
TBARS (μM)	63.0 \pm 15.2 ^a	201.2 \pm 19.4 ^d	103.4 \pm 18.9 ^c	148.0 \pm 17.3 ^c	142.0 \pm 32.0 ^c	31.1 \pm 13.3 ^b
Glutathione (μM)	84.5 \pm 7.7 ^d	19.9 \pm 2.5 ^a	41.2 \pm 6.6 ^b	48.3 \pm 6.9 ^b	65.2 \pm 6.8 ^c	47.0 \pm 7.9 ^b
Superoxide dismutase (nmol/min)	18.1 \pm 2.4 ^d	0.9 \pm 0.2 ^a	13.8 \pm 3.1 ^d	7.1 \pm 1.6 ^c	3.2 \pm 0.7 ^b	14.5 \pm 2.4 ^d
Catalase ($\mu\text{mol/min}$)	3.6 \pm 0.8 ^{bc}	1.5 \pm 0.4 ^a	2.8 \pm 0.1 ^b	3.8 \pm 0.4 ^c	2.4 \pm 0.5 ^b	3.9 \pm 0.3 ^c

Results are expressed as mean \pm SD (n=5). ^{a-d}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

5.5.5 Discussion

The present study demonstrated that the butanol fraction of *P. biglobosa* leaves possess potent *in vivo* anti-oxidative activity in a T2D model of rats.

Glutathione is the most important *in vivo* antioxidant biomolecule and serves as the first line of defense against reactive oxygen species whereas SOD and catalase are the two major antioxidant defense enzymes that catalyze the detoxification superoxide radicals to hydrogen peroxides which is subsequently decompose to water [71]. The PBBF treatment was found to increase the levels of glutathione, SOD and catalase in the serum and organs of diabetic rats which suggests that the fraction possesses bioactive phytochemicals that could boost these endogenous anti-oxidant reserves under diabetic condition as well as prevent T2D-induced lipid peroxidation in the blood and various organs of the body.

Oxidative stress has been reported to play a major role in insulin resistance, hepatic and kidney damages associated with diabetes [71, 40] and in our previous study, the PBBF was found to significantly reduce

the severity of these T2D-associated complications (see 5.4 above). Thus, the ability of the PBBF to retard the development of oxidative stress in the liver and kidney of diabetic animals suggests that the amelioration of the T2D-induced insulin resistance, hepatic and kidney damages by the fraction was mediated through an anti-oxidative dependent mechanism.

Cardiovascular complications are associated with T2D and are usually caused by alterations in serum lipids profile during diabetes [72]. Thus, exploration of the biochemical events occurring at cardiac tissues during diabetes may ultimately provide a clue to the management of cardiovascular diseases associated with T2D. The observed effects of PBBF on the heart anti-oxidant parameters suggest that the fraction was able to reduce an oxidative attack to the heart resulting from increased serum lipids. Hyperlipidemia has been reported to be an important factor that enhances the formation of lipid peroxides and the development of oxidative stress in T2D [73] and in our previous study, we observed an anti-hyperlipidemic activity of the PBBF in diabetic rats (see 5.4 above). Based on the foregoing, it is possible to surmise that the detrimental effects of the T2D-induced hyperlipidemia to the cardiac tissues were reversed via an anti-oxidative dependent mechanism.

A number of studies have reported that pancreas has a very limited amount of ROS detoxifying enzymes (catalase and superoxide dismutase) which makes it highly susceptible to oxidative damage [38, 74]. Indeed, this phenomenon is a major event responsible for pancreatic β -cell death and subsequent decline in insulin secretion [75]. Interestingly, our findings also suggest that the pancreas has a lower amount of anti-oxidant enzymes compared to other organs. The observed effects of the PBBF on the pancreatic antioxidant parameters indicate that the fraction is capable of reducing the number of pancreatic β -cell death and associated hypoinsulinemia. Thus, this observation could provide a biochemical rationale for the higher serum insulin levels, β -cell function (HOMA- β) and β -cell number in PBBF-treated diabetic rats compared to diabetic controls (see 5.4 above).

Taken the results of this study as a whole, it also appeared that the low dose treatment (150 mg/kg bw) confer better protection against T2D-oxidative stress than the high dose (300 mg/kg bw) which corroborates with the findings on the anti-diabetic effects of the two doses (see 5.4 above). This seemingly linear relationship is indeed very interesting because it provides strong evidence of a linkage between the anti-diabetic effects and *in vivo* anti-oxidative activity which further supports the earlier assertions on the involvement of anti-oxidative dependent processes in the overall anti-diabetic effects of the fraction.

In conclusion, findings from this study revealed that PBBF, especially at a low dose of 150 mg/kg bw, is therapeutically active in alleviating T2D-induced oxidative stress in various organs of the body and this phenomenon plays a vital role in the overall anti-T2D effects of the fraction.

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CHAPTER 6

6.0 THE ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITY OF *Khaya senegalensis* *IN VITRO* AND *IN VIVO*

Preface: This sub section (6.1.1-6.1.5) on the brief review of *Khaya senegalensis* is a synopsis of an accepted book chapter that I wrote during the course of this PhD study. However, due to space constraints, the entire book chapter was not presented in this dissertation. The reference to the book chapter is as follows:

Ibrahim M. A. and Islam M. S. (2013). Biological activities, phytochemistry and industrial potentials of *Khaya senegalensis* (Meliaceae) A. Juss. In Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics, Volume 3: page 213-234. M/S Daya Publication house, New Delhi, India.

6.1.1 *Khaya senegalensis* (Meliaceae) A. Juss



Figure 6.1: *Khaya senegalensis* (Meliaceae) A. Juss: African Mahogany (English); Cailcedrat (French); mkangazi (Swahili); Ogonwo (Yoruba, Nigeria); Madachi (Hausa, Nigeria); Ono (Igbo, Nigeria). Photo: Mohammed Auwal Ibrahim (2013), Zaria, Nigeria

6.1.2 Background

Khaya senegalensis is an evergreen tree that makes up to 15-30 m high. The leaves are compound approximately 13-33 cm. It occurs naturally in the tropics of 19 central African countries discontinuously from Senegal and neighboring countries in the west (mostly between about 8°N and 15°N but extending to about 6°N in Nigeria) through to Sudan and Uganda in the east where it extends southward to about

2°45'N. This distribution covers a wide range of climatic, altitudinal, ecological and edaphic conditions. Throughout its natural range, *K. senegalensis* has been exploited for timber, which is used for fine furniture, construction, and fuel. It was one of the first west African forest tree species to be imported by Europeans due to its excellent timber and veneer [1]. Apart from the wood and timber uses of this plant, more relevant to this dissertation, is the medicinal application of this species in the treatment of various tropical diseases.

6.1.3 Ethnomedicinal uses

Almost all parts of the plant are claimed to cure a multitude of tropical diseases prevalent in the areas where it grows and as such, it is considered as a primary health care facility to millions of Africans. In an ethnobotanical survey, Atawodi et al. [2] reported that among all other plants, *K. senegalensis* was the most commonly used plant for the treatment of trypanosomiasis in Kaduna state of Nigeria where the disease is prevalent. Similarly, in a survey of Ghanaian herbal market, *K. senegalensis* was among the medicinal plants that had highest market demand where the bark is used as blood tonic, aphrodisiac and for treating fever and malaria [3]. Reports from northern Cote-d'Ivoire also indicate that the stem bark of *K. senegalensis* is among the highly utilized medicinal plants for the treatment of wounds, diarrhea and dysentery [4]. Furthermore, a study on herbal remedies among the Tem tribe of Togo also suggests that *K. senegalensis* was among the list of plants used to cure several diseases such as malaria, anemia and gastrointestinal diseases [5]. Another ethnobotanical study from north western Nigeria also indicated that *K. senegalensis* is highly ranked in the list of medicinal plants used for the traditional management of diabetes mellitus [6].

6.1.4 Biological activities

The most widely scientifically validated pharmacological property of *K. senegalensis* extracts is perhaps, the antitrypanosomal activity. Atawodi et al. [7] and Wurochekke and Nok [8] demonstrated that extracts from *K. senegalensis* had the best *in vitro* antitrypanosomal activity using different species of trypanosomes. Subsequently, an *in vivo* activity of stem bark extracts of the plant against *T. brucei* [9] and *T. evansi* [10] was reported. More recently, Ibrahim et al. [11] reported the *in vivo* antitrypanosomal activity of a phenolics rich fraction of the stem bark where phloroglucinol and 3,4-(dihydroxyphenyl) acetic acid were identified as the possible bioactive compounds. The anti-malarial and antihelminthic activities of extracts of this plant were also validated where the stem bark extracts were found to inhibit the proliferation of *Plasmodium falciparum* [12] as well as mediate the killing of both intestinal and abdominal gastrointestinal nematode parasites [13]. Furthermore, the antibacterial and anti-inflammatory activities of the plant were also investigated. For example, Kone et al. [4] which demonstrated that ethanolic extract of *K. senegalensis* stem bark was among the ten most active plant extracts (out of fifty) against some antibiotic resistant bacteria strains and a freeze dried aqueous extract of the plant was

shown to reduce carrageenan- and croton oil-induced oedema [14,15]. The antioxidant activity of the stem bark, root and leaf samples of the plant has also been reported using 2-deoxyguanosine and hypoxanthine/xanthine assays and the stem bark possessed the highest antioxidant activity [16]. Preliminary investigations on the larvicidal [17], pesticidal [18], anti-cancer [19] and anti-anemic [20] activities of the plant's extracts have also been reported. Recently, preliminary anti-diabetic study was performed on the plant where the aqueous stem bark extract of *K. senegalensis* was found to reduce the fasting blood glucose levels of alloxan induced type 1 diabetes model of rats [21].

6.1.5 Phytochemistry

The major bioactive compounds isolated from different parts and extracts of *K. senegalensis* are limonoids. The first limonoid isolated from *K. senegalensis* in 1996 was khayanolide limonoids [22] which was followed by the isolation of a novel limonoid, 2,6-dihydroxyfissinolide and two known limonoids, fissinolide and methyl 3b-acetoxy-6-hydroxy-1-oxomeliac-14-enoate from the bark of the plant [23]. In 2001, another research group identified the presence of two mexicanolide-type limonoids named khayanone and 2-hydroxyseneganolide as well as one rearranged phragmalin limonoid 1-O-acetylkhayanolide A [24]. This is followed by the isolation of three new modified limonoids from the acetone extract of *K. senegalensis* bark which were named khayanolide D, khayanolide E and khayanoside [25]. Subsequently, a number of related limonoids were isolated and characterized from various parts of the plant [26-28]. Apart from limonoids, the only group of phytochemicals isolated from *K. senegalensis* are dimeric proanthocyanidins fisetinidol-(4 α ,6)-catechin (proanthocyanidin B3) and catechin-(4 α ,8)-catechin [29].

In order to elaborately validate the folkloric claim for anti-diabetic activity of this plant (as earlier mentioned), a systematic and comprehensive investigation on the anti-diabetic activity of this plant using *in vitro* and *in vivo* models was carried out. Initially, various solvent crude extracts of the stem bark, root and leaves of the plant were subjected to detailed *in vitro* anti-oxidative studies (using four models) from where the ethanol extract of the root was found to have the best activity. Subsequently, the ethanol extract of the root was fractionated across various solvents and the fractions were subjected to *in vitro* anti-oxidative and α -glucosidase and α -amylase inhibitory studies where the butanol fraction was found to have the best anti-oxidative and enzymes inhibitory activities. Thus, the butanol fraction was investigated for *in vivo* anti-diabetic activity in a type 2 diabetes model of rats as well as the possible mechanism of action. A pure bioactive anti-diabetic compound was also isolated from the butanol fraction.

6.2 Anti-oxidative activity and inhibition of key enzymes linked to type 2 diabetes (α -glucosidase and α -amylase) by *Khaya senegalensis*

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Preface: The *in vitro* anti-oxidative activities of the stem bark, root and leaves of the plant were investigated in this article. Subsequently, the crude extract was partitioned and the recovered solvent fractions were investigated for anti-oxidative and α -glucosidase and α -amylase inhibitory activities. This article has been submitted for publication to ‘‘Acta Pharmaceutica’’ and is currently under review.

6.2.1 Abstract

This study evaluated the *in vitro* anti-oxidative activities of *Khaya senegalensis* extracts and the inhibitory effects of some solvent fractions on α -glucosidase and α -amylase activities. The stem bark, root and leaf samples of the plant were sequentially extracted with ethyl acetate, ethanol and water and then tested for anti-oxidative activity in a series of *in vitro* models. Our findings revealed that the ethanolic extract of the root had the most potent reducing power and could scavenge DPPH, hydroxyl and nitric oxide radicals. Solvent-solvent fractionation of the root ethanolic extract yielded a butanol fraction that possessed more potent anti-oxidative activity than other (aqueous, ethyl acetate and dichloromethane) fractions. Furthermore, the butanol fraction had significantly higher ($P < 0.05$) α -glucosidase and α -amylase inhibitory activities among other fractions with IC_{50} values of 2.89 ± 0.46 and 97.51 ± 5.72 $\mu\text{g/ml}$ for α -glucosidase and α -amylase inhibitions respectively. Enzyme kinetic studies indicated that the butanol fraction is a non-competitive inhibitor for α -glucosidase with an inhibition binding constant (K_i) of 1.30 $\mu\text{g/ml}$ and a competitive inhibitor of α -amylase with a K_i of 7.50 $\mu\text{g/ml}$. GC-MS analysis revealed that the butanol fraction contains pyrogallol and phloroglucinol derivatives. Data from this study suggest that the butanol fraction derived from the ethanolic extract of *K. senegalensis* root possessed excellent anti-oxidative as well as α -glucosidase and α -amylase inhibitory activities while pyrogallol and phloroglucinol derivatives could be the bioactive constituents. The results provide a strong motivation for further detailed *in vivo* study on the fraction.

6.2.2 Introduction

Diabetes mellitus is one of the major global public health problems and its prevalence is currently increasing at an alarming rate. According to the International Diabetes Federation (IDF), about 366 million people are living with diabetes and this figure is projected to increase to 552 million by the year

2030 [30]. Between the two major types of diabetes, type 2 diabetes (T2D) is most prevalent and accounts for > 95% of the total diabetic population worldwide. Type 2 diabetes (T2D) is a heterogeneous disorder characterized by a progressive decline in insulin action (insulin resistance), followed by the inability of pancreatic β -cells to compensate for insulin resistance (β -cell dysfunction) leading to hyperglycemia [31] and oxidative stress is a major contributor to the β cell damage [32].

Oxidative stress refers to the existence of products called free radicals (molecules possessing an unpaired electron) and reactive oxygen species (ROS), which are formed in normal physiological processes but become deleterious when they are not quenched by a cascade of antioxidants systems. This can result either from an overproduction of ROS or from the inactivation of the antioxidants (AO), thereby shifting the ROS/AO balance in favor of stress [33]. In T2D, hyperglycemia induces generation of free radicals, including ROS, hydroxyl and nitric oxide (NO) radicals [34] which are responsible for oxidative stress induced pancreatic β cell destruction as well as the activation of all major pathways underlying the different components of chronic vascular diabetic complications such as glycation, sorbitol pathways among others [33]. The concept of oxidative stress as an important trigger, and postprandial hyperglycemia in the onset and progression of diabetes offer a unique therapeutic strategy for the treatment of T2D and for reducing chronic vascular complications [35]. Current therapeutic strategy for the control of postprandial hyperglycemia is the inhibition of two members of exo-acting glycoside hydrolase (α -glucosidase and α -amylase), resulting in aggressive delay of carbohydrate digestion to absorbable monosaccharides [36]. Starch and other complex polysaccharides are hydrolyzed by α -amylase to oligosaccharides that are further hydrolyzed to liberate glucose by intestinal α -glucosidase before being absorbed into the intestinal epithelium and entering blood circulation. Therefore, α -glucosidase and α -amylase inhibitors will ultimately reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, diminishing the postprandial hyperglycemia. However, the leading glucosidase inhibitors, acarbose and miglitol, are often reported to produce diarrhea and other intestinal disturbances, with corresponding bloating, flatulence, cramping and abdominal pain [37]. Randomized controlled trials with glucosidase inhibitors report these gastrointestinal side effects as the most common reason for noncompliance and early subject withdrawal [38]. Interestingly, plant based agents were reported to be a more acceptable source of glucosidase inhibitors due to their low cost and relatively better safety levels, including a lower incidence of serious gastrointestinal side effects [39]. The foregoing therefore makes research on diabetes therapy to focus attention on the search for alternative agents with potent anti-oxidative properties which could also decrease postprandial hyperglycemia, thereby providing a holistic avenue to control hyperglycemia and other diabetic complications resulting from oxidative stress.

Khaya senegalensis (Desr.) A. Juss, also known as African mahogany, is a member of the Meliaceae family and highly reputed for numerous medicinal effects. Ethnopharmacological surveys revealed that the stem bark, root and leaves of the plant are used in the traditional treatment of diabetes, malaria, anemia, diarrhea, gastrointestinal diseases, fever amongst others [3, 6]. The antitrypanosomal [9], antioxidant [16] and anticancer [19] activities of different parts of the plant have been demonstrated. Furthermore, some preliminary investigations have reported the anti-hyperglycemic activity of the aqueous stem bark extract of this plant in a type 1 diabetes model of rats [21] as well as an *in vitro* α -amylase inhibitory potential [40]. However, the complete anti-oxidative and α -glucosidase or α -amylase inhibitory activity of the extracts from different parts of this plant has not been evaluated till today.

Hence, in this study, we conducted a comprehensive and systematic investigation on the *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory activities of various extracts and solvent fractions of *K. senegalensis* using different *in vitro* models.

6.2.3 Materials and Methods

Please refer to chapter two sub-sections 2.3-2.6, page 35-40 for detailed materials and methods that affect *K. senegalensis*

6.2.4 Results

The ethanolic extracts were found to contain a significantly ($P < 0.05$) higher amount of total phenolics than other extracts with the highest amount being present in the ethanolic extract of the stem bark (**Table 6.2.1**). The total reducing power (in terms of percentage gallic acid equivalent) of the various extracts from the stem bark, root and leaves of *K. senegalensis* was compared to ascorbic acid and trolox (**Figure 6.2.1**). While all the solvent extracts from the various parts of *K. senegalensis* demonstrated $\text{Fe}^{3+} - \text{Fe}^{2+}$ reductive ability but the ethanolic extract of the root exhibited a significantly ($P < 0.05$) higher reducing power compared to the other extracts and the standard antioxidants used.

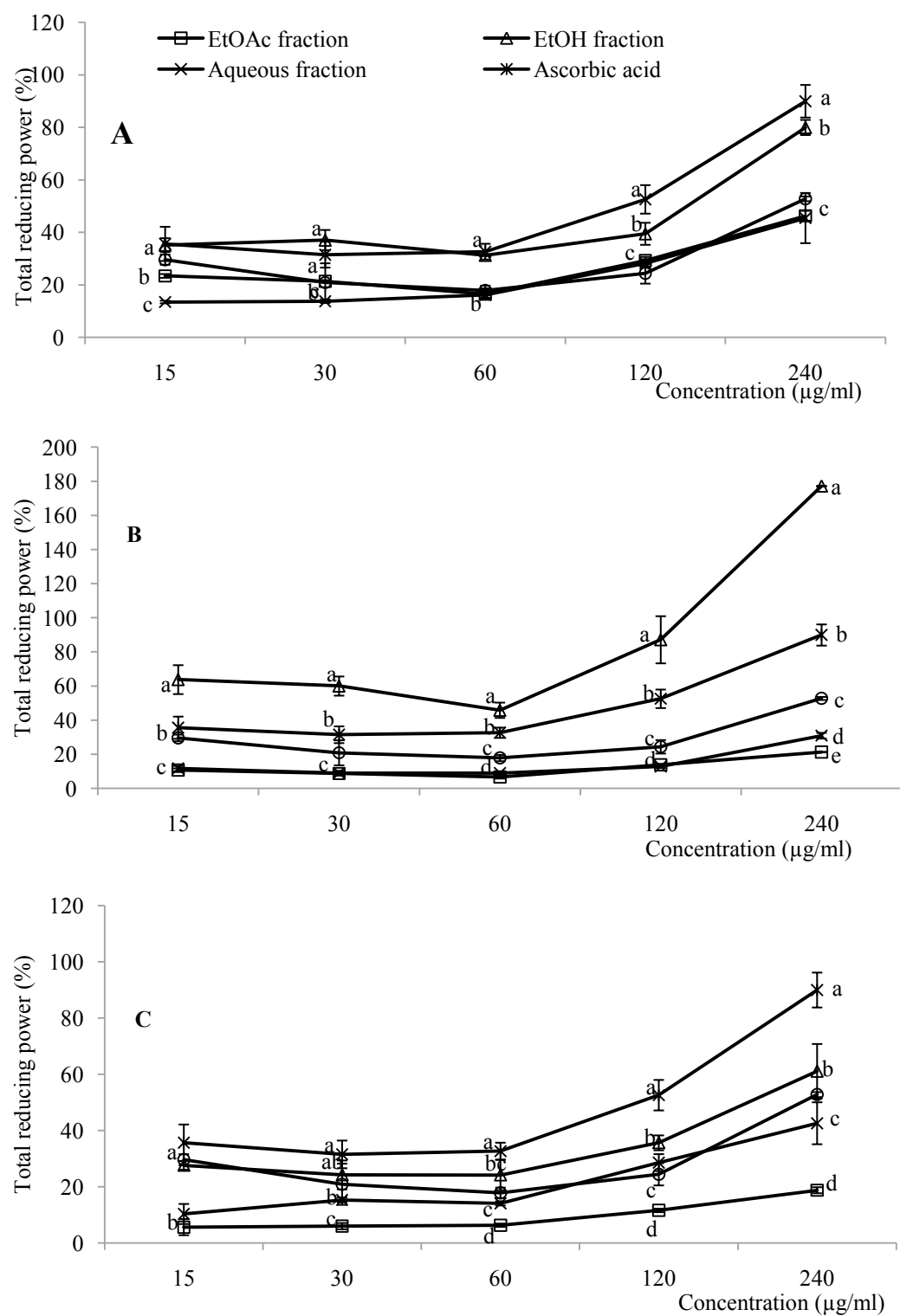


Figure 6.2.1: Total reducing power (relative to gallic acid) of stem bark (A), root (B) and leaves (C) extracts of *K. senegalensis*. Data are presented as mean \pm SD of triplicate determinations. ^{a-d}Different alphabets presented for a given concentration of each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The anti-oxidative activities (using DPPH, HRS and NO models) of the extracts from the different parts of *K. senegalensis* are summarized in **Table 6.2.1**. All the extracts from the plant scavenged DPPH radicals, however the ethanolic extracts of the stem bark and root as well as the aqueous extract of the leaves displayed a statistically similar but significantly ($P < 0.05$) higher DPPH radical scavenging activity than all other extracts from the plant. Furthermore, all the extracts could scavenge hydroxyl radicals generated by the Fenton's reaction except the ethyl acetate extracts of the stem bark and leaves which showed pro-oxidative tendencies in this model. The ethyl acetate extract of the root showed the best activity with the ethanolic extracts also showing good activity. The highest NO scavenging activity was observed with the ethanolic extract of the leaves followed by the stem bark aqueous extract and ethanolic extract of the root. Thus, a close analysis of the results obtained from the total reducing power assay and the three models for anti-oxidative studies revealed that the ethanolic extract of the root had a high anti-oxidative activity that reasonably cuts across all the models used and therefore, it was selected for the next step of the experiment.

Table 6.2.1: Percentage yield, total phenolics and IC_{50} values of various extracts of *K. senegalensis* parts in different anti-oxidative models

Samples	% Yield	Total phenolics (mg/g GAE)	IC ₅₀ (µg/ml)		
			DPPH	HRS	NO
Stem bark					
Ethyl acetate	1.36	50.62 ± 0.08 ^c	7.15 ± 2.12 ^c	P	3205.41 ± 760.44 ^h
Ethanol	4.75	107.31 ± 3.05 ^g	1.99 ± 0.87 ^a	2.08 ± 0.28 ^a	63.75 ± 4.81 ^c
Aqueous	3.51	59.64 ± 1.03 ^d	195.01 ± 45.60 ^f	15.13 ± 1.25 ^d	28.50 ± 7.71 ^b
Root					
Ethyl acetate	0.98	13.70 ± 0.37 ^b	48.20 ± 3.47 ^e	1.68 ± 0.27 ^a	95.32 ± 6.08 ^d
Ethanol	2.91	97.76 ± 0.66 ^f	1.59 ± 0.64 ^a	2.18 ± 0.45 ^a	29.23 ± 1.87 ^b
Aqueous	2.66	58.32 ± 1.76 ^d	22.71 ± 3.47 ^d	4.85 ± 0.61 ^c	192.70 ± 33.91 ^e
Leaves					
Ethyl acetate	1.43	12.08 ± 0.12 ^a	44.67 ± 4.62 ^e	P	47.75 ± 5.92 ^c
Ethanol	2.99	77.38 ± 1.27 ^e	3.25 ± 0.57 ^b	2.44 ± 0.53 ^{ab}	6.92 ± 1.42 ^a
Aqueous	3.82	14.08 ± 0.74 ^b	1.40 ± 0.22 ^a	59.31 ± 12.43 ^e	183.45 ± 15.18 ^e
Ascorbic acid	-	-	2.56 ± 0.26 ^{ab}	ND	26.40 ± 6.46 ^b
Gallic acid	-	-	1.40 ± 0.43 ^a	ND	1053.48 ± 239.92 ^g
Trolox	-	-	8.47 ± 2.88 ^c	3.23 ± 0.49 ^b	629.76 ± 63.98 ^f

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-h}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). ND means not determined and P means the extract showed pro-oxidative properties in the experimental model.

Using solvent-solvent fractionation, the ethanolic extract of the root was fractionated to obtain a dichloromethane, ethyl acetate, butanol and aqueous fractions. The butanol fraction had a significantly ($P < 0.05$) higher total reducing power (**Figure 6.2.2**) and DPPH radical scavenging activity (**Table 6.2.2**)

than other fractions. However, the ethyl acetate and dichloromethane fractions had significantly higher ($P<0.05$) hydroxyl and NO radicals scavenging activities respectively, than other fractions (**Table 6.2.2**).

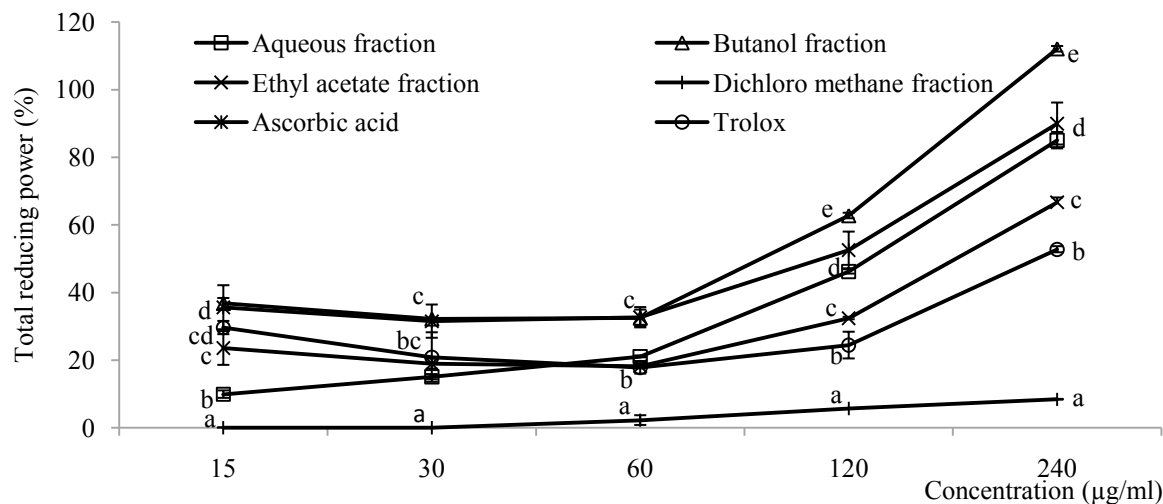


Figure 6.2.2: Total reducing power (relative to gallic acid) of different solvent fractions of *K. senegalensis* root ethanolic extract. The results are expressed as mean \pm SD of triplicate determinations. ^{a-d}Different alphabets presented for a given concentration for each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

Table 6.2.2: IC₅₀ values of various solvent fractions of ethanolic extract of *K. senegalensis* root in different anti-oxidative models

Fractions	Total phenolics (mg/g GAE)	IC ₅₀ (µg/ml)		
		DPPH	Hydroxyl radical	Nitric oxide
Aqueous	128.23 \pm 1.73 ^b	27.71 \pm 0.87 ^b	62.45 \pm 0.72 ^c	6.92 \pm 0.4 ^{c*}
Butanol	201.24 \pm 4.64 ^d	3.11 \pm 0.77 ^a	41.20 \pm 2.27 ^b	35.89 \pm 3.24 ^{d*}
Ethyl acetate	182.15 \pm 2.78 ^c	3.78 \pm 1.12 ^a	31.53 \pm 4.22 ^a	3.58 \pm 0.5 ^{b*}
Dichloromethane	7.71 \pm 3.30 ^a	91.00 \pm 1.09 ^c	58.31 \pm 4.74 ^c	1042.84 \pm 40.28 ^a

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-d}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$). *Unit was expressed in mg/ml.

Figure 6.2.3 shows the inhibition of α -glucosidase and α -amylase by the fractions of ethanolic extract of the root. All the fractions inhibited α -glucosidase and α -amylase activities *in vitro* in a dose dependent pattern. The more polar (aqueous and butanol) fractions consistently maintained higher inhibitory activities against the enzymes than the less polar (ethyl acetate and dichloromethane) fractions. Furthermore, as shown by the IC₅₀ values, the butanol fraction had a significantly ($P<0.05$) higher inhibitory activity than other fractions with IC₅₀ values of 2.89 ± 0.46 and 97.51 ± 5.72 µg/ml for α -glucosidase and α -amylase inhibitions respectively (**Table 6.2.3**).

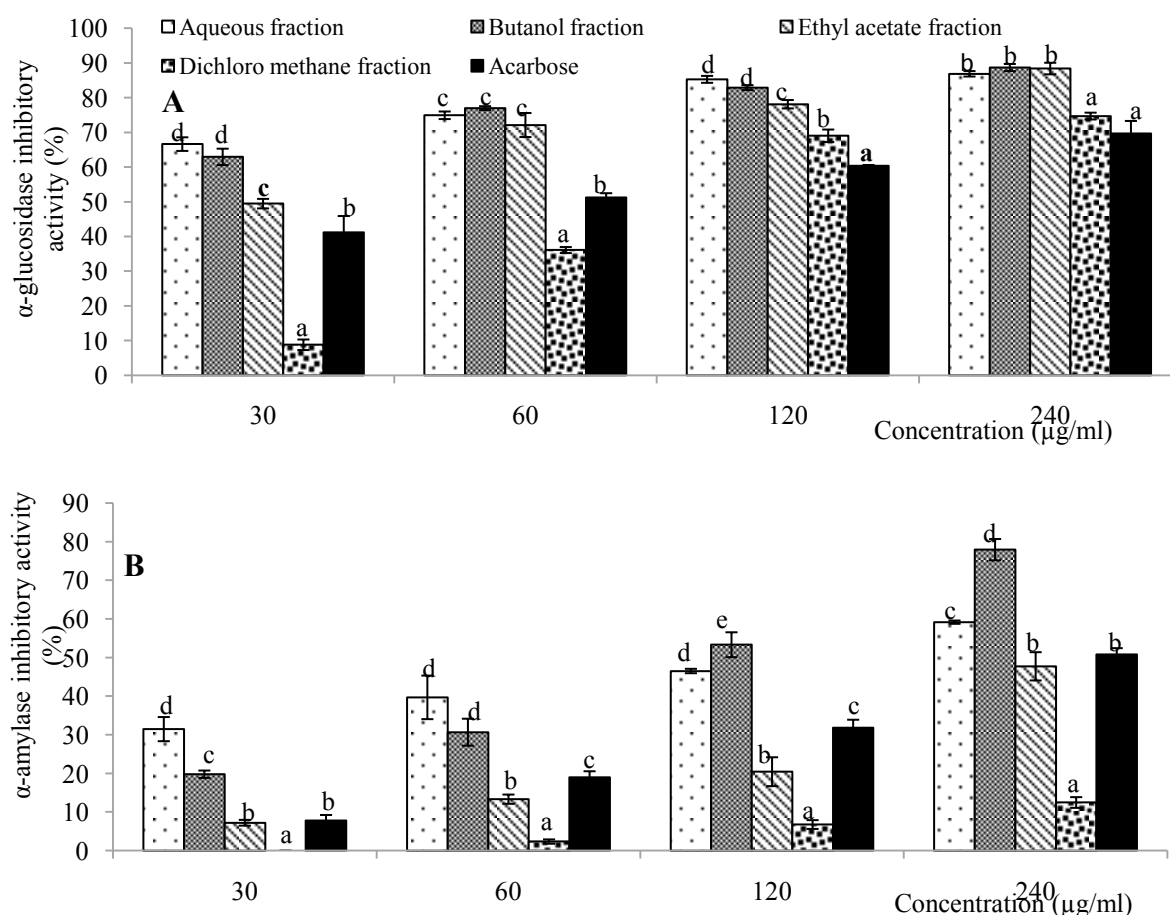


Figure 6.2.3: α -glucosidase (A) and α -amylase (B) inhibitory activities of different solvent fractions of ethanolic extract of *K. senegalensis* root. The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different superscripts alphabets over the bars for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 6.2.3: IC_{50} values for the inhibition of α -glucosidase and α -amylase by various solvent fractions of ethanolic extract of *K. senegalensis* root

Fractions/standard	IC_{50} ($\mu\text{g/ml}$)	
	α -glucosidase	α -amylase
Aqueous	5.15 ± 1.10^b	130.23 ± 12.90^b
Butanol	2.89 ± 0.46^a	97.51 ± 5.72^a
Ethyl acetate	23.89 ± 0.70^c	394.97 ± 13.46^d
Dichloromethane	91.23 ± 2.17^c	$154.64 \pm 6.67^{c*}$
Acarbose	55.59 ± 5.22^d	256.66 ± 20.52^c

The results are expressed as mean \pm SD of triplicate determinations. ^{a-c}Different superscripts alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). *Unit was expressed in mg/ml.

The enzyme kinetic studies revealed that the butanol fraction is a non-competitive inhibitor of α -glucosidase with V_{\max} decreasing from 655.09 $\mu\text{mol}/\text{min}$ to 27.38 $\mu\text{mol}/\text{min}$. The K_M remained unchanged at 2.00 mM and the computed K_i value for inhibiting α -glucosidase was 1.30 $\mu\text{g}/\text{ml}$ (**Figure 6.2.4A**). Conversely, the mechanism of inhibition of α -amylase by the butanol fraction revealed a competitive inhibition pattern with a K_M value of 1.25% and 0.25% in the presence and absence of the inhibitor respectively. The V_{\max} of the α -amylase was not affected (33.70 $\mu\text{mol}/\text{min}$) and K_i for inhibiting α -amylase by the fraction was 7.50 $\mu\text{g}/\text{ml}$ (**Figure 6.2.4B**).

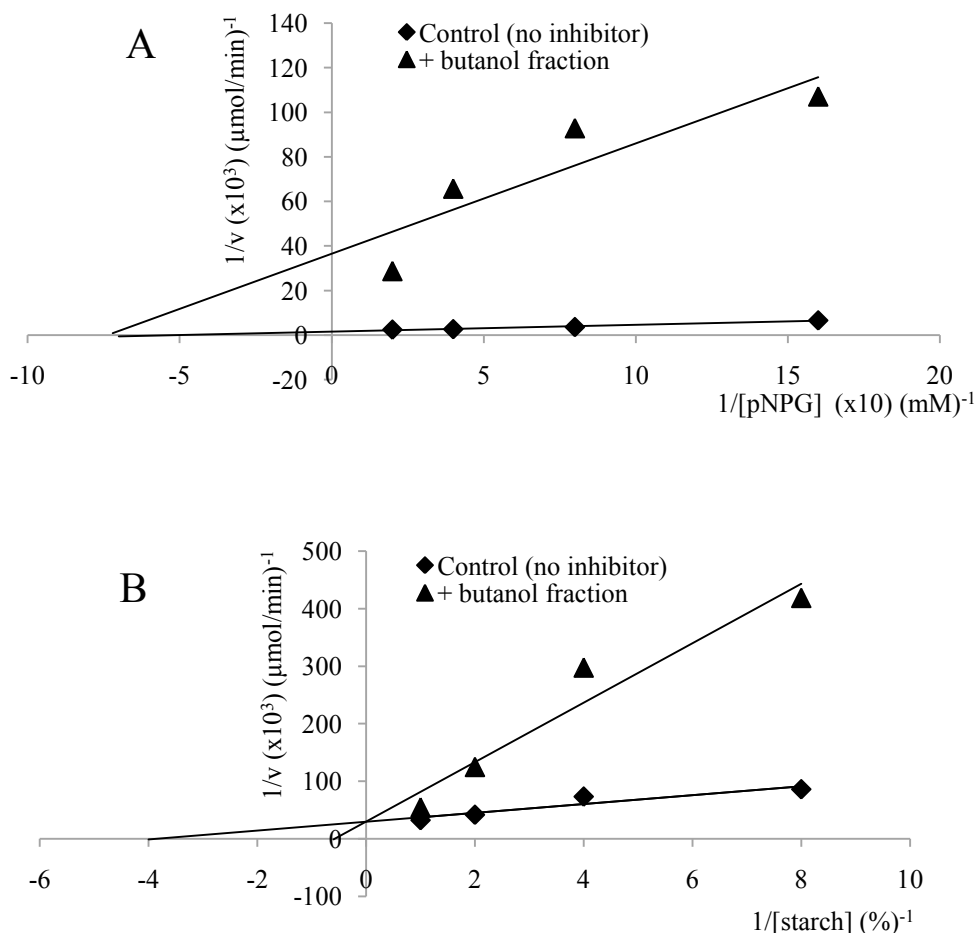


Figure 6.2.4: Lineweaver-Burke's plot of α -glucosidase (A) and α -amylase (B) catalysed reactions in the presence and absence of the butanol fraction derived from the *K. senegalensis* root ethanolic extract

The GC-MS chromatogram of the butanol fraction is presented in **Figure 6.2.5**. The major peaks detected in the chromatogram of the butanol fraction were those of the chromones 7,8-dihydroxy-2,3-dihydrochromone (**1**), 5,7-dihydroxychromone (**2**), *p*-anilinophenol hydrochloride (**3**) and 3-ethyl-5-(3-ethyl-(3H)-benzothiazol-2-ylidene)-2-(*p*-tolylvinylamino)-4-thiazolidinone (**4**) identified by the their

fragmentation patterns and in conjunction with the NIST library (**Figure 6.2.6**). The retention time and molecular mass of the detected phytochemicals are provided in **Table 6.2.4**.

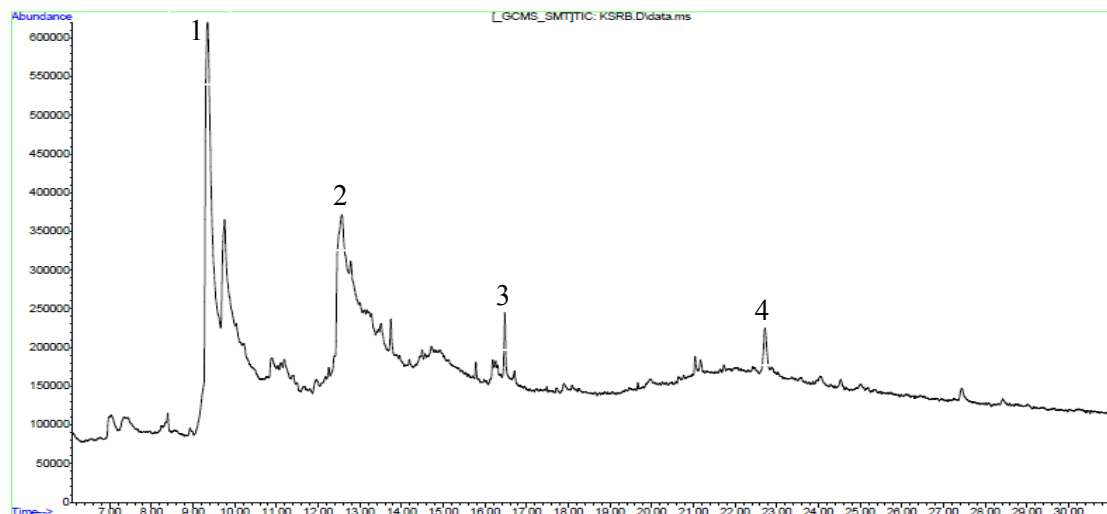


Figure 6.2.5: GC-MS chromatogram of butanol fraction of *K. senegalensis* root ethanolic extract

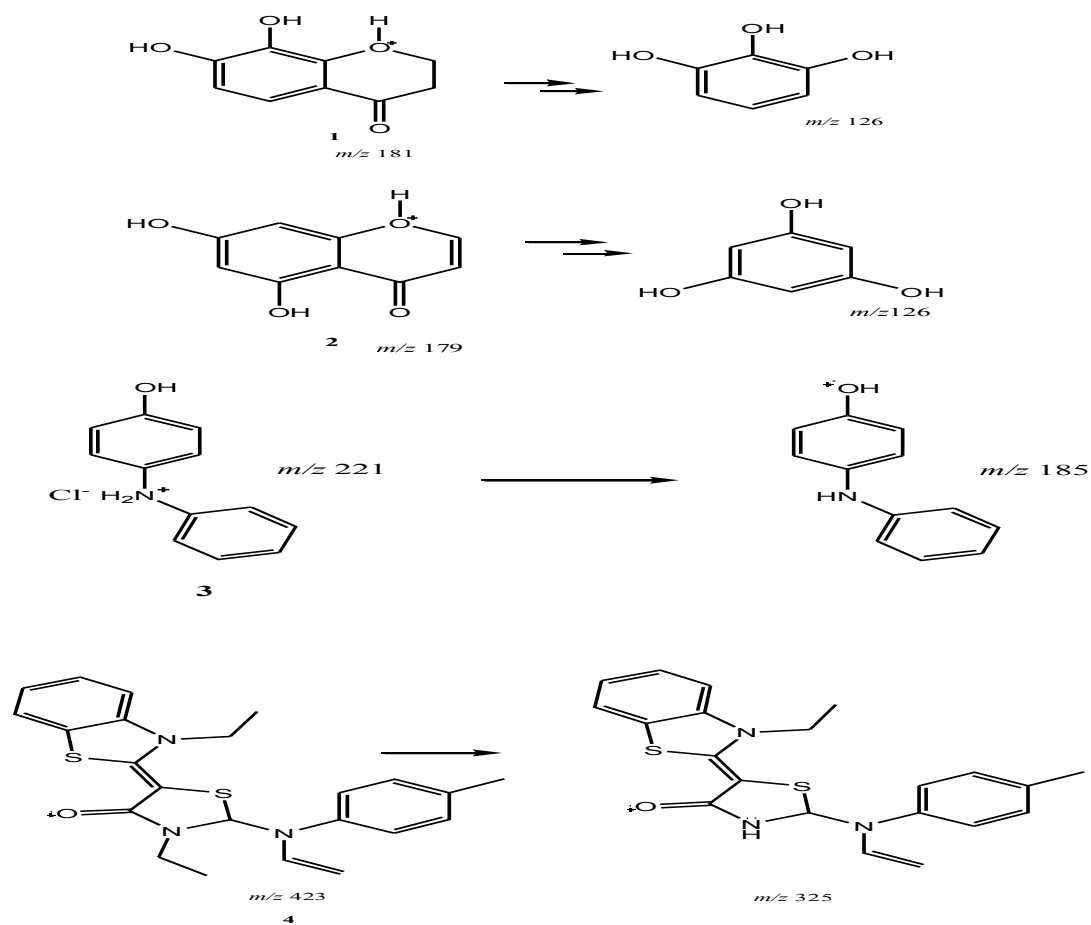


Figure 6.2.6: Structures of components identified by GC-MS

Table 6.2.4: Identified components of butanol fraction of *K. senegalensis* root ethanolic extract by GC-MS

§	Compound	Retention time (min)	Molecular mass
1	7,8-dihydroxy-2,3-dihydrochromone	9.35	181
2	5,7-dihydroxychromone	12.53	179
3	<i>p</i> -anilinophenol hydrochloride	16.49	221
4	3-ethyl-5-(3-ethyl-(3H)-benzothiazol-2-ylidene)-2-(<i>p</i> -tolylvinylamino)-4-thiazolidinone	22.76	423

§ means annotated peak number on the GC-MS chromatogram

6.2.5 Discussion

The roles of oxidative stress and hyperglycemia as underlying factors in the pathogenesis of T2D and associated complications have been fully elucidated in several previous studies [41, 42]. Therefore, the use of potent anti-oxidative agents and inhibitors of carbohydrate digesting enzymes (α -glucosidase and α -amylase) will provide a holistic therapeutic strategy for the control of postprandial blood glucose levels, T2D and chronic vascular complications. This study revealed that the butanol fraction of ethanolic extract of the root of *K. senegalensis*, a plant commonly used for the treatment of diabetes in Nigeria [6] contained powerful anti-oxidative agents as well as inhibitors of α -glucosidase and α -amylase.

Four experimental models for *in vitro* anti-oxidative studies were used because a single model cannot give a full evaluation of the anti-oxidative capabilities of the different extracts tested due to the involvement of multiple mechanisms. The total reducing power was measured by the reduction of Fe^{3+} – Fe^{2+} in the presence of the extracts using the method of Oyaizu [43]. The reducing capacity of an extract/compound may serve as a significant indicator of its potential antioxidant activity. Using this method, the ethanolic extract of the root displayed a consistently higher reducing power than the other extracts. Free radicals are known to be a major factor contributing to biological damages and DPPH has been used to evaluate the free radical scavenging activity of natural AO. Hydroxyl radicals are also extremely reactive species capable of damaging any biological molecule found in living systems [44] and NO is an unstable species which reacts with oxygen to generate nitrite and peroxynitrite anions [45] All these radicals are implicated in the pathogenesis of T2D [34] From the present study, the ethanolic extract of the root contain better group(s) of phytochemical(s) which could reasonably scavenge all the various forms of the reactive species tested. This further suggests that the phytochemical components of this plant with high redox potentials, at least in this part, are ethanol extractable. Guided by these activities, solvent fractionation of the ethanolic extract of the root was carried out.

The butanol fraction partitioned from the ethanolic extract of the root was shown to possess better antioxidant and scavenging activities than other fractions across the experimental models used. This is indicative of the presence of powerful phytochemical(s) that might have the ability to inhibit free radical upsurge as well as oxidative stress. These phytochemicals, either in pure form or in combination could be useful therapeutic agents for treating oxidative stress based metabolic disorders.

The control of postprandial hyperglycemia is believed to be important in the treatment of diabetes and prevention of cardiovascular complications. One of the therapeutic approaches to this control is to retard glucose absorption by inhibiting carbohydrate hydrolysing enzymes, such as α -amylase and α -glucosidase, in the digestive organs [36]. All the fractions from the ethanol extract of the root inhibited α -glucosidase in a dose dependent manner. Inhibition of this enzyme would slow down the breakdown of disaccharides to liberate glucose; thereby reducing glucose absorption from the small intestine [46]. However, the butanol fraction exhibited stronger α -glucosidase inhibitory activity with especially remarkable IC_{50} value ($2.89 \pm 0.46 \mu\text{g/ml}$). In spite of abundant reports on the α -glucosidase inhibitory activities of extracts and pure compounds from plants in different parts of the world, only a few authors [47] reported such a low IC_{50} value as found with the butanol fraction in our study. This observation might not be unconnected to the high phenolics content recorded in this fraction because polyphenolic fractions from plants have been shown to inhibit α -glucosidase activity allowing for tighter control of blood glucose [48]. Furthermore, Mai et al. [49] reported a strong positive correlation between polyphenolic content and α -glucosidase inhibitory effects of 28 extracts from Vietnamese edible plants but also suggested that the enzymatic inhibition might depend on the type of the polyphenolics. In the same vein, the butanol fraction had the highest α -amylase inhibitory activity which suggests the presence of better group(s) of phytochemicals that could inhibit the breakdown of complex carbohydrates to oligosaccharides, thereby diminishing the effect of carbohydrate consumption on postprandial hyperglycemia. Moreover, the observed higher α -glucosidase inhibitory activity of the butanol fraction over the corresponding α -amylase activity is of great pharmaceutical interest. This is because some of the side effects associated with the currently available drugs for the management of T2D are linked with the excessive inhibition of α -amylase activity [35].

The mechanism of α -glucosidase inhibition by the butanol fraction revealed a non-competitive inhibition pattern which indicates that the fraction binds the α -glucosidase at separate site(s) of the enzyme (rather than the active site) but caused conformational modification at the active site, thereby preventing effective binding of the pNPG and consequently reduced the α -glucosidase activity. It further suggests that the fraction contains some phytochemicals that are capable of interacting with the α -glucosidase-pNPG complex [50]. On the contrary, the pattern of α -amylase inhibition by the fraction suggests that the active site of the enzyme is directly involved in the inhibitory action and the fraction most likely contains some

phytochemical(s) that could serve as substrate analogues, thereby competing for the active site of α -amylase [51].

Phenolics are very important constituents among the secondary metabolites of plants because of their multiple biological effects and direct contribution to anti-oxidative and α -glucosidase inhibitory activities [49]. While not discounting the possible contributions of the other detected phytochemicals, it is possible to suggest that the bioactive agents of the butanol fraction contain phenolic groups such as those present in the two chromones **1** and **2** identified by GC-MS.

The results of this study suggests that the butanol fraction derived from the ethanolic extract of *K. senegalensis* root contain the most potent α -glucosidase and α -amylase inhibitory agents and the identified phenolics could be the bioactive agents hence can be investigated as novel anti-diabetic therapeutic.

Postscript: From the above studies, the butanol fraction had the best α -glucosidase and α -amylase effects and therefore it was selected for detailed *in vivo* anti-diabetic study

6.3 Anti-diabetic activity of butanol fraction of *Khaya senegalensis* root in a type 2 diabetes model of rats and the isolation of bicyclo [2.2.0]hexane-2,3,5-triol as a possible bioactive compound

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Preface: This article reports the detailed *in vivo* anti-diabetic studies of the butanol fraction and the isolation of the possible bioactive anti-diabetic compound. This section is not yet submitted for publication.

6.3.1 Abstract

The present study was conducted to examine the anti-diabetic activity of butanol fraction of *Khaya senegalensis* root (KSBF) in a type 2 diabetes (T2D) model of rats as well as the possible bioactive compound from the fraction. T2D was induced by feeding rats with 10 % fructose solution *ad libitum* for two weeks followed by intraperitoneal injection of 40 mg/kg bw streptozotocin and the animals were treated with 150 and 300 mg/kg bw of the fraction for five days in a week. The KSBF treatment, at 300 mg/kg bw, significantly ($P < 0.05$) lowered blood glucose level, improved oral glucose tolerance ability and β cell function (HOMA- β), decreased insulin resistance (HOMA-IR), stimulated hepatic glycogen synthesis, ameliorated serum lipids alterations and prevented hepatic and renal damages compared to untreated diabetic rats. Additionally, the fraction was found to insignificantly ($P > 0.05$) improve weight gain, decrease feed and fluid intakes, stimulate insulin secretions and lower serum fructosamine concentrations compared to untreated diabetic rats. Bioassay guided fractionation of the fraction led to the isolation of bicyclo [2.2.0]hexane-2,3,5-triol which strongly inhibited α -glucosidase and α -amylase with IC_{50} values of 45.87 ± 6.46 and 63.28 ± 10.10 μ g/ml respectively. Further kinetic studies revealed that the compound is a non-competitive inhibitor of α -glucosidase and α -amylase activities. Data from the study suggests that orally administered KSBF, at 300 mg/kg bw, possess remarkable anti-T2D activity and could ameliorate some diabetes-associated complications while bicyclo [2.2.0]hexane-2,3,5-triol is an important bioactive anti-diabetic agent in the fraction.

6.3.2 Introduction

Diabetes mellitus is a heterogeneous group of metabolic disorder characterized by high blood glucose levels (hyperglycemia) with alterations in carbohydrate, lipid and protein metabolism resulting from defects in insulin secretion and/or action [52]. Current estimation for the prevalence of diabetes stands at

366 million people which is projected to reach 552 million by the year 2030 [30] while type 2 diabetes (T2D) accounts for >90% of all the diabetic cases. T2D is a complex, heterogenous and polygenic disease characterized mainly by insulin resistance and pancreatic β cell dysfunction [53]. A number of theories have been proposed to explain the pathogenesis of insulin resistance and pancreatic β cell dysfunction but oxidative stress, through a single unifying mechanism of reactive oxygen species production, is regarded as the common biological player for the two main pathogenic features of T2D and also plays vital roles in other aspects of diabetes pathology [54-56], thereby making it a potential therapeutic target. At present, T2D is predominantly managed with sulfonylureas and biguanides but α -glucosidase inhibitors are still considered as vital therapeutic option especially in the developing world [36]. Unfortunately, none of the multiple therapeutic agents designed for the management of diabetes is not without undesirable side effects such as diarrhea, nausea and liver failure [37].

For centuries, several medicinal plants or their extracts are traditionally used in the treatment of diabetes with limited side effects. In this modern era, these medicinal plants also continue to play an essential role in modern drug discovery. Indeed, the widely used anti-diabetic drug metformin was originally developed from a medicinal plant, *Galega officinalis* [57]. Apart from providing chemical leads for novel anti-T2D drugs, some of these medicinal plants are currently available for sale in some African markets as standardized herbal products (in crude form) after approval by relevant authorities [58]. Based on the above, there is growing research interest on African traditional herbal medicines for possible exploitation either directly as standardized anti-diabetic herbal products or indirectly by providing novel chemical leads for the development of newer alternative therapies for the treatment of T2D.

Khaya senegalensis A. Juss (Meliaceae), also known as African mahogany, is a large tree growing mainly in the sub-Saharan Africa and highly reputed for numerous medicinal activities [27]. The plant has been reported to produce an array of limonoids with fascinating pharmacological activities such as antiplasmodial, anticancer, antifeedant and antifungal properties [25-27]. Polyphenolic compounds such as catechin, rutin and procyanidins with significant antioxidant activities were also identified in different parts of the plant by HPLC [16]. More recently, Ibrahim et al. [11] reported that phloroglucinol and 3,4-(dihydroxyphenyl) acetic acid-enriched fraction from the stem bark of the plant elicited antitrypanosomal activity and ameliorated the trypanosome-induced anemia and organ damage.

Ethnobotanical surveys have revealed that *K. senegalensis* is among the most commonly used plants for the traditional treatment of diabetes in northwestern part of Nigeria and central region of Togo [6]. Preliminary *in vitro* α -amylase inhibitory potential as well as anti-hyperglycemic activity of the stem bark aqueous extract of the plant in a type 1 diabetes model of rats have been demonstrated [21, 40]. However, a comprehensive anti-diabetic study on any part of this plant has not yet been conducted either in humans or experimental models of T2D.

In a preliminary screening, we subjected different solvent crude extracts of the stem bark, root and leaves of the plant to anti-oxidative activity assays using several models and found that the ethanolic extract of the root had the best anti-oxidative activity among all other extracts. Hence, in the present study, the ethanolic extract of the root was further fractionated across solvents of different polarity and a butanol fraction derived from it, was subjected to a comprehensive *in vivo* anti-diabetic study in a T2D model of rats. Additionally, we isolated a pure bicyclo [2.2.0] hexane-2,3,5-triol as a possible bioactive anti-diabetic compound from the fraction.

6.3.3 Materials and methods

Please refer to chapter two sub-sections 2.3-2.4 (pages 35-36); 2.7.1-2.7.7 (pages 40-43) and 2.8.3 page 46 for detailed materials and methods that affect *K. senegalensis*. However, the group codes DFL, DFH and NFT used in the chapter two are replaced with DKL, DKH and NKT respectively. KSBF refers to the butanol fraction of *K. senegalensis* root.

6.3.4 Results

The mean feed and fluid intake as well as weekly body weight changes of all animals were monitored during the experiment. Compared with the normal rats, significantly ($P < 0.05$) higher feed and fluid intakes with lower body weight gains were recorded in the diabetic rats. Administration of KSBF to the diabetic rats insignificantly ($P > 0.05$) decreased the feed and fluid intake compared to the untreated diabetic rats (**Figure 6.3.1**). However, the body weight loss recorded in diabetic rats was insignificantly restored in the DKH group only while DKL group had relatively lower body weight gain than DBC group in the last four weeks of the experiment (**Figure 6.3.2**).

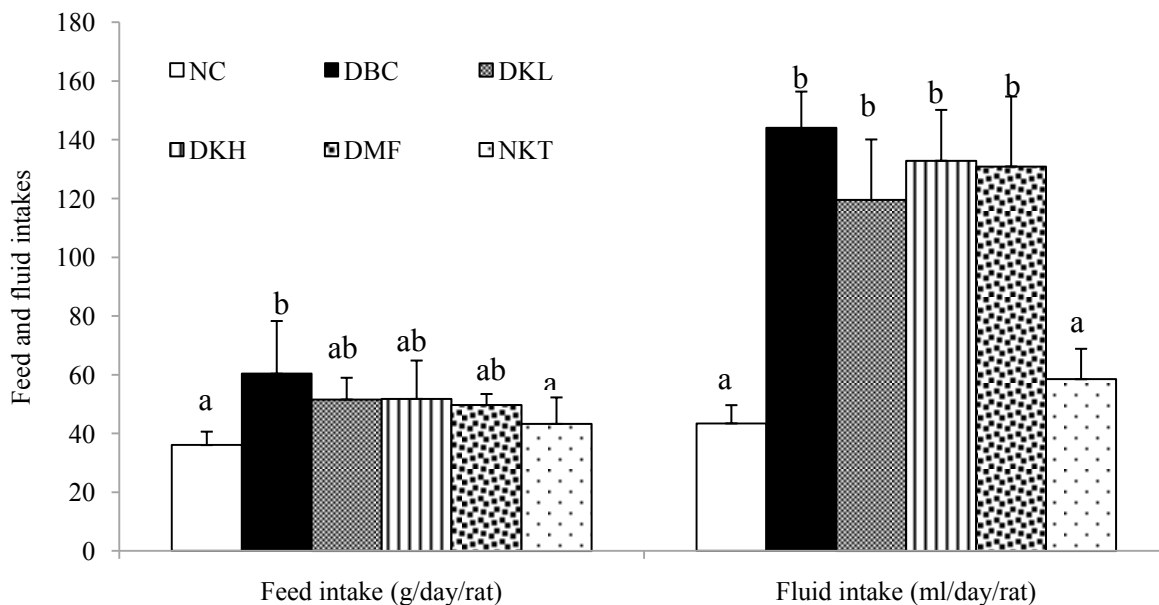


Figure 6.3.1: Feed and fluid intakes of different groups of during the experimental period. Data are presented as the mean \pm SD of eight animals. ^{a-b}Different superscript alphabets over the bars for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

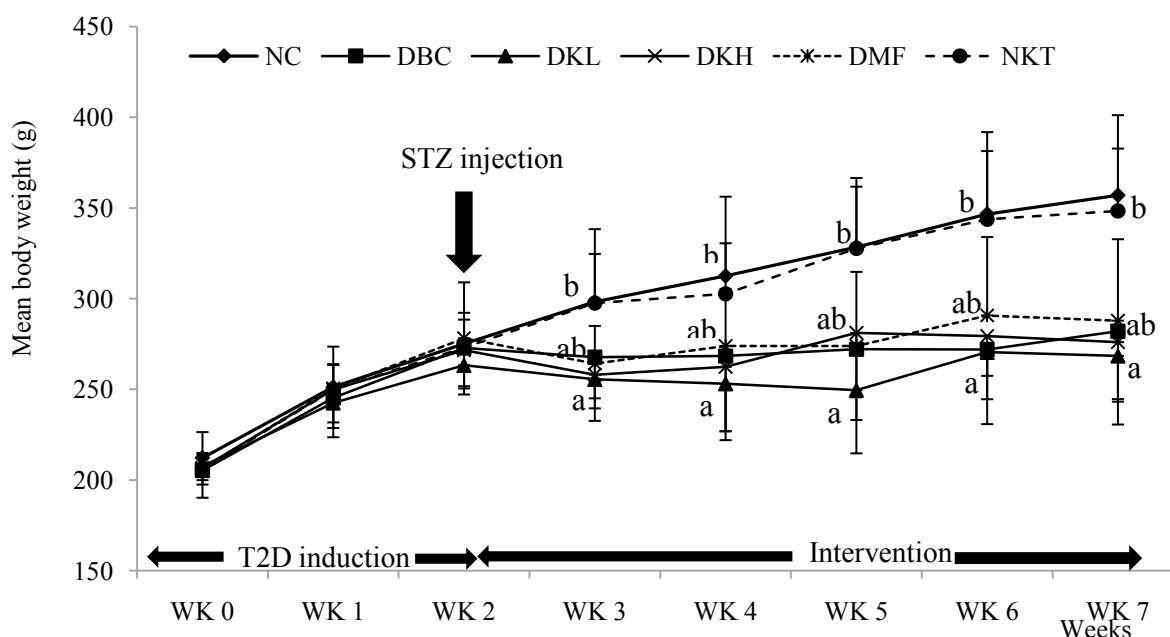


Figure 6.3.2: Mean body weight gain for all groups of experimental animals over the seven weeks experimental period. The results are expressed as the mean \pm SD of eight animals. ^{a-c}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The profiles of weekly NFBG levels in all groups of experimental rats indicated that normal rats (NC and NKT groups) maintained an NFBG level of about 7.0 mmol/L throughout the experiment while all diabetic rats had an elevated NFBG level. However, treatment of the diabetic rats with KSBF decreased the T2D-induced increase in NFBG. The effect of the treatment on NFBG is more pronounced in DKH than DKL groups (**Figure 6.3.3**). Indeed, a sharp and significant decrease ($P < 0.05$) in the FBG level was observed in the DKH group compared to the DBC group at the last week of the experiment. The data for OGTT are shown in **Figure 6.3.4**. At 0 and 30 min, the blood glucose concentrations of the diabetic groups were significantly ($P < 0.05$) higher than the NC group. There was no significant difference ($P < 0.05$) between the glucose tolerance ability of DKL and DBC groups during the entire experimental period but the DKH group had a significantly ($P < 0.05$) better glucose tolerance ability compared to the DBC group. In fact, statistically similar glucose tolerance ability was observed between DKH and DMF groups during the entire experimental period.

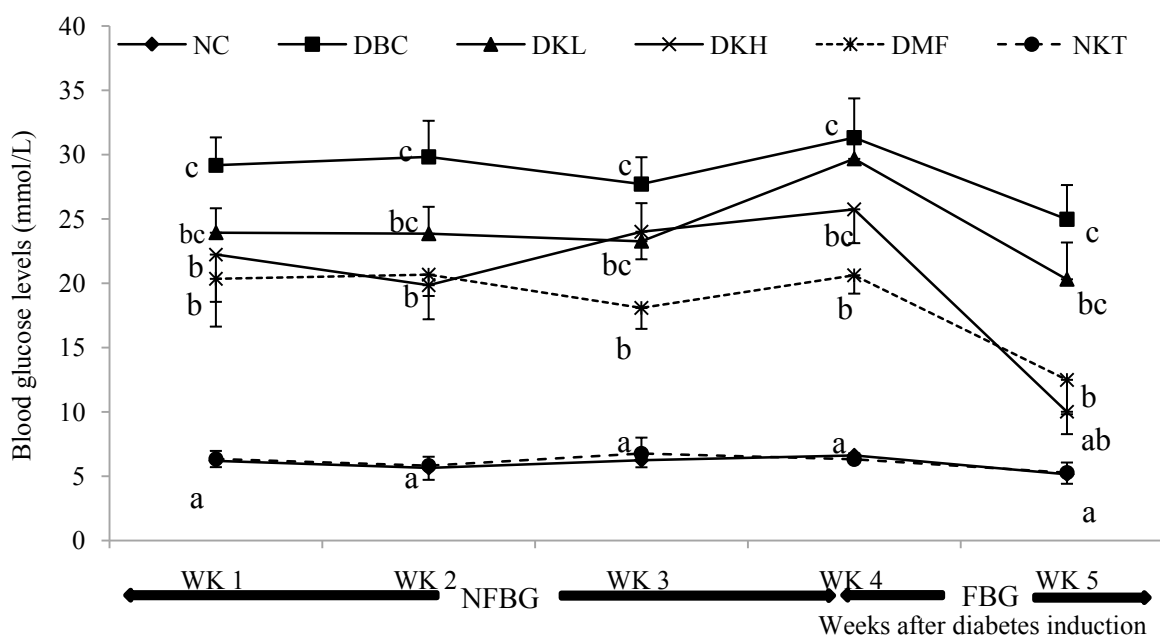


Figure 6.3.3: Weekly blood glucose concentrations (post induction) of different animal groups. The results are presented as the mean \pm SD of eight animals. ^{a-c}Different alphabets for a given week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

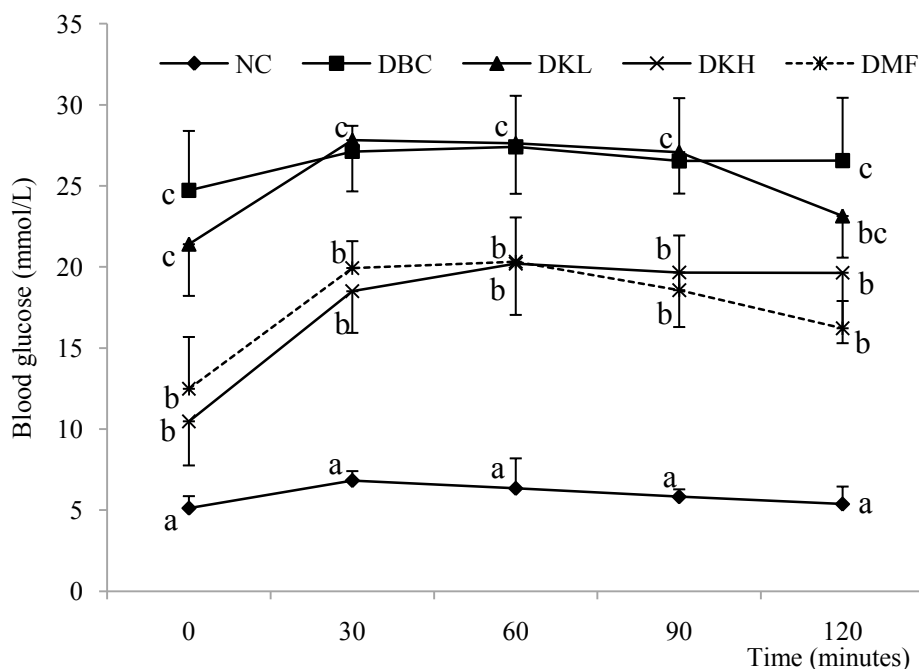


Figure 6.3.4: Oral glucose tolerance test (OGTT) for all groups of animals in the last week of experimental period. The results are expressed as the mean \pm SD of eight animals. ^{a-c}Different alphabets for a given time indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The serum insulin concentrations of the DBC group were significantly ($P < 0.05$) lowered compared to NC group but the KSBF-treated diabetic groups recorded an insignificantly ($P > 0.05$) higher insulin levels

than the DBC group (**Table 6.3.1**). HOMA-IR score was significantly ($P<0.05$) higher while HOMA- β score was significantly lower in the DBC group compared to the NC group. However, the DKH group had a significantly ($P<0.05$) ameliorated HOMA-IR and HOMA- β scores while statistically similar, though relatively better, results were recorded in the DKL group compared to DBC group. Also, T2D caused a significant ($P<0.05$) elevation in serum fructosamine concentration which was not significantly ($P>0.05$) reversed in the KSBF treated diabetic groups.

Table 6.3.1: Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores for different animal groups at the end of the experimental period

	NC	DBC	DKL	DKH	DMF	NKT
Serum insulin (pmol/L)	153.1 \pm 9.9 ^c	64.2 \pm 29.9 ^a	74.5 \pm 20.3 ^a	84.6 \pm 18.9 ^a	119.2 \pm 13.0 ^b	162.3 \pm 7.5 ^c
Serum fructosamine (μ mol/L)	196.3 \pm 5.6 ^a	258.4 \pm 10.9 ^b	245.6 \pm 8.8 ^b	239.2 \pm 2.3 ^b	253.8 \pm 30.4 ^b	209.1 \pm 20.9 ^{ab}
HOMA-IR	4.96 \pm 1.0 ^{ab}	16.2 \pm 5.7 ^c	11.8 \pm 2.3 ^c	3.3 \pm 0.53 ^a	9.8 \pm 3.8 ^c	5.3 \pm 0.6 ^b
HOMA- β	304.07 \pm 53.72 ^c	9.7 \pm 5.2 ^a	15.8 \pm 3.7 ^a	27.5 \pm 4.4 ^b	54.9 \pm 27.5 ^b	289.9 \pm 107.8 ^c

The results are expressed as the mean \pm SD of eight animals. ^{a-c}Different alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$)

HOMA-IR = [(Fasting serum insulin in U/L x Fasting blood glucose in mmol/L) / 22.5]

HOMA- β = (Fasting serum insulin in U/L x 20 / Fasting blood glucose in mmol/L – 3.5)

Conversion factor: insulin (1U/L = 7.174 pmol/l)

There were no significant differences in the absolute liver weights of all groups of experimental rats but the relative liver weights of the diabetic groups were higher than NC and NKT groups (**Table 6.3.2**). The KSBF treatments did not significantly ($P>0.05$) affect the relative liver weights in all the experimental animals. The liver glycogen content was significantly ($P<0.05$) depleted in the DBC group compared to the NC group but the KSBF treatment significantly ($P<0.05$) boosted the liver glycogen reserves in the diabetic animals.

Table 6.3.2: Liver weights and liver glycogen concentrations in different animal groups at the end of the experimental period

	NC	DBC	DKL	DKH	DMF	NKT
Liver weight (g)	11.8±2.4	10.9±1.9	10.4±0.9	11.1±1.8	10.4±1.7	11.7±1.4
Relative liver weight (%)	3.3±0.3 ^a	3.9±0.2 ^b	3.9±0.2 ^{ab}	4.0±0.3 ^b	3.7±0.1 ^a	3.3±0.3 ^a
Liver glycogen (mg/g tissue)	3.5±0.8 ^b	1.5±0.1 ^a	3.4±1.0 ^b	2.9±1.1 ^b	2.6±0.4 ^b	3.3±0.5 ^b

The results are expressed as the mean ± SD of eight animals. ^{a-b}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

The serum lipid profiles and other biochemical parameters are presented in **Table 6.3.3**. The DBC group had relatively higher levels of total and LDL cholesterol (compared to the NC group) which were reduced in the DKL and DKH groups. Conversely, the DBC group had a significantly (P<0.05) lower level of HDL-cholesterol which was boosted in the KSBF treated groups. Furthermore, significantly (P<0.05) higher triglycerides concentration was recorded in the DBC group which was also decreased in the KSBF-treated diabetic rats. Results of other indices of diabetic complications indicated that the serum level of AST was neither affected by the T2D nor the KSBF treatments but serum ALT, ALP and urea levels were significantly elevated (P<0.05) in the DBC group compared to the NC group. However, the KSBF treatments significantly (P<0.05) ameliorated the T2D-induced increase in ALP and urea levels whereas the increase in ALT level was significantly ameliorated in the DKH group only. It is noteworthy that the KSBF-treated groups had a significantly lower urea levels than the NC group. Relatively lower serum creatinine concentration was observed in the DBC group which was boosted in the DKL and DKH groups.

Table 6.3.3: Serum lipid profiles and other biochemical parameters in different animal groups at the end of the experimental period

	NC	DBC	DKL	DKH	DMF	NKT
Total cholesterol (mg/dl)	66.3±6.4 ^{ab}	76.3±10.62 ^b	54.2±8.5 ^a	68.8±12.7 ^{ab}	74.6±12.1 ^b	62.8±11.3 ^{ab}
HDL cholesterol (mg/dl)	31.0±9.6 ^b	19.8±1.6 ^a	23.5±6.8 ^{ab}	24.2±5.6 ^{ab}	28.1±5.4 ^{ab}	34.4±8.6 ^b
LDL cholesterol (mg/dl)	19.9±5.4 ^{ab}	27.5±4.3 ^b	22.4±8.2 ^{ab}	25.6±8.8 ^{ab}	25.9±7.4 ^{ab}	16.3±5.9 ^a
Triglycerides (mg/dl)	102.5±27.5 ^a	184.8±40.9 ^b	163.8±37.7 ^{ab}	105.0±12.9 ^a	120.1±27.4 ^{ab}	92.0±30.5 ^a
AST (U/L)	88.0±14.8	73.6±7.6	80.0±15.5	82.0±18.0	88.7±17.9	81.9±6.8
ALT (U/L)	56.3±12.6 ^a	77.8±4.6 ^b	69.0±6.2 ^{ab}	67.2±4.8 ^{ab}	63.3±18.2 ^{ab}	47.0±5.5 ^a
ALP (U/L)	188.8±19.9 ^a	898.6±174.5 ^c	547.3±196.2 ^b	378.6±124.1 ^b	472.0±74.9 ^b	174.1±35.1 ^a
Urea (mg/dl)	48.0±6.2 ^b	93.0±16.4 ^c	22.7±6.0 ^a	25.8±3.9 ^a	42.0±11.8 ^b	20.9±6.6 ^a
Creatinine (µg/dl)	582.0±70.5 ^{ab}	485.0±40.4 ^a	560.0±71.1 ^{ab}	554.0±26.1 ^b	675.0±95.2 ^b	551.7±75.7 ^{ab}

The results are expressed as the mean ± SD of eight animals. ^{a-c}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05)

Histopathological examination of the pancreatic sections revealed a reduction in the size of pancreatic islets as well as the number of β cells per islet count in the DBC group compared to the NC group. However, the KSBF treatments protected the T2D-associated destruction of β cells because larger islets with higher number of β -cell were detected in the DKL and DKH groups compared to DBC group, although the result was better in the DKH group (**Figure 6.3.5**).

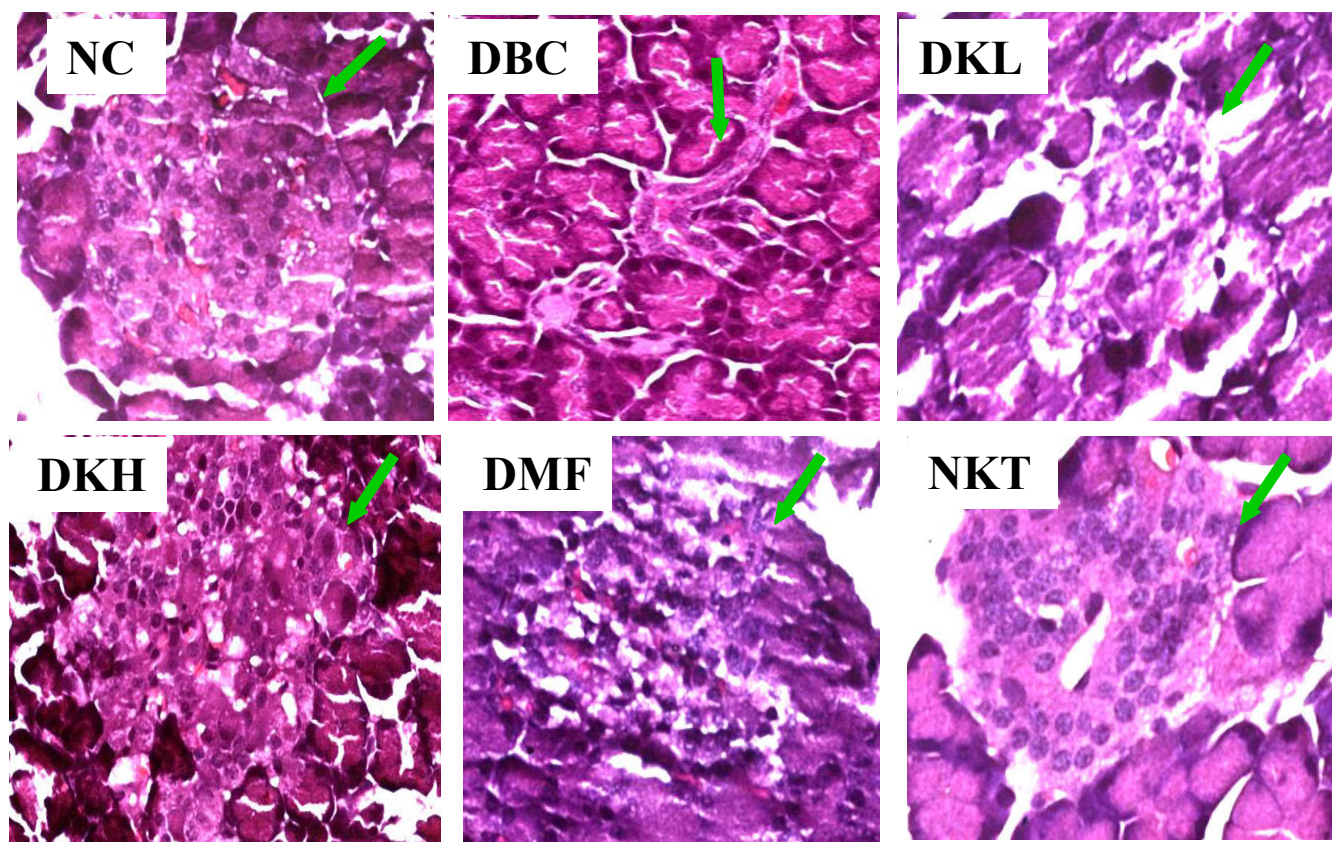


Figure 6.3.5: Histopathological pictures of the pancreas of different experimental groups at the end of the experiment. The NC had large islets with high number of β -cell while the DBC had smaller islets and morphologically deformed β -cells. The DKL, DKH and DMF groups had relatively larger islets with higher number of β -cells compared to DBC.

The isolated compound was identified as bicyclo[2.2.0]hexane-2,3,5triol (**Figure 6.3.6**) through detailed spectral analysis that includes ^1H NMR, ^{13}C NMR, ^1H - ^1H COSY, ^1H - ^1H NOESY, HSQC and HMBC NMR spectra (Appendix 5-11) as well as mass spectroscopy. The ^1H NMR spectrum showed the presence of a triplet at δ_{H} 0.83 (2H-6, $J = 7.4$ Hz) which corresponded to a methylene carbon resonance at δ_{C} 14.3 in the ^{13}C NMR spectrum. The proton resonance is actually two coalesced doublets (a doublet for each of the protons in the methylene group) since the two protons are non-equivalent. This resonance was seen coupled to the multiplet methine resonance at δ_{H} 1.28 (H-1), which was coupled in turn to the other methine resonance at δ_{H} 1.46 (H-4), leading to a C6-C1-C4 system. There were two other multiplet peaks in the oxygenated region of the spectrum at δ_{H} 3.39 (2H) and δ_{H} 3.55 (1H). The H-4 resonance was coupled to the resonance at δ_{H} 3.39, which was assigned to the two oxygenated methine groups at H-3 and H-5. This proton peak had corresponding carbon resonances at δ_{C} 72.2 and 73.3 (C-3 and C-5 respectively). The other multiplet resonance at δ_{H} 3.55 was assigned to H-2. HMBC correlations between C-1 and H-3/5, C-4 and H-3/5, and C-2 and H-3/5 as well as correlations between C-1 and 2H-6 and C-4 and 2H-6 support the proposed structure.

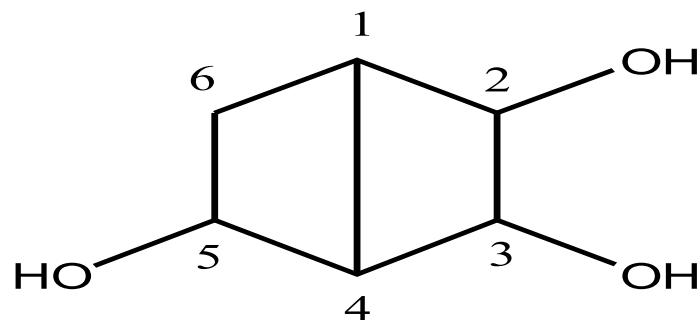


Figure 6.3.6: Structure of the possible anti-diabetic agent (bicyclo [2.2.0] hexane-2,3,5-triol) isolated from the butanol fraction of *K. senegalensis* root through bioassay guided isolation

Analysis of α -glucosidase and α -amylase inhibitory activity of the bicyclo [2.2.0] hexane-2,3,5-triol revealed that the compound is a potent inhibitor of α -glucosidase and α -amylase activity with IC_{50} values of 45.87 ± 6.46 and 63.28 ± 10.10 $\mu\text{g/ml}$ respectively. Furthermore, enzyme kinetics studies revealed that the compound exerts non-competitive inhibition pattern on both enzymes thereby decreasing the V_{max} of the enzymes while the K_M remained unchanged (**Figure 6.3.7**). For α -glucosidase, the V_{max} was reduced from 655.09 $\mu\text{mol/min}$ to 66.52 $\mu\text{mol/min}$ and the K_M remained unchanged at 2mM while for α -amylase, the V_{max} was reduced from 33.70 $\mu\text{mol/min}$ to 0.86 $\mu\text{mol/min}$ and the K_M remained unchanged at 0.25%.

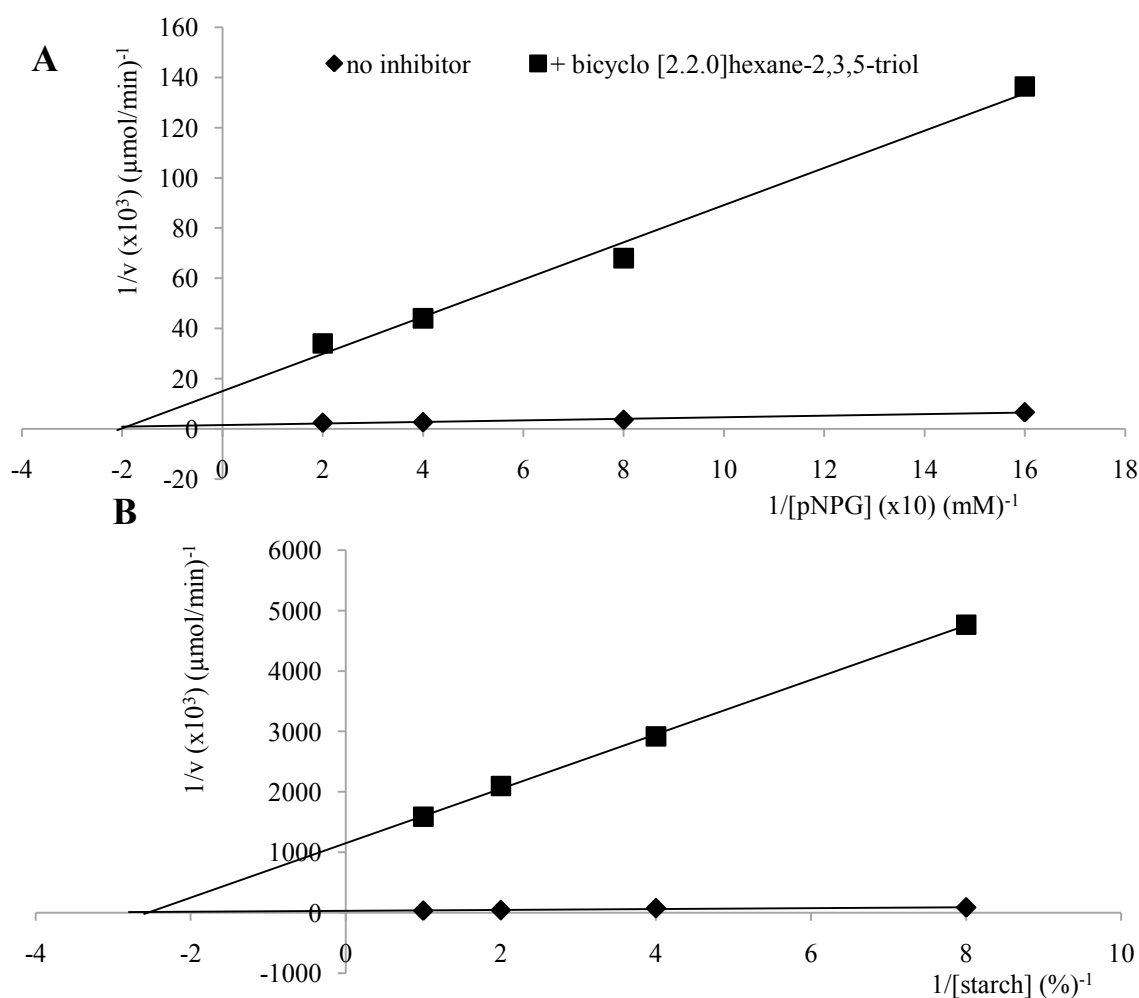


Figure 6.3.7: Lineweaver-Burke's plot for α -glucosidase (A) and α -amylase (B) catalysed reactions in the presence and absence of bicyclo [2.2.0] hexane-2,3,5-triol as an inhibitor

6.3.5 Discussion

Khaya senegalensis is traditionally used in the management of diabetes in some West African countries [6]. Earlier workers have reported preliminary studies on the *in vitro* α -amylase inhibitory activity as well as an anti-hyperglycemic activity of the stem bark of the plant in a type 1 diabetes model of rats [21,40]. In the present study, we performed a detailed study on the *in vivo* anti-diabetic activity of the butanol fraction of *K. senegalensis* root in a newly developed model of T2D as well as isolation of a possible bioactive anti-diabetic agent from the fraction.

Polyphagia and polydipsia with concomitant reduction of body weight are major symptoms of diabetes mellitus [59] which were also evidently observed in the diabetic groups of our experiment. Treatment with KSBF did not completely prevent the T2D-induced changes in these physico-metabolic parameters possibly because the experimental period was short for their reversal to near-normal.

Elevated fasting or postprandial hyperglycemia is the hallmark of T2D and causes life-threatening complications linked to the disease. Consequently, maintaining glucose homeostasis is essential in preventing the detrimental effects of hyperglycemia and its associated complications [60]. In our study, at a dose of 300 mg/kg bw, the KSBF displayed remarkable anti-hyperglycemic, and not hypoglycemic, activity because the fraction did not cause any decrease in the blood glucose level of normal rats. Furthermore, the fraction improved glucose tolerance abilities, lowered peripheral insulin resistance, stimulated pancreatic β cell function with mild insulintropic effect as well as ameliorated the diabetes-induced damage to pancreatic architecture. Taken together, it seems that, in addition to the potent α -glucosidase inhibitory activity of the fraction, the anti-diabetic activity of the fraction is mediated through both pancreatic (protection and modulation of β -cells to increase insulin secretion) and other extra pancreatic (peripheral utilization of glucose) mechanisms. The anti-diabetic activity of a number of plant materials were reported to be through interference with gastrointestinal glucose absorption [61], insulintropic action [62] or by promoting the regeneration of pancreatic β -cells [63].

Another important feature of experimentally induced diabetes is a reduction in the liver glycogen level [64] which is caused by reduced activity of glycogen synthase and increased glycogen phosphorylase activity during the disease. Previous studies have demonstrated that a number of plant materials [65, 66] elicited an anti-diabetic activity partly through stimulation of hepatic glycogenesis. Thus, the significantly higher liver glycogen content recorded in the KSBF treated groups compared to the DBC group (**Table 6.3.2**) indicated that the anti-diabetic activity of the KSBF was also mediated not only through the earlier postulated mechanisms but also by stimulating hepatic glycogenesis and/or inhibiting glycogenolysis, thereby reducing an upsurge in blood glucose levels (**Figure 6.3.2**).

Type 2 diabetes is associated with profound changes in the serum lipid and lipoprotein profile with an increased risk in coronary heart diseases [67]. The LDL transports cholesterol to the peripheral tissues where it is deposited while HDL transports cholesterol from peripheral tissues to the liver and facilitate its metabolism as well as excretion. Thus, elevation of serum LDL-cholesterol indicates the development of atherogenic process while increased HDL-cholesterol decrease the risk of coronary diseases [68]. Thus, the ability of KSBF to relatively reduce total cholesterol, triglycerides, LDL-cholesterol and elevate HDL-cholesterol in the serum of diabetic animals suggests that, in addition to the anti-diabetic activity, the fraction also has a tendency to reduce T2D-associated complications such as coronary heart disease. This is partly supported by the lowered serum fructosamine level detected in the KSBF treated diabetic animals which also suggest that the fraction interrupts proteins glycation cascades, thereby reducing the risk of diabetic complications [69]. Results from other indices of diabetic complications also indicated that the T2D caused a significant increase in serum levels of ALT and ALP indicating impaired liver function as well as urea which denotes renal function. Interestingly, the fraction also restored these

biomarkers (to varying degrees) suggesting the protection of hepatic and renal damages associated with diabetic complications. On the other hand, lower serum creatinine concentration was found in the DBC group compared to NC group (**Table 6.3.3**) which corroborates with findings in human based studies [70]. Lower serum creatinine level was reported to be a predictor of insulin resistance [70]. It is thus plausible to suggest that the ability of KSBF to effectively reduce insulin resistance could be linked to the relatively elevated creatinine levels detected in the KSBF-treated diabetic groups compared to the DBC group.

After the anti-T2D of the KSBF was investigated by *in vivo* studies, phytochemical analysis for the potential bioactive anti-diabetic agent in the fraction was further conducted which led to the isolation of bicyclo [2.2.0]hexane-2,3,5-triol. This is the first time the compound was isolated from any part of *K. senegalensis*. Indeed, apart from a report on the isolation of dimeric proanthocyanidins [29] from the stem bark of the plant, all other studies on the phytochemistry of this plant reported mainly on limonoids [25-27]. Interestingly, the compound was found to be a potent inhibitor of the two enzymes (α -glucosidase and α -amylase) in *in vitro* models suggesting that the compound could lead to a reduction in postprandial hyperglycemic shoot up. Furthermore, kinetic delineation of α -glucosidase and α -amylase inhibitions indicated non-competitive patterns for both enzymes. These indicate that bicyclo [2.2.0]hexane-2,3,5-triol binds α -glucosidase and α -amylase at separate site(s) of the enzyme (rather than the active sites) but caused conformational modification at the active sites thereby preventing effective binding of the substrates. This type of inhibition could further suggest that the compound could interact with the enzyme-substrate complexes [50].

In conclusion, data from the study revealed that the butanol fraction of *K. senegalensis* root (at a dose of 300 mg/kg bw) has anti-T2D activity which is mediated possibly through modulation of β -cell function, stimulation of peripheral insulin sensitivity and hepatic glycogenesis. Furthermore, the fraction was able to ameliorate some of the diabetes-associated complications and bicyclo [2.2.0]hexane-2,3,5-triol is a possible anti-diabetic agent in the fraction. Our future work will focus on detailed studies on the molecular mechanism(s) for the observed results.

Postscript: In order to further explore the possible mechanism of anti-diabetic action of the butanol fraction, the serum, liver, kidney, heart and pancreas were investigated for *in vivo* anti-oxidative parameters

6.4 Attenuation of *in vivo* antioxidant status could be involved in the anti-diabetic activity of the butanol fraction of *Khaya senegalensis* root in a type 2 diabetes model of rats

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Preface: This article describes the *in vivo* anti-oxidative status of the serum and organs collected from the rats in the above experiment to give an insight into the possible mechanism of action from oxidative stress point of view. This sub section is also not yet submitted for publication.

6.4.1 Abstract

The effects of the butanol fraction of *Khaya senegalensis* root (KSBF) on the anti-oxidant status of the liver, kidney, heart, pancreas and serum of type 2 diabetic rats were investigated. Rats were induced with type 2 diabetes (T2D) and treated with 150 and 300 mg/kg body weight (bw) of the KSBF. The levels of thiobarbituric acid reactive substances (TBARS), glutathione, superoxide dismutase (SOD) and catalase in the selected organs and serum were determined at the end of the experiment. The T2D was found to significantly ($P<0.05$) increase the TBARS levels and significantly ($P<0.05$) decrease the glutathione, SOD and catalase in the serum and organs compared to normal rats. However, the KSBF treatment caused a dose dependent decrease in the TBARS levels of the serum and organs of type 2 diabetic rats. Furthermore, the KSBF treatment ameliorated the T2D-induced depletion in glutathione, SOD and catalase in the serum and organs in a dose dependent fashion. It was concluded that the KSBF alleviated T2D-induced oxidative stress which could be relevant for the treatment of T2D by the fraction.

6.4.2 Introduction

Diabetes mellitus is a complex polygenic disease with multiple aetiologies that poses threat to the global health system. At present, oxidative stress is considered to be the most serious contributor to the onset and progression of the disease [35]. Our body is naturally endowed with a number of defense systems against oxidative damage but impairment of glutathione metabolism and other endogenous non-protein anti-oxidants as well as attenuation of anti-oxidant enzymes occur in diabetic conditions [33] and this is regarded as a vital pathogenic mechanism for the disease [41].

Khaya senegalensis A. Juss (Meliaceae) is an African medicinal plant commonly used in the traditional treatment of several metabolic diseases including diabetes mellitus in Nigeria [6]. Preliminary *in vitro* α -amylase inhibitory potential as well as anti-hyperglycemic activity of the stem bark aqueous extract of the plant in a type 1 diabetes model of rats have been demonstrated [21, 40]. However, our interest is to

comprehensively study the anti-diabetic potentials of this plant as well as the possible molecular mechanism(s) involved in the observed activity of the plant. Hence, in a previous study (see 6.2 above), we subjected the various crude solvents extracts from the stem bark, root and leaf samples of the plant to a series of *in vitro* anti-oxidative assays and reported that the ethanolic extract of the root had the most potent anti-oxidative activities that reasonably cuts across various models. Subsequently, the crude ethanolic extract of the root was subjected to solvent-solvent partitioning and found that the butanol fraction contain the most potent anti-oxidative and α -glucosidase and α -amylase inhibitory phytochemicals in *in vitro* models (see 6.2 above). Based on the foregoing observations, the butanol fraction was investigated for *in vivo* anti-diabetic activity in a type 2 diabetes model of rats where it was found to lower blood glucose levels, improve oral glucose tolerance ability and β -cell function (HOMA- β), stimulate insulin secretions, decrease peripheral insulin resistance (HOMA-IR), ameliorate serum lipids alterations as well as prevent liver and kidney damages associated with the disease (see 6.3 above). Thus, considering the crucial role of oxidative stress in the pathogenesis of diabetes and its complications, the present study was conducted to investigate, whether or not, attenuation of *in vivo* antioxidant status plays a role in the observed anti-diabetic activity of the butanol fraction of *K. senegalensis* root.

6.4.3 Materials and methods

Please refer to the chapter two sub sections 2.3-2.4 (pages 35-36); 2.7.1-2.7.5 (pages 40-42); and 2.7.8 (2.7.8.1-2.7.8.4) pages 44-45 for detailed material and methods that affect *K. senegalensis*.

6.4.4 Results

The TBARS levels in the serum and all organs investigated in this study were significantly ($P<0.05$) higher in the DBC group compared to the NC group while the glutathione, SOD and catalase levels were significantly ($P<0.05$) lower in the DBC group than the NC group. The DKL and DKH groups had significantly ($P<0.05$) lower levels of hepatic TBARS and significantly ($P<0.05$) higher levels of hepatic glutathione and catalase than the DBC group. SOD was also significantly ($P<0.05$) boosted in the DKH group and not the DKL group (**Table 6.4.1**).

Table 6.4.1: Effects of KSBF on hepatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DKL	DKH	DMF	NKT
TBARS (μM)	10.6 \pm 3.0 ^a	33.9 \pm 4.9 ^c	27.8 \pm 2.5 ^c	22.2 \pm 2.0 ^b	35.9 \pm 9.5 ^{bc}	13.6 \pm 4.8 ^a
Glutathione (μM)	120.8 \pm 6.0 ^d	29.9 \pm 9.3 ^a	90.2 \pm 3.6 ^c	98.3 \pm 6.2 ^c	73.6 \pm 10.2 ^b	104.5 \pm 9.9 ^d
Superoxide dismutase (nmol/min)	25.5 \pm 5.3 ^b	12.4 \pm 1.6 ^a	16.5 \pm 5.7 ^{ab}	19.1 \pm 4.4 ^b	17.5 \pm 5.2 ^{ab}	24.2 \pm 3.1 ^b
Catalase ($\mu\text{mol/min}$)	16.5 \pm 0.9 ^c	1.54 \pm 0.3 ^a	6.6 \pm 1.6 ^b	8.3 \pm 1.8 ^b	1.7 \pm 0.6 ^a	9.8 \pm 1.8 ^b

The results are expressed as the mean \pm SD of five animals. ^{a-d}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Also, the DKL and DKH groups had significantly ($P < 0.05$) lower levels of kidney TBARS but significantly ($P < 0.05$) higher levels of SOD and catalase than the DBC group. The glutathione level in the kidney was significantly ($P < 0.05$) elevated in the DKH and not DKL group (**Table 6.4.2**).

Table 6.4.2: Effects of KSBF on kidney lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DKL	DKH	DMF	NKT
TBARS (μM)	14.1 \pm 5.8 ^a	40.8 \pm 12.5 ^d	33.4 \pm 5.6 ^c	26.0 \pm 1.9 ^b	33.4 \pm 10.0 ^{bc}	17.9 \pm 5.3 ^a
Glutathione (μM)	68.2 \pm 20.8 ^{ab}	46.0 \pm 8.6 ^a	40.6 \pm 7.3 ^a	67.9 \pm 9.7 ^b	70.8 \pm 10.4 ^b	63.3 \pm 5.5 ^b
Superoxide dismutase (nmol/min)	16.1 \pm 3.4 ^{bc}	6.8 \pm 1.5 ^a	10.2 \pm 1.5 ^b	11.6 \pm 2.2 ^b	11.5 \pm 4.4 ^{ab}	18.1 \pm 3.8 ^c
Catalase ($\mu\text{mol/min}$)	1.7 \pm 0.3 ^b	0.9 \pm 0.2 ^a	1.7 \pm 0.4 ^b	2.1 \pm 0.4 ^{bc}	1.6 \pm 0.4 ^b	2.9 \pm 0.3 ^c

The results are expressed as the mean \pm SD of five animals. ^{a-d}Different superscript alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The levels of the TBARS and anti-oxidant parameters in the heart of the DKL group was not significantly ($P > 0.05$) different from the DBC group. However, the DKH group recorded significantly ($P < 0.05$) lower levels of TBARS and higher levels of glutathione than the DBC group. Relatively higher levels of SOD and catalase were recorded in the DKH group compared to the DBC group (**Table 6.4.3**).

Table 6.4.3: Effects of KSBF on lipid peroxidation and anti-oxidant status in the heart of type 2 diabetic rats

	NC	DBC	DKL	DKH	DMF	NKT
TBARS (μM)	9.36 \pm 2.60 ^a	24.60 \pm 8.83 ^b	13.50 \pm 3.68 ^{ab}	8.28 \pm 1.98 ^a	33.92 \pm 5.89 ^b	8.03 \pm 2.06 ^a
Glutathione (μM)	122.23 \pm 22.00 ^c	48.57 \pm 14.60 ^a	74.03 \pm 14.64 ^{ab}	91.94 \pm 5.25 ^b	79.21 \pm 18.30 ^{ab}	94.97 \pm 6.32 ^{bc}
Superoxide dismutase (nmol/min)	15.24 \pm 4.02 ^b	5.76 \pm 1.23 ^a	8.40 \pm 2.55 ^a	9.07 \pm 2.43 ^{ab}	12.83 \pm 2.01 ^b	16.24 \pm 3.84 ^b
Catalase ($\mu\text{mol/min}$)	2.10 \pm 0.48 ^b	0.93 \pm 0.20 ^a	1.29 \pm 0.25 ^a	1.34 \pm 0.36 ^a	1.44 \pm 0.42 ^a	3.29 \pm 1.06 ^b

The results are expressed as the mean \pm SD of five animals. ^{a-c}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The TBARS levels in the pancreas was insignificantly ($P > 0.05$) lowered, but the glutathione and catalase levels were significantly ($P < 0.05$) higher in the DKL and DKH groups than the DBC group (**Table 6.4.4**).

Table 6.4.4: Effects of KSBF on pancreatic lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DKL	DKH	DMF	NKT
TBARS (μM)	6.0 \pm 1.2 ^a	35.7 \pm 9.1 ^c	26.1 \pm 8.6 ^{bc}	22.6 \pm 2.4 ^{bc}	18.4 \pm 4.4 ^b	7.8 \pm 1.9 ^a
Glutathione (μM)	80.0 \pm 0.09 ^{cd}	6.2 \pm 3.5 ^a	58.7 \pm 11.1 ^c	86.5 \pm 6.8 ^d	37.3 \pm 8.6 ^b	71.7 \pm 12.2 ^{cd}
Superoxide dismutase (nmol/min)	2.6 \pm 0.5 ^c	0.4 \pm 0.2 ^a	0.4 \pm 0.02 ^a	0.9 \pm 0.2 ^b	1.0 \pm 0.5 ^b	2.8 \pm 0.6 ^c
Catalase ($\mu\text{mol/min}$)	0.7 \pm 0.2 ^c	0.35 \pm 0.04 ^a	0.43 \pm 0.03 ^b	1.0 \pm 0.3 ^{cd}	1.1 \pm 0.2 ^d	1.5 \pm 0.4 ^d

The results are expressed as the mean \pm SD of eight animals. ^{a-d}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

In the serum, a significantly higher ($P < 0.05$) levels of glutathione, SOD and catalase were observed in the DKL and DKH groups than the DBC group whereas TBARS level was significantly ($P < 0.05$) lower in the DKH group than the DBC group (**Table 6.4.5**).

Table 6.4.5: Effects of KSBF on serum lipid peroxidation and anti-oxidant status of type 2 diabetic rats

	NC	DBC	DKL	DKH	DMF	NKT
TBARS (μM)	63.0 \pm 15.2 ^a	201.2 \pm 19.4 ^c	147.9 \pm 36.6 ^{bc}	111.9 \pm 18.9 ^b	142.0 \pm 32.0 ^b	83.5 \pm 12.9 ^a
Glutathione (μM)	84.5 \pm 7.7 ^c	19.9 \pm 2.5 ^a	77.8 \pm 6.1 ^{bc}	79.5 \pm 3.4 ^{bc}	65.2 \pm 6.8 ^b	67.3 \pm 13.9 ^b
Superoxide dismutase (nmol/min)	18.1 \pm 2.42 ^d	0.9 \pm 0.2 ^a	8.2 \pm 2.6 ^c	11.2 \pm 2.0 ^c	3.2 \pm 0.7 ^b	15.9 \pm 3.8 ^{cd}
Catalase ($\mu\text{mol/min}$)	3.6 \pm 0.8 ^c	1.5 \pm 0.4 ^a	2.5 \pm 0.3 ^b	2.7 \pm 0.6 ^{bc}	2.4 \pm 0.5 ^b	3.1 \pm 0.9 ^b

The results are expressed as the mean \pm SD of eight animals. ^{a-d}Different superscripts alphabets along a row indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

6.4.5 Discussion

One of the possible mechanisms of anti-diabetic action of phytochemicals is via attenuation of *in vivo* anti-oxidative status. Therefore, the anti-oxidant parameters of serum and vital body organs of KSBF treated diabetic rats were investigated to unravel the possible involvement of anti-oxidant activity to the overall anti-diabetic activity of the fraction.

Analysis of the entire data generated in this study indicates that the KSBF effectively ameliorated the T2D-induced oxidative stress in the blood and vital body organs in a dose dependent pattern. Interestingly, similar pattern was observed for the anti-T2D of the fraction in the previous study (see 6.3 above), suggesting a possible correlation between the two phenomenon.

The attenuation of the hepatic and kidney anti-oxidant parameters by the KSBF indicates that the fraction could reduce the oxidative damage to these vital organs and consequently might ameliorate oxidative stress- associated pathological complications in diabetes such as liver and kidney damages as well as insulin resistance. In the previous anti-diabetic study (see 6.3 above), we found that KSBF was able to ameliorate the above mentioned complications in type 2 diabetic rats. Hyperlipidemia has been reported to be an important contributory factor for the development of oxidative stress as well as cardiovascular complications in T2D [71]. Thus, the observed effects of KSBF on the heart anti-oxidant parameters could indicate the beneficial effects of KSBF in alleviating the hyperlipidemia-induced oxidative stress which might consequently be useful in the management of cardiovascular diseases associated with T2D. The KSBF treatment also boosted the endogenous anti-oxidant reserves in the pancreas which could go a long way to reduce the susceptibility of the organ to oxidative attack and consequently improved its function. Interestingly, this hypothesis is supported by the mild insulinitropism, improved β -cell function and higher number of β -cells demonstrated in KSBF treated type 2 diabetic rats (see 6.3 above). Based on the above discussion coupled with the vital role of oxidative stress in the pathogenesis of pancreatic β -cell

dysfunction [72], we speculated that the mode of action of KSBF in the pancreas of diabetic animals is related to the attenuation of the anti-oxidants status.

We thus concluded that KSBF treatment attenuated the oxidative stress in various organs of type 2 diabetic animals and this mechanism could be involved in the overall anti-diabetic activity of the fraction.

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CHAPTER 7

7.0 THE *IN VITRO* ANTI-OXIDATIVE AND ANTI-DIABETIC ACTIVITY OF *Vitex doniana*

7.1.1 *Vitex doniana* (Verbanaceae)

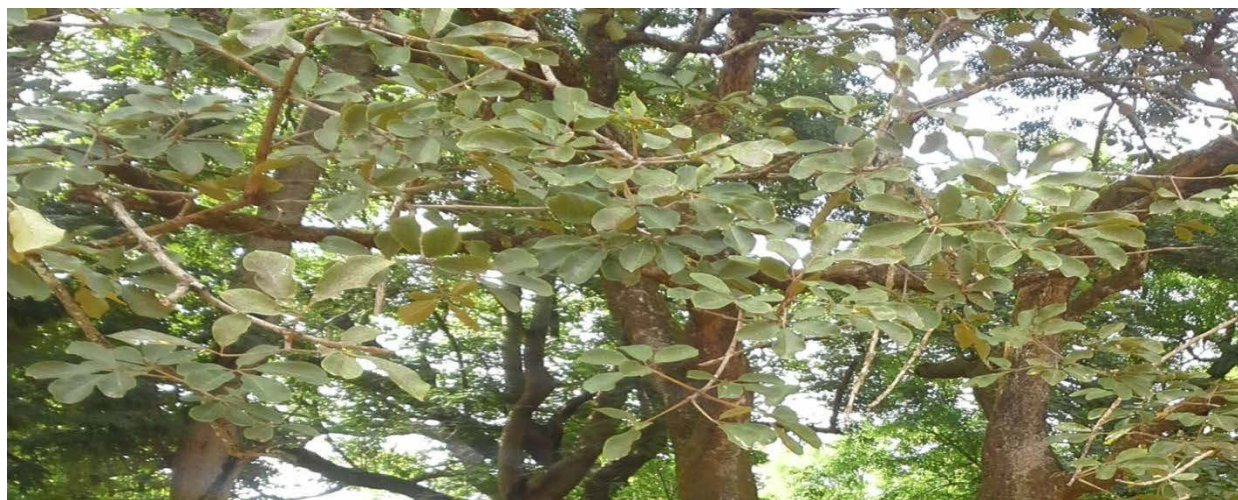


Figure 7.1: *Vitex doniana* (Verbanaceae); common names: African black plum (English); Dinya (Hausa, Nigeria); Orin la (Yoruba, Nigeria); mfudu (Swahili, Tanzania); kashilumbulu (Lunda, Zambia and Angola); munyamazi (Luganda, Uganda); utakiri (Igbo, Nigeria), mfutu (Nyanja, Malawi). Photo: Mohammed Auwal Ibrahim (2013), Zaria, Nigeria

7.1.2 Background

Vitex doniana is a widespread deciduous tree that makes up to 8 -18 m high. The stem bark of the plant is usually rough and pale brown whereas the leaves are usually pale greyish-green of approximately 14-34 cm long. The fruits are drupe, black, edible and sweet which made them highly palatable for consumption as snack [1]. Indeed, the fruits are sold in large quantities at various local markets. *Vitex doniana* is widely distributed in tropical West African countries such as Nigeria, Niger and Senegal. It also extended eastward to Uganda, Kenya and Tanzania [2]. The plant is also available in some Southern African countries such as Angola, Namibia, Zambia and Botswana.

7.1.3 Ethnomedicinal uses

Different parts of *Vitex doniana* are used in folkloric medicine for the treatment of a number of diseases. Aqueous extract of the leaves have been reported to be useful in the traditional treatment of stomachache, diarrhea, dysentery, epilepsy and other psychiatric disorders [2-4]. The stem bark of the plant is used for the traditional management of postpartum bleeding in nursing mothers [5] and also as chewing sticks [6].

Ethnopharmacological survey from northwestern Nigeria indicates that the stem bark of the plant is used for the traditional treatment of diabetes mellitus [7].

7.1.4 Biological activities

Different parts of *V. doniana* have been investigated for a number of pharmacological activities. The antibacterial activity of the stem bark was reported where the extract demonstrated potent activity against medically and dentally relevant bacteria [6]. Different solvent extracts of the leaves of the plant were reported to possess antibacterial activity against *S. typhi* and *E. coli* [8]. In a comparative study, the crude ethanol extracts of various parts of the plant were found to have a broad spectrum antibacterial activity. Upon fractionation, only fractions retained most of the activity which was attributed to the presence of cardiac glycosides and tannins [9]. Other biological investigations on the stem bark extract of the plant demonstrated a non-dose dependent anti-diarrhoeal activity of the methanol extract [5] and a dose dependent hepatoprotective activity of an aqueous extract [1]. Furthermore, the aqueous extract was found to induce graded uterine muscle contractions and potentiated the contractile effects of prostaglandins and oxytocin [5]. The *in vitro* antitrypanosomal activity of the stem bark extracts were also reported [10]. For the root part of the plant, an aqueous extract was found to induce potent depressant activity on peripheral and central nervous system [11] whereas the methanol extract was found to reduce exploratory and stereo-typic behavior but potentiated pentobarbital sleeping time [6]. Leaves of *V. doniana* have also been investigated for several biological activities. The anti-helminthic and hepatoprotective activities of the aqueous extracts were reported [1, 12]. Also, the leaves exhibited anti-inflammatory and analgesic activities through the inhibition of prostaglandin synthesis [2]; a myeloprotective activity in Wistar rats [13] and an antioxidant activity [14]. In a reproductive study, consumption of the plant by wild baboons was linked to a reduced reproductive function [15]. For the fruits of *V. doniana*, preliminary investigations revealed that the fruits possess anti-oxidative activity [16].

7.1.5 Phytochemistry

Information on detailed isolation and structural elucidation of a compound from any part of *V. doniana* does not appear in the literature. However, preliminary analysis on the isolation of α -terpineol has been described [9]. Qualitative phytochemical screening of the leaves indicates the presence of saponins, tannins, flavonoids, terpenoids, anthraquinones and alkaloids [2, 14].

In order to validate the folkloric claim for anti-diabetic activity of this plant (as earlier mentioned), a systematic and comprehensive investigation on the anti-diabetic activity of this plant using *in vitro* and *in vivo* models was intended but due to unforeseen circumstances in terms of the availability of the plant material, only the *in vitro* anti-oxidative and α -glucosidase and α -amylase inhibitory studies of the plant were investigated.

7.2 Anti-oxidative, α -glucosidase and α -amylase inhibitory activity of *Vitex doniana*: possible exploitation in the management of type 2 diabetes

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Preface: The *in vitro* anti-oxidative activities of the stem bark, root and leaves of the plant were investigated in this article. Subsequently, the crude extract was partitioned and the recovered the solvent fractions were investigated for anti-oxidative and α -glucosidase and α -amylase inhibitory activities. The phytochemical content of the most active fraction was analyzed by GC-MS. This article has been submitted for publication to ‘Records of Natural Product’ and is currently under review.

7.2.1 Abstract

Vitex doniana is an important African medicinal plant traditionally used for the treatment of many diseases including type 2 diabetes (T2D). In this study, ethyl acetate, ethanol and aqueous extracts of the stem bark, root and leaf of *V. doniana* were analyzed for *in vitro* anti-oxidative activity and the results indicated that the ethanolic extract of the leaves had the best anti-oxidative activity. Subsequently, the ethanolic extract of the leaves was partitioned between hexane, dichloromethane, ethyl acetate and water. The aqueous fraction had a significantly ($P < 0.05$) higher phenolic content and also showed the best anti-oxidative activity within the fractions. Furthermore, the aqueous fraction demonstrated significantly ($P < 0.05$) more potent inhibitory activities against α -glucosidase and α -amylase than other fractions. Steady state kinetic analysis revealed that the aqueous fraction inhibited both α -glucosidase and α -amylase activities in a non-competitive manner with inhibition binding constant (K_i) values of 5.93 and 167.44 $\mu\text{g/ml}$, respectively. Analysis of the aqueous fraction by GC-MS showed the presence of resorcinol, 4-hydroxybenzoic acid, 3,4,5 trimethoxy phenol and 2,4'-dihydroxychalcone identified by their mass fragmentation patterns and comparison to standard spectra. The results obtained from this study showed that *V. doniana* leaves have a good anti-T2D potential possibly elicited through phenolics.

7.2.2 Introduction

Despite extensive research efforts, the incidence of diabetes is still increasing at an alarming rate with over 346 million people affected worldwide and the number is expected to rise to 544 million people in 2030 [17, 18]. Prolonged diabetes leads to serious damage to many of the body's physiological processes and causes a number of medical complications, such as cardiovascular disease, stroke, atherosclerosis, blindness, kidney damage, lower-limb amputations among many others [18,19]. Among the two major types of diabetes, type 2 diabetes (T2D) accounts for 90-95% of the total diabetic patients worldwide. It is a heterogeneous disorder characterized by a progressive decline in insulin action (insulin resistance), followed by the inability of pancreatic β -cells to compensate for insulin resistance (β -cell dysfunction) which leads to hyperglycemia [20].

Prolonged hyperglycemia leads to the auto-oxidation of glucose and formation of advanced glycated end products which are involved in the generation of reactive oxygen species (ROS) that cause lipid peroxidation and play an important role in the production of secondary complications in T2D [21]. Oxidative stress is believed to be a common pathway linking diverse mechanisms for the pathogenesis of microvascular and macrovascular complications of diabetes [22]. Therefore, the use of antioxidants could be exploited in the treatment of T2D and prevention of its chronic vascular complications [23]. Another therapeutic approach commonly exploited in the management of T2D is decreasing the postprandial rise of blood glucose level by impeding glucose absorption in the digestive tract through inhibition of carbohydrate hydrolyzing enzymes (α -glucosidase and α -amylase) [24]. Combined actions of α -glucosidase and α -amylase enzymes are responsible for hydrolysis of starch, complex carbohydrates and oligosaccharides to glucose and other monosaccharides which are then absorbed in the intestinal epithelium and enter into the blood circulation [25]. Therefore, α -glucosidase and α -amylase inhibitors will ultimately reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, diminishing the postprandial hyperglycemia.

At present, many clinical drugs are used as α -glucosidase inhibitors; however, severe side effects such as diarrhea, flatulence, lactic acid intoxication and other gastrointestinal problems are associated with their intake [26, 27]. Randomized controlled trials with glucosidase inhibitors report these gastrointestinal side effects as the most common reason for noncompliance and early subject withdrawal [28]. Interestingly, plant based agents were reported to be a more acceptable source of glucosidase inhibitors due to their low cost and non-toxic nature, including a low incidence of serious gastrointestinal side effects [29, 30]. On the other hand, synthetic anti-oxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tertibutylhydroxytoluene exhibit potent free radical scavenging effects but they induce liver damage and carcinogenesis in laboratory animals [31, 32]. Based

on the above, recourse to plants as sources of antioxidants and α -glucosidase inhibitors becomes an appealing alternative.

Vitex doniana (Verbanaceae) commonly called African black plum is widely distributed in tropical West Africa. Various parts of the plant are used by traditional medicine practitioners in Nigeria for the management and treatment of several disorders which include rheumatism, hypertension, cancer, and inflammatory diseases. An ethnobotanical study also revealed that the plant leaves are used in the treatment of diabetes mellitus in northern Nigeria [7]. The myelo- and hepatoprotective as well as the analgesic and antitrypanosomal activities of the leaf extracts have also been reported in several recent studies [1-2; 10, 13]. The stem bark of the plant was found to induce graded uterine muscle contractions [5] and demonstrated antidiarrhoeal and antibacterial activities [3, 6, 9]. Furthermore, natural consumption of the plant was linked to reduced reproductive function of wild baboons [15]. A recent preliminary study also reported the antioxidant potential of the methanolic extract from the leaves of this plant [14]. However, the detailed anti-oxidative effects of different parts of this plant using various extracts and solvent fractions as well as the inhibitory effects on the carbohydrate digesting enzymes are still unknown, despite the traditional use of the plant in the management of diabetes.

Hence our current study conducted a comprehensive and systematic investigation on the *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory activities of various extracts and solvent fractions of *V. doniana* with a view to find new alternative agent(s) that could provide a holistic avenue to control postprandial hyperglycemia and other diabetic complications.

7.2.3 Materials and Methods

Please refer to chapter two sub-sections 2.3-2.6 page 35-38; 2.6.1-2.6.6 page 38-40 for detailed materials and methods that affect *V. doniana*

7.2.4 Results

The ethanolic extract of the leaves was found to contain a significantly ($P<0.05$) higher amount of total phenolics than other crude extracts from the different parts of the plant (**Table 7.2.1**). All the stem bark extracts displayed a weak and statistically similar ($P<0.05$) total reducing power (GAE) compared to ascorbic acid and trolox. For the roots, the ethyl acetate extract had significantly ($P<0.05$) higher reducing power than other extracts at all concentrations. The ethanolic extract of the leaves demonstrated a significantly ($P<0.05$) higher Fe^{3+} - Fe^{2+} reducing ability than all other extracts and trolox (**Figure 7.2.1**).

Table 7.2.1: Percentage yield and total polyphenol concentrations of various solvent fractions of *V. doniana* parts

Crude extracts	% Yield	Total polyphenol (mg/g GAE)
Stem bark		
Ethyl acetate	0.26	0.86 ± 0.03^a
Ethanollic	0.53	4.38 ± 0.38^c
Aqueous	0.67	10.65 ± 0.62^d
Root		
Ethyl acetate	0.11	17.75 ± 0.64^e
Ethanollic	0.42	29.04 ± 3.88^f
Aqueous	0.51	19.92 ± 2.15^e
Leaves		
Ethyl acetate	0.29	9.76 ± 1.83^d
Ethanollic	0.22	50.90 ± 6.06^g
Aqueous	0.38	2.62 ± 0.94^b

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-g}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

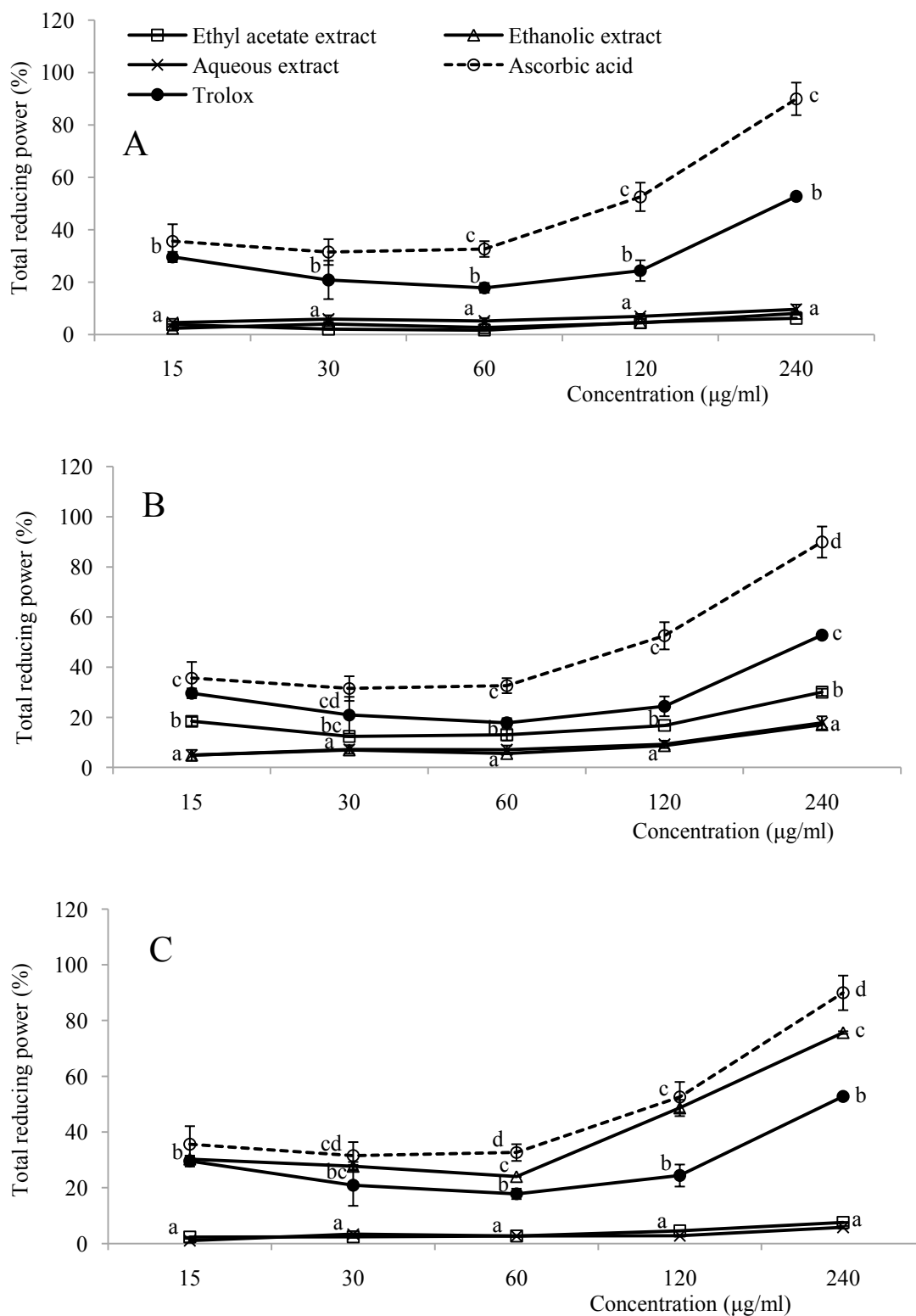


Figure 7.2.1: Percentage total reducing power (gallic acid equivalent) of crude extracts of stem bark (A), root (B) and leaves (C) of *Vitex doniana* parts. Data are presented as mean \pm SD of triplicate determinations. ^{a-d} Different alphabets presented for a given concentration of each extract indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

The calculated IC_{50} values for the different anti-oxidative models are presented in **Table 7.2.2**. According to the IC_{50} values, the DPPH radical scavenging activities of the various extracts of *V. doniana* parts indicated that the stem bark extracts had weaker free radicals quenching ability than extracts from other parts of the plant (**Table 7.2.2**). Crude ethanolic and aqueous extracts of the root as well as the ethanolic extract of the leaves demonstrated the strongest and statistically similar ($P < 0.05$) free radical scavenging activity among all the crude extracts from the plant. The best HRS activity was observed with ethyl acetate extract of the root ($IC_{50} = 2.22 \pm 0.15 \mu\text{g/ml}$) and the ethanolic extract of the leaves ($IC_{50} = 2.72 \pm 0.60 \mu\text{g/ml}$). Furthermore, in the NO scavenging assay, the ethanolic extract of the leaves showed a significantly higher ($P < 0.05$) scavenging activity towards the generated NO radicals than other extracts from the plant (**Table 7.2.2**). A close analysis of the results obtained from the four different models for anti-oxidative studies indicated that the ethanolic extract of the leaves had the highest anti-oxidative activity that reasonably cuts across all the models used and therefore, was partitioned.

Table 7.2.2: IC₅₀ values of various extracts of *V. doniana* parts in different anti-oxidative models

Samples	IC ₅₀ (μg/ml)		
	DPPH	HRS	NO
Stem bark			
Ethyl acetate	13.70 ± 1.18 ^{h*}	2.43 ± 0.22 ^{i**}	298.79 ± 56.05 ^e
Ethanol	170.88 ± 12.40 ^f	7.51 ± 0.90 ^c	4.47 ± 0.13 ^{i*}
Aqueous	128.93 ± 19.41 ^e	467.54 ± 63.40 ^g	192.10 ± 31.16 ^d
Root			
Ethyl acetate	47.38 ± 7.84 ^d	2.22 ± 0.15 ^a	10.02 ± 1.52 ^{k*}
Ethanol	2.36 ± 0.34 ^b	37.66 ± 5.15 ^d	549.87 ± 58.84 ^f
Aqueous	1.96 ± 0.16 ^{ab}	99.66 ± 10.25 ^e	691.22 ± 6.53 ^{fg}
Leaves			
Ethyl acetate	103.63 ± 5.45 ^e	371.86 ± 23.28 ^f	2.17 ± 0.22 ^{i*}
Ethanol	2.64 ± 0.76 ^b	2.72 ± 0.60 ^a	45.94 ± 3.04 ^b
Aqueous	6.49 ± 0.10 ^{g*}	1.37 ± 0.08 ^{h*}	76.39 ± 6.45 ^c
Ascorbic acid	2.56 ± 0.26 ^b	ND	26.40 ± 6.46 ^a
Gallic acid	1.40 ± 0.43 ^a	ND	1.05 ± 0.23 ^{h*}
Trolox	8.47 ± 2.88 ^c	3.23 ± 0.49 ^{ab}	629.76 ± 63.98 ^f

The results are expressed as mean ± SD values of triplicate determinations. ^{a-k}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05). ND means not determined. *Unit was expressed in mg/ml. **Unit was expressed in g/ml.

Using solvent-solvent partitioning, hexane, dichloromethane, ethyl acetate and aqueous fractions were obtained. All the fractions possessed reducing power ability but the aqueous fraction displayed the best activity in this model (**Figure 7.2.2**). However, the results of the DPPH radical scavenging activity showed that the ethyl acetate fraction had a significantly higher (P<0.05) free radical scavenging activity than the aqueous and dichloromethane fractions (**Table 7.2.3**). The results also indicated that the hexane fraction did not contain free radical scavenging phytochemicals. Among the four fractions, only the aqueous and ethyl acetate fractions scavenged hydroxyl and NO radicals. However, the results were not

significantly ($P>0.05$) different from each other in the HRS activity assay whereas the aqueous fraction showed a significantly higher ($P<0.05$) NO scavenging activity than the ethyl acetate fraction (**Table 7.2.3**).

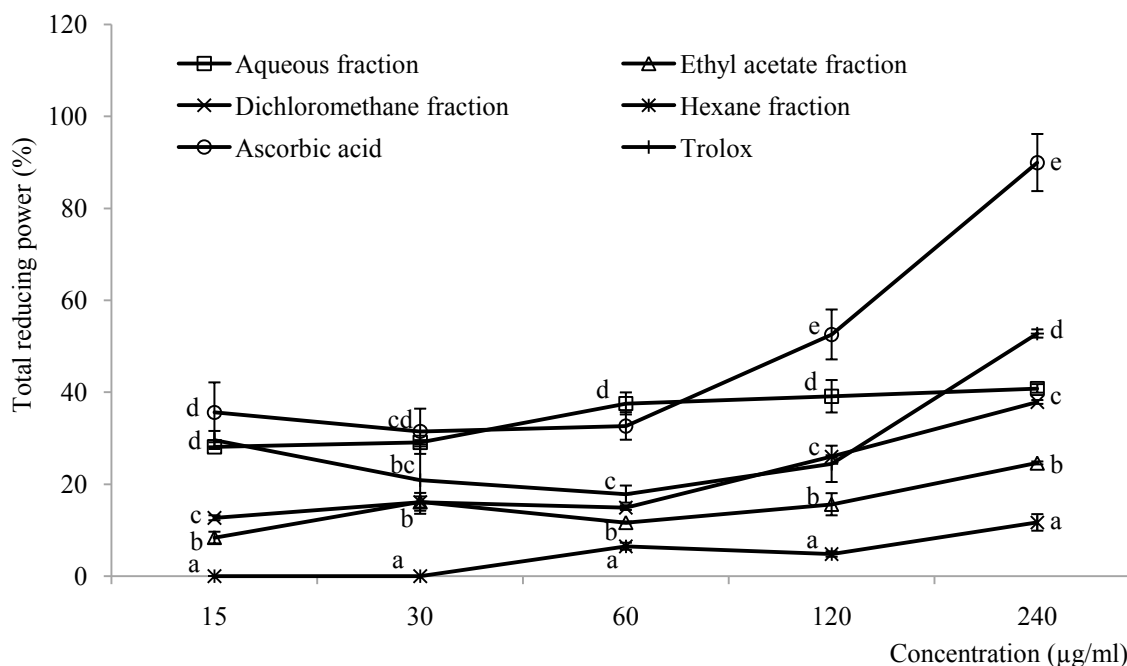


Figure 7.2.2: Percentage total reducing power (gallic acid equivalent) of different solvent fractions of ethanolic extract of *V. doniana* leaves. Data are presented as mean \pm SD of triplicate determinations. ^{a-c}Values with different letters presented for a given concentration are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

Table 7.2.3: IC₅₀ values of various solvent fractions of ethanolic extract of *V. doniana* leaves in different anti-oxidative models

Fractions	Total phenolics (mg/g GAE)	IC ₅₀ (µg/ml)		
		DPPH	HRS	NO
Aqueous	32.57 \pm 2.11 ^a	67.42 \pm 1.54 ^b	81.26 \pm 1.92	210.29 \pm 12.20 ^a
Ethyl acetate	24.81 \pm 0.68 ^b	52.73 \pm 0.39 ^a	77.93 \pm 4.44	697.49 \pm 27.71 ^b
Dichloromethane	9.27 \pm 1.34 ^c	92.06 \pm 0.44 ^c	NIL	NIL
Hexane	NIL	NIL	NIL	NIL

The results are expressed as mean \pm SD values of triplicate determinations. ^{a-c}Different superscript alphabets along a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P<0.05$).

The α -glucosidase and α -amylase inhibitory activities of the different solvent fractions of the ethanolic extract of the leaves are shown in **Figure 7.2.3**. With the exception of hexane fraction, all other fractions inhibited α -glucosidase and α -amylase *in vitro* (**Figure 7.2.3**) but judging from the IC₅₀ values, the aqueous fraction also displayed a significantly ($P < 0.05$) higher α -glucosidase and α -amylase inhibitory activities than other fractions (**Table 7.2.4**).

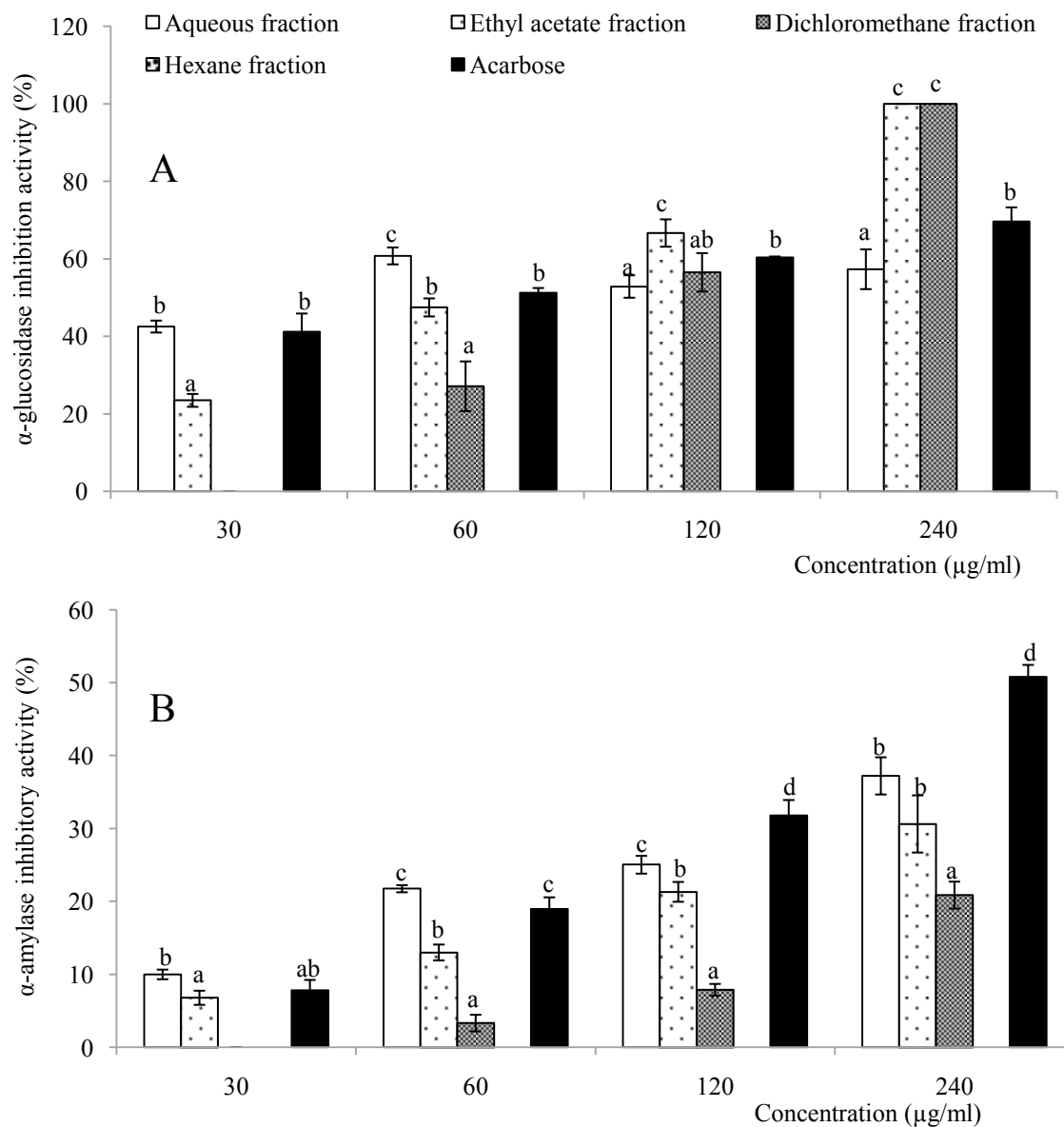


Figure 7.2.3: α -glucosidase (A) and α -amylase (B) inhibitory activities of different solvent fractions of ethanolic extract of *V. doniana* leaves. The results are expressed as mean \pm SD of triplicate determinations. ^{a-d}Different alphabets over the bars for a given concentration indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$)

Table 7.2.4: IC₅₀ values for the inhibition of α -glucosidase and α -amylase by various solvent fractions of ethanolic extract of *V. doniana* leaves

Fractions/standard	IC ₅₀ (μ g/ml)	
	α -glucosidase	α -amylase
Aqueous	41.26 \pm 4.25 ^a	729.31 \pm 145.76 ^b
Ethyl acetate	61.98 \pm 4.81 ^b	1.67 \pm 0.10 ^{c*}
Dichloromethane	91.37 \pm 5.22 ^c	7.27 \pm 0.33 ^{d*}
Hexane	NIL	NIL
Acarbose	55.59 \pm 5.22 ^b	256.66 \pm 20.52 ^a

The results are expressed as mean \pm SD of triplicate determinations. ^{a-d}Different superscript alphabets within a column for a given parameter indicate significant difference (Tukey's-HSD multiple range *post hoc* test, P<0.05). *Unit was expressed in mg/ml.

Steady state kinetic analysis from the initial velocity studies of α -glucosidase using pNPG as substrate gave a K_M and Vmax of 2.00 mM and 655.09 μ mol/min respectively whereas a K_M and Vmax of 0.25 % and 33.70 μ mol/min respectively, were computed for α -amylase using starch as the substrate. Delineation of the type of inhibition exerted by the aqueous fraction revealed that α -glucosidase and α -amylase are non-competitively (**Figure 7.2.4**) inhibited, albeit at different rates, with Ki values of 5.93 and 167.44 μ g/ml respectively (**Table 7.2.5**).

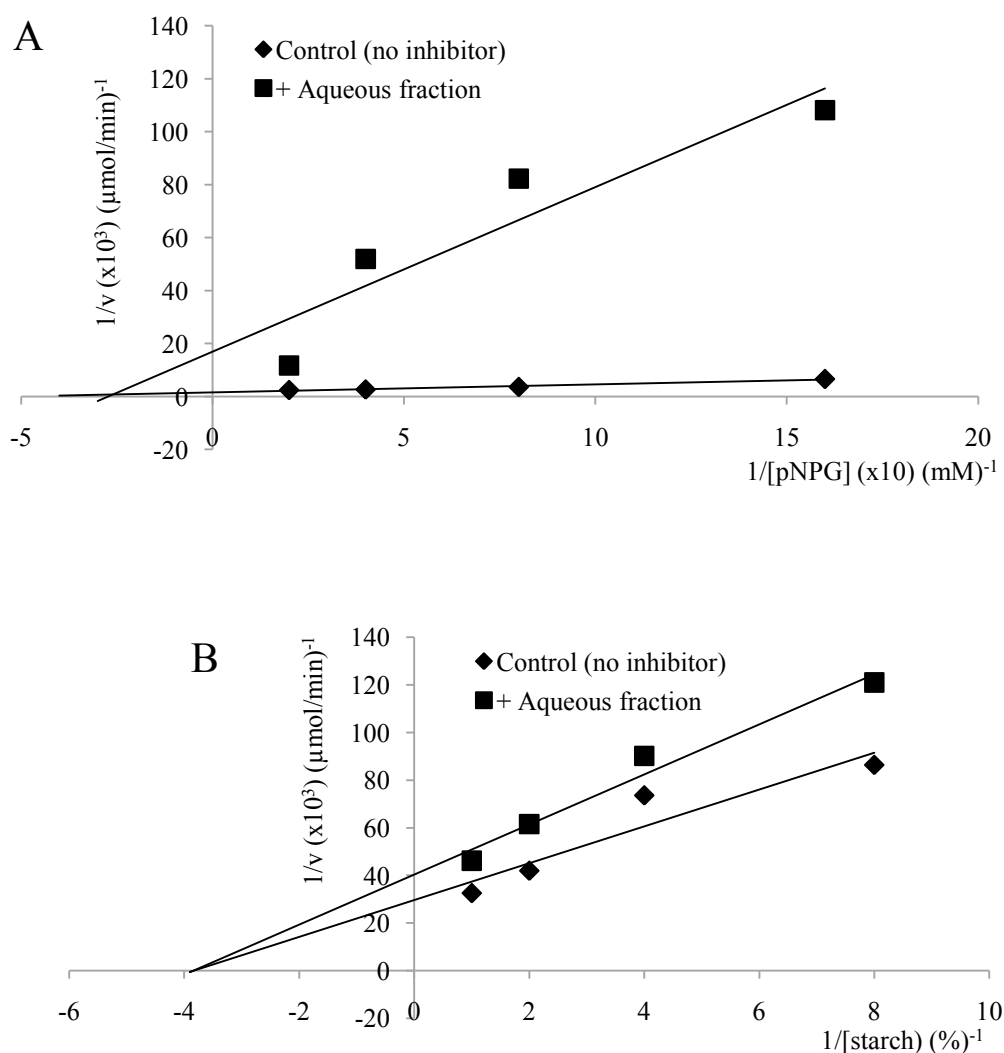


Figure 7.2.4: Lineweaver-Burke's plot of α -glucosidase (A) and α -amylase (B) catalyzed reactions in the presence and absence of the aqueous fraction derived from the *V. doniana* leaves ethanolic extract

Table 7.2.5: Effect of aqueous fraction (60 $\mu\text{g/ml}$) of ethanolic extract of *V. doniana* leaves on some kinetic parameters of α -glucosidase and α -amylase

Kinetic parameters	α -glucosidase		α -amylase	
	Control	+ aqueous fraction	Control	+ aqueous fraction
K_M	2.00*	2.00*	0.25 [#]	0.25 [#]
V_{\max} ($\mu\text{mol/min}$)	655.09	58.96	33.70	24.81
K_i ($\mu\text{g/ml}$)	-	5.93	-	167.44

The units for K_M were mM (*) and % ([#])

Based on the above results, the aqueous fraction was subjected to GC-MS analysis in order to identify the components of the fraction. From the experiment, the reasonably identified compounds in the fraction were mainly phenolic compounds such as resorcinol, 4-hydroxybenzoic acid, 3,4,5 trimethoxy phenol and 2,4'-dihydroxychalcone (**Figure 7.2.5 and Table 7.2.6**).

Table 7.2.6: Components of the aqueous fraction of *V. doniana* leaves ethanolic extract identified through their mass fragmentation pattern

Retention time (min)	Compound	Molecular mass
8.05	Resorcinol	110.1
11.43	4-hydroxy benzoic acid	138.1
12.36	3,4,5-trimethoxy phenol	184.2
20.94	2,4'-dihydroxychalcone	240.2

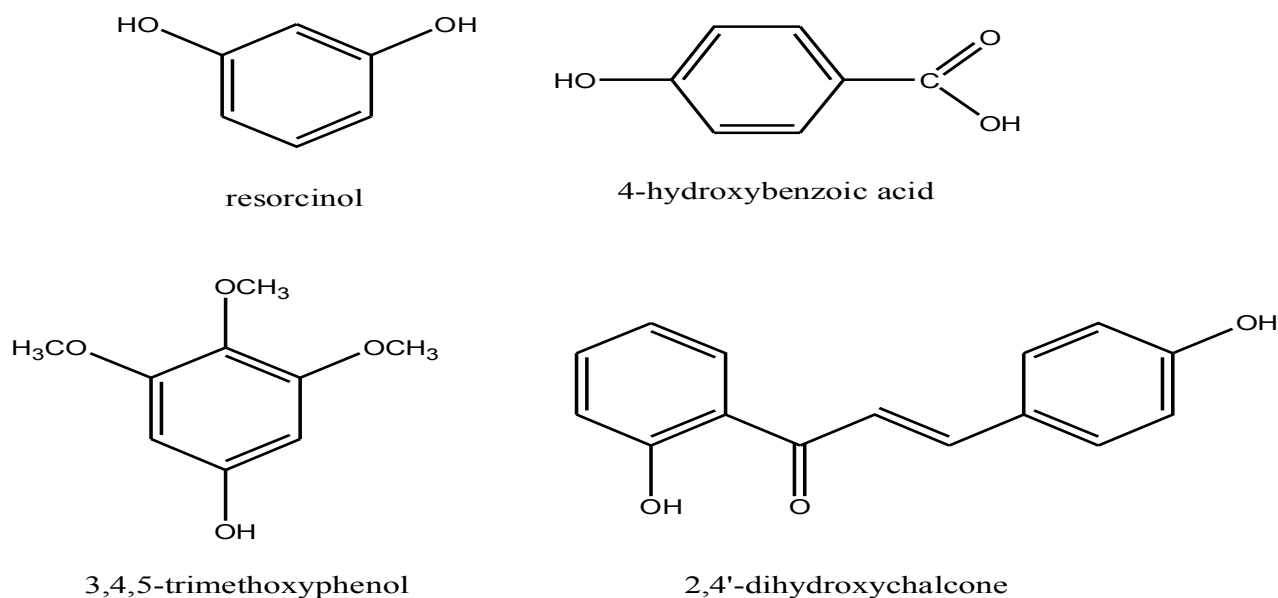


Figure 7.2.5: The chemical structures of the compounds identified in the aqueous fraction of the ethanolic extract of *V. doniana* leaves.

7.2.5 Discussion

Type 2 diabetes is linked to oxidative stress-mediated complications as well as hyperglycemia which are regarded as important underlying factors for the pathogenesis of the disease [33]. Thus, agents that can scavenge free radicals and have strong antioxidant as well as α -glucosidase and α -amylase inhibitory properties play a significant role in the treatment and prevention of T2D and related complications. In the present study, the stem bark, root and leaves of different solvent extracts of *V. doniana* were evaluated for their anti-oxidative property which led to the fractionation of the ethanolic extract of the leaves as well as investigation of the anti-oxidative and α -glucosidase and α -amylase inhibitory activity of the fractions.

Four complementary assays were performed to evaluate the anti-oxidative activities of the extracts because a single model cannot give a full evaluation of the anti-oxidative capabilities of the different extracts tested due to the involvement of multiple mechanisms. The total reducing power of a compound serves as a significant indicator of its antioxidant potential. The reductants terminate the free radical chain reaction by donating hydrogen atoms to the radical molecules. Free radicals are known to be a major factor in cellular damages in biological systems and DPPH method has been used to evaluate the free radical scavenging activity of natural antioxidants. On the other hand, hydroxyl radicals are also extremely reactive species capable of damaging any biological molecule found in living systems [34] and NO is an unstable species which reacts with oxygen to generate the reactive nitrite and peroxynitrite anions [35]. All these radicals are implicated in the pathogenesis of T2D [36]. Findings from this investigation revealed that among the various solvent crude extracts of the different parts of *V. doniana*, the ethanolic extract of the leaves possessed better antioxidant activity having reasonably high scavenging activity toward the various forms of radicals. This observation supports the traditional use of the leaves of *V. doniana* by traditional healers for the management of T2D and other radical pathologies as opposed to the other parts of the plant. Hence, the ethanolic extract of the leaves was selected for further fractionation based on its anti-oxidative activities.

Evaluation of the different fractions from the ethanol extract of the leaves indicated that the aqueous fraction had the best anti-oxidative activity amongst the fraction but the activity was less potent than what was observed for the crude ethanolic extract. This suggests that not all anti-oxidative components were concentrated in the aqueous fraction and other less polar components also contribute to the overall anti-oxidative activity. Nevertheless, at least among the fractions, the aqueous fraction seems to contain the most powerful phytochemical(s) that could be useful therapeutic agents for treating oxidative stress based metabolic disorders including T2D. Moreover, the observed anti-oxidative activity is linked to the highest phenolic content recorded in this fraction because phenolic compounds are the major constituents in plants reported to possess anti-oxidative activity [37].

Analysis of the α -glucosidase inhibitory activity of the fractions also demonstrated that the aqueous fraction contains the most powerful inhibitors against this enzyme which could slow down the breakdown of disaccharides to liberate glucose; thereby reducing glucose absorption from the small intestine [38]. This consequently suppresses the postprandial rise in the blood glucose level. The observation could be linked to the high phenolic content recorded in this fraction since polyphenolic fractions from plants have been shown to inhibit α -glucosidase activity and allow for a tight control of blood glucose [39]. Indeed, some isolated phenolics have been reported to be the main bioactive anti-diabetic agents of *Brickellia cavanillesii* [40] and *Garcinia mangostana* [41] and the activity was mediated through the inhibition of α -glucosidase. Furthermore, Mai et al. [42] reported a strong positive correlation between polyphenolic content and α -glucosidase inhibitory effects of 28 extracts from Vietnamese edible plants. In the same vein, the aqueous fraction had the highest α -amylase inhibitory activity amongst the fractions. Humans have five α -amylase genes, three encoding salivary α -amylase and two encoding pancreatic α -amylase. Both salivary and pancreatic α -amylases are composed of a single polypeptide chain of 496 amino acids with high degree of amino acid sequence similarity of 97% overall identical residues and 92% in the catalytic domain [24]. These similarities suggest that the aqueous fraction could interact with both isozymes through a similar pattern to slow down the breakdown of complex carbohydrates to oligosaccharides, thereby diminishing the effect of carbohydrates consumption on postprandial hyperglycemia. Although the α -amylase inhibitory activity of the aqueous fraction was lower than the reference drug acarbose, but keeping in mind the multiple health benefits of herbal medicines, this finding may provide further scope for investigation.

Kinetic delineation of the α -glucosidase and α -amylase inhibitions indicated that the aqueous fraction inhibited the enzymes in a non-competitive manner suggesting that the bioactive ingredient(s) bind to the enzymes at separate site(s) of the enzyme (rather than the active site) but caused conformational modification at the active site which prevented the effective binding of the substrates to the enzymes. It could further suggest that the fraction contains some phytochemicals capable of interacting with the α -glucosidase-pNPG and α -amylase-starch complexes [43]. Similarly, recent studies have shown that some isolated phenolics such as 6-hydroxyacetyl-5-hydroxy-2,2-dimethyl-2H-chromene, fucofuroeckol A and Dioxinodehydroeckol exhibited potent α -glucosidase inhibitions in a non-competitive manner [40,44]. Interestingly, our GC-MS analysis also revealed that the aqueous fraction mainly contains phenolic compounds such as resorcinol, 4 hydroxybenzoic acid and 3,4,5 trimethoxy phenol and 2,4'-dihydroxychalcone. It could thus imply that the observed α -glucosidase and α -amylase inhibitions of this fraction is mediated by these phenolics acting individually or synergistically. The identification of the chalcone in the fraction is also interesting in that chalcones are biosynthetic precursors to the flavonoids and themselves have a range of medicinal properties [45].

The data obtained from this study suggest that the *V. doniana* leaves contain phenolic compounds that could serve as anti-oxidative agents and inhibitors of α -glucosidase and α -amylase which could be exploited for the development of a holistic therapeutic strategy for the control of postprandial blood glucose levels, T2D and chronic vascular complications. However, the determination of the specific role of each phenolic compound awaits further work on the isolation of these compounds and conducting detailed intervention trials in a T2D model of rats.

Acknowledgements

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Postscript: From the above studies, the aqueous fraction derived from the crude ethanolic extract of the leaves had the best *in vitro* anti-diabetic potentials. Unfortunately however, due to the low yield and unavailability of the leaves of this plant (for more extraction) as at the time needed, this plant was not subjected to further *in vivo* studies.

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CHAPTER 8

8.0 GENERAL DISCUSSIONS AND CONCLUSIONS

8.1 General discussions

Diabetes mellitus affects more than 14 million people in Africa, accounting for approximately 4.3% of average adults and responsible for about 401 000 death in the continent [1]. It inflicts a major socio economic problem, especially to the patients. Currently, different therapeutic approaches are used to control the disease using synthetic anti-diabetic drugs in addition to life style modification. Unfortunately however, most of the available anti-diabetic drugs are costly and not readily affordable to the majority of the affected population. Interestingly, the continent is endowed with tremendous medicinal plants that are explored for the folkloric treatment of the disease. These medicinal plants include *Ziziphus mucronata*, *Cassia singueana*, *Parkia biglobosa*, *Khaya senegalensis* and *Vitex doniana* [2, 3]. Thus, this study was undertaken to validate the traditional use of these plants in the treatment of diabetes which would provide a scientific basis for the subsequent standardization and commercialization of these medicinal plants as anti-diabetic herbal products (in crude form) after approval by relevant authorities. Furthermore, the study was aimed to identify the possible bioactive anti-diabetic compounds from these medicinal plants as a prelude to the development of novel plant-derived anti-diabetic drugs which are considered safer than chemically-originated drugs.

In order to achieve the set goals, the stem bark, root and leaves of each of the afore-mentioned plants were initially subjected to a thorough investigation for *in vitro* anti-oxidative activity. This is because of the vital role of oxidative stress to the two main pathogenic features of diabetes (insulin resistance and pancreatic β -cell dysfunction) as well as other aspects of diabetes complications [4-6]. Thus, the best anti-oxidative crude extracts were selected for further studies. The selection was made in comparison to other extracts within a plant and not across plants.

Ziziphus mucronata was the first plant under study which had a crude ethanolic extract of the root with remarkable *in vitro* anti-oxidative activity. Upon fractionation, this crude extract yielded a butanol fraction with high *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory activities. In spite of these activities of the butanol fraction, the *in vivo* anti-diabetic activity of this fraction was not as powerful as expected. Indeed, among all the four fractions investigated for *in vivo* anti-diabetic activity in this study, this butanol fraction demonstrated the weakest anti-diabetic effects despite its ability to ameliorate oxidative stress in some organs of diabetic animals. These observations suggest that the alleviation of oxidative stress is not strong enough to reverse the alterations of diabetes related parameters possibly because some phytochemicals might have lost their activity due to xenobiotic metabolic processes that could convert bioactive compounds to inactive ones [7]. It is also possible that the

bioavailability of the bioactive phytochemicals was highly limited. In the GC-MS analysis, phenolics were identified as the main bioactive phytochemicals and coincidentally, a pure phenolic compound (2,7-dihydroxy-4*H*-1-benzopyran-4-one) with potent α -glucosidase and α -amylase inhibitory activities was subsequently isolated from the fraction and characterized. The 2,7-dihydroxy-4*H*-1-benzopyran-4-one inhibited α -glucosidase and α -amylase in uncompetitive and non-competitive patterns respectively, which were different from the mixed inhibition patterns observed for the entire fraction. This observation suggests that other phytochemical components of the fraction contribute to the inhibition of the two enzymes. Based on the above discussions, it appears that standardisation and commercialisation of this part of the plant (in crude form) as anti-diabetic herbal remedy might not be a promising venture because of the weak activity. However, on a positive note, the fraction yielded a pure bioactive compound that could be exploited for the development of novel plant-derived anti-diabetic drugs.

Cassia singueana was the second plant under study. The crude ethyl acetate extract of the stem bark yielded an acetone fraction with higher *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory effects compared to other solvent fractions. Subsequently, the fraction retained its anti-diabetic effects in a dose dependent fashion with no toxic effects *in vivo* and was also able ameliorate most of the diabetes associated complications which were linked to the observed results under *in vitro* conditions. The foregoing suggests the stability and safety of the bioactive compounds of the fraction. Although the GC-MS analysis detected mainly phenolics as the bioactive ingredients but the bioassay guided isolation led to a pentacyclic triterpene, 3 β -*O*-acetyl betulinic acid. This underscores the need to isolate, characterize and test a pure compound before a definite statement is made on the bioactive phytochemicals in a plant material. The difference in the mechanism of α -glucosidase and α -amylase inhibitions could also attest to the earlier assertion. Thus, taken the results of *in vitro* and *in vivo* studies as a whole, the *C. singueana* stem bark has the potentials for standardisation as anti-diabetic herbal product.

For *P. biglobosa*, crude ethanolic extract of the leaves had the best anti-oxidative activity and upon fractionation, a butanol fraction had the best *in vitro* anti-oxidative as well as α -glucosidase and α -amylase inhibitory effects compared to other solvent fractions. In the *in vivo* studies, this fraction demonstrated remarkable anti-diabetic effects. More interestingly, the fraction seems to work via multiple mechanisms that involve the established modes of actions of most of the anti-diabetic therapies. This could be a trace of evidence for synergism among the phytochemical constituents of the fraction. It is also noteworthy to state that, in comparison to the fractions from other plants under study, this fraction demonstrated the best *in vivo* anti-diabetic effects. However, from the phytochemistry view point, similar observations to the *C. singueana* fraction were made where the GC-MS analysis detected mainly phenolics and sugars but a pentacyclic triterpene, lupeol was isolated as the α -glucosidase and α -amylase inhibitory phytochemical. Furthermore, the mechanism of α -glucosidase and α -amylase inhibitions between the fraction and lupeol was different which agrees with the earlier suggestion on possible

synergism. Considering the fact that this fraction was derived from the leaves and coupled with its remarkable anti-diabetic effects, this part of the plant presents a good opportunity for possible standardisation as anti-diabetic herbal remedy which could subsequently be commercialised. The fraction also further confirmed that lupeol has an anti-diabetic potential.

The butanol fraction derived from the crude ethanolic extract of *K. senegalensis* root was the last fraction in the *in vivo* studies because it had the best *in vitro* activities among other solvent fractions. The *in vivo* studies also demonstrated that the fraction has dose-dependent anti-diabetic effects which were also mediated through multiple mechanisms. However, the anti-diabetic effects are generally not as potent as those of *P. biglobosa* fraction. The GC-MS analysis detected an array of phenolic compounds as the phytochemical constituents of the fraction and subsequently, the bioassay guided isolation led to a phenolic-like, simple (6 carbon) and rarely-isolated compound, bicyclo [2.2.0] hexane-2,3,5-triol. This compound inhibited α -glucosidase, but not α -amylase, in the same pattern as the butanol fraction which also indicate possible synergism with other phytochemicals. However, the synergism in this fraction did not seem to be as powerful as what was observed in the *P. biglobosa* fraction because in this case, at least the pattern of inhibition against one enzyme is similar. Generally, results from this plant also demonstrated its potential for further standardization. More interestingly, this fraction yielded a rarely encountered compound in natural product chemistry and it possessed potent α -glucosidase and α -amylase inhibitory activities.

8.2 General conclusions

Among the five plants solvent fractions investigated in this work, three of the fractions have demonstrated remarkable and more effective, in some cases, similar *in vivo* anti-diabetic effects than a commonly used standard anti-diabetic drug, metformin. These fractions did not show signs of toxicity for most of the parameters measured in this investigation. The study identified the possible bioactive anti-diabetic compounds (in their pure form) from the selected fractions. The findings are important for the relevant government agencies, pharmaceutical industries, scientific community and poor diabetic patients because it might open an avenue for the development of viable and cost effective anti-diabetic herbal products and/or novel plant-derived anti-diabetic drugs.

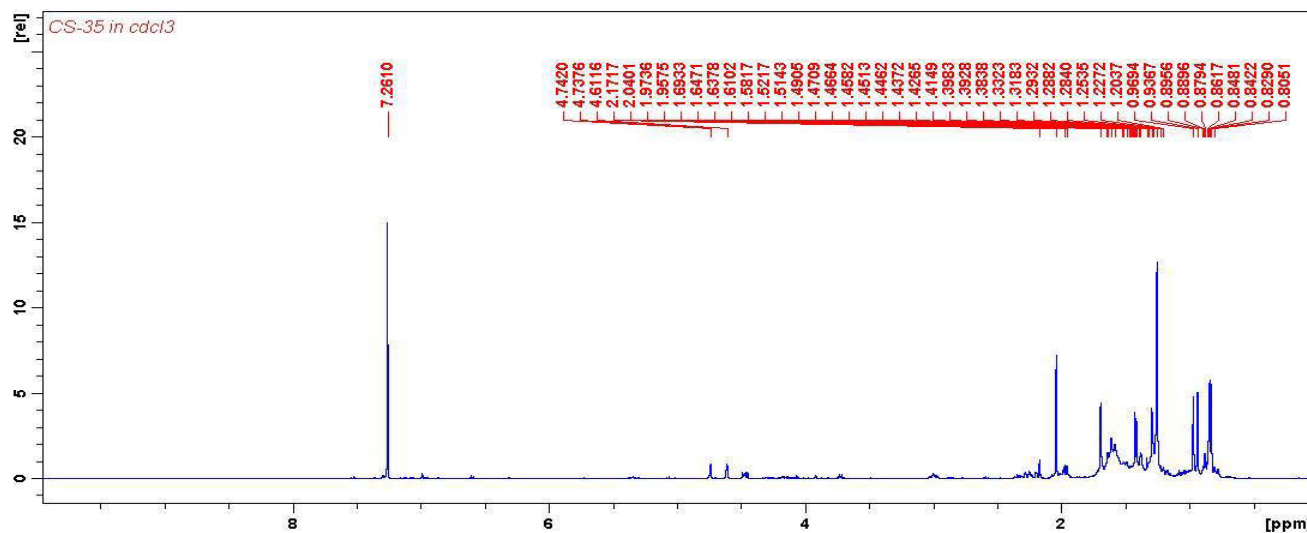
8.3 Recommendations

Massive investment of human and financial resources to conduct a large human based clinical study may be worthwhile as it will confirm the efficacy of the plant samples in humans for eventual standardisation and commercialization as herbal products. The identified bioactive compounds should be subjected to a thorough *in vivo* anti-diabetic study as conducted with the fractions in this study. This may eventually lead to development of novel anti-diabetic drugs.

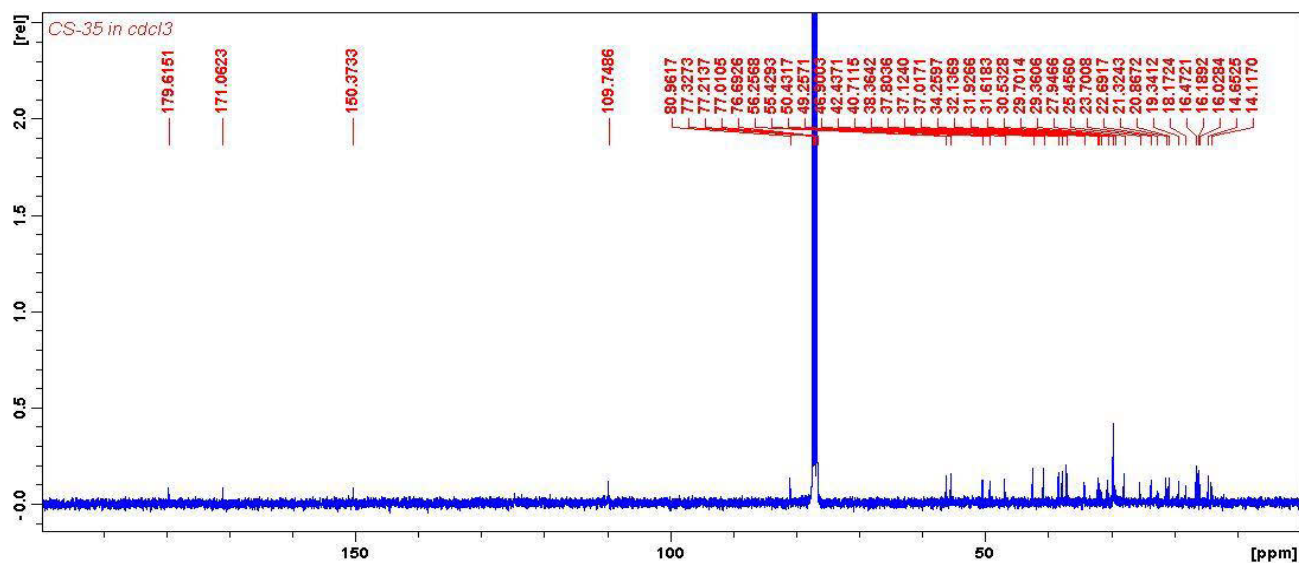
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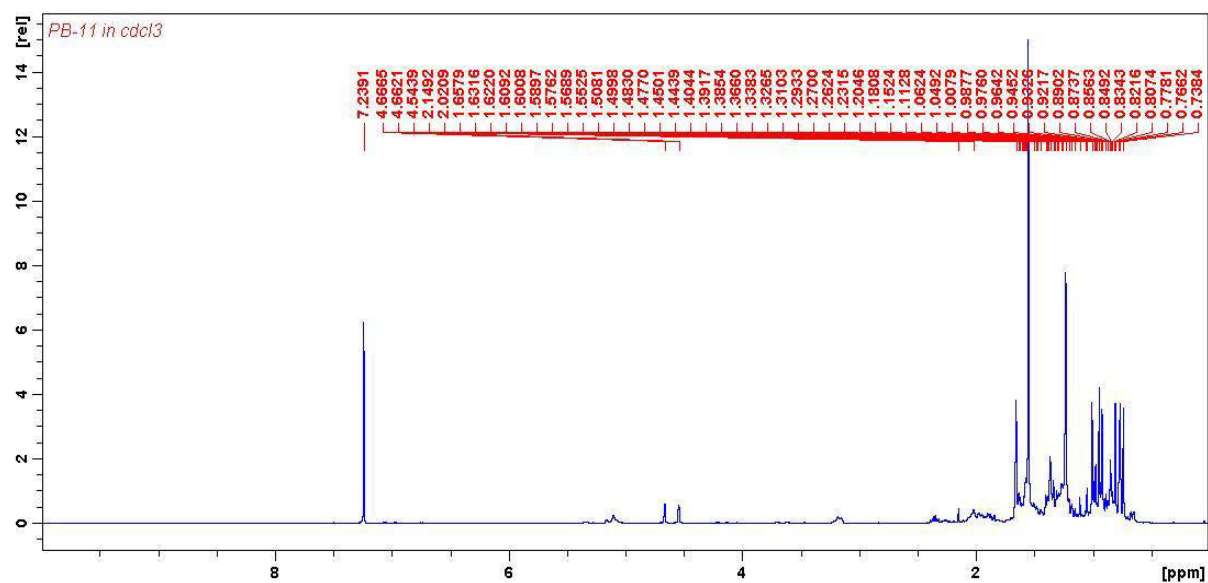
APPENDIX



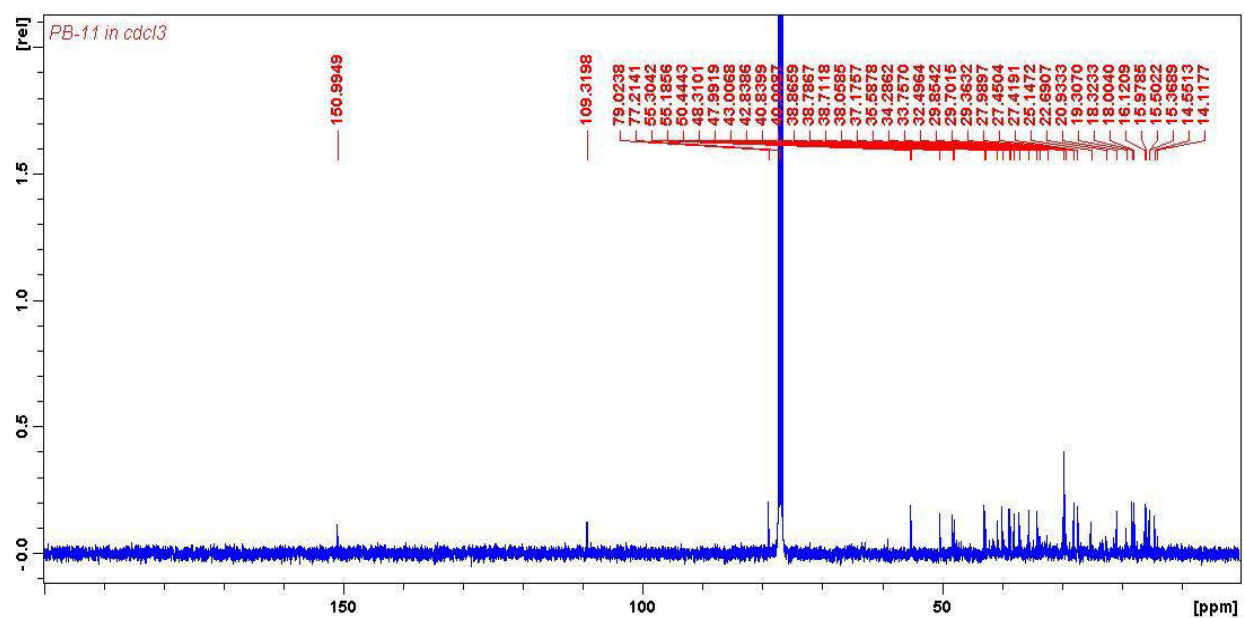
Appendix 1: ^1H NMR spectrum of 3 β -O-acetyl betulinic acid isolated from the acetone fraction of *C.singueana* stem bark.



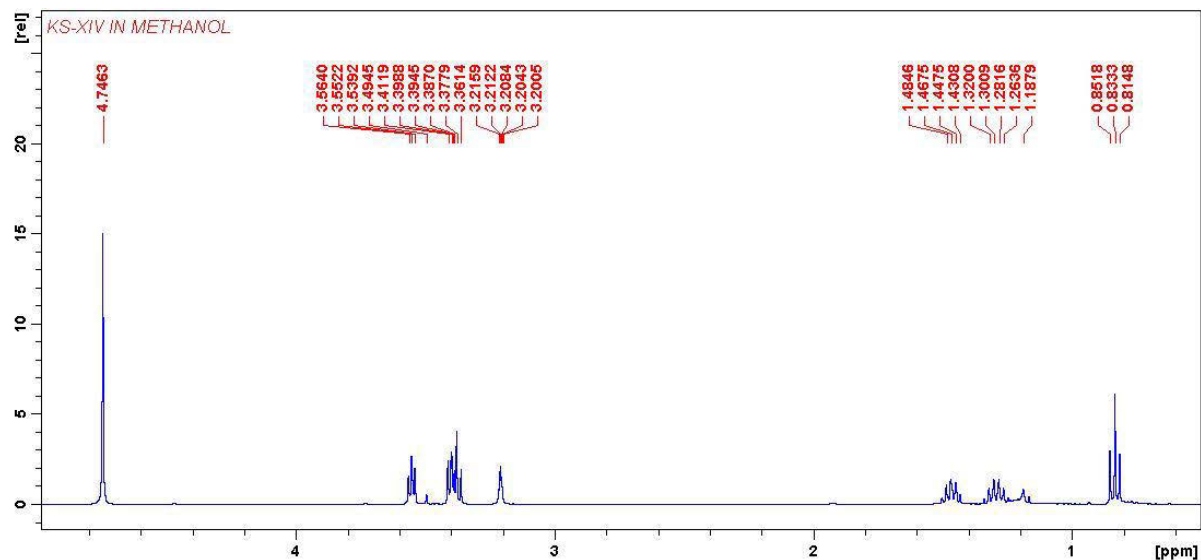
Appendix 2: ^{13}C NMR spectrum of 3 β -O-acetyl betulinic acid isolated from the acetone fraction of *C.singueana* stem bark.



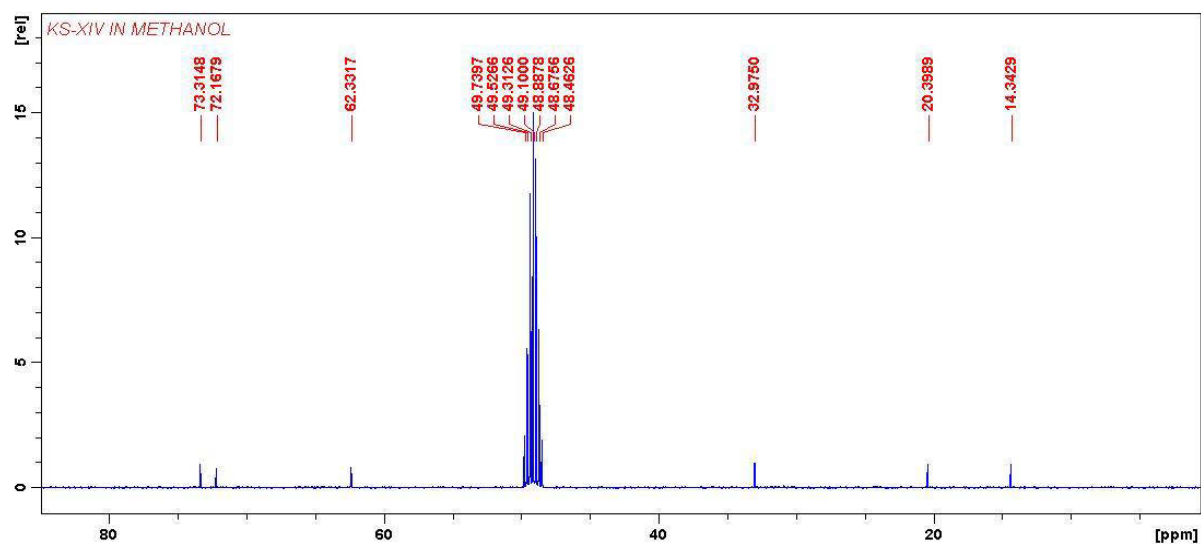
Appendix 3: ^1H NMR spectrum of lupeol isolated from the butanol fraction of *P. biglobosa* leaves



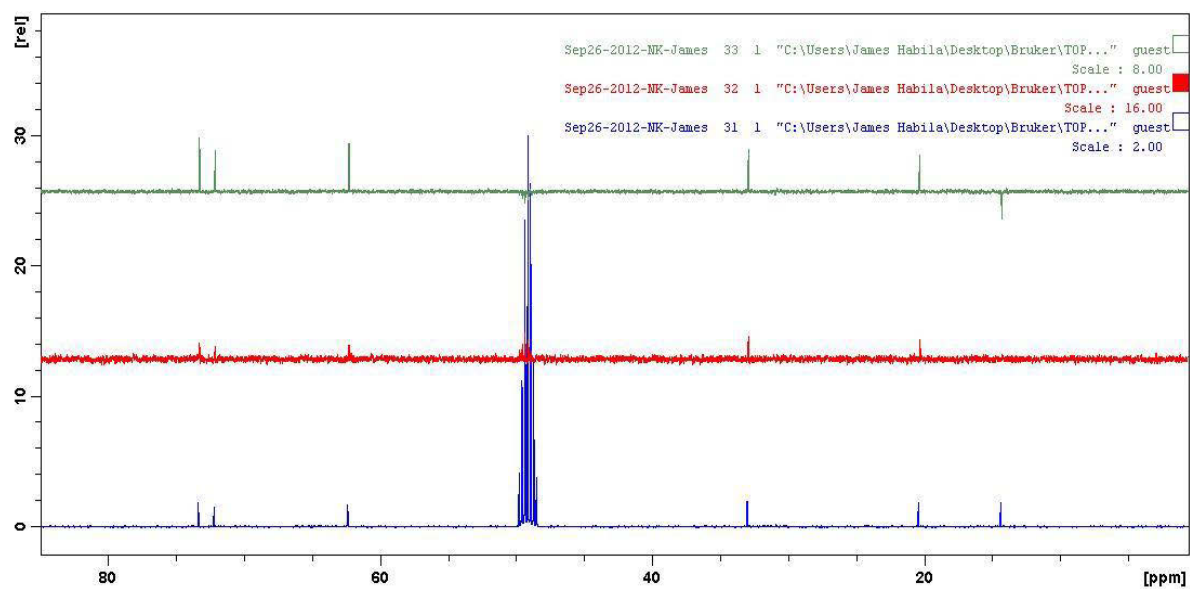
Appendix 4: ^{13}C NMR spectrum of lupeol isolated from the butanol fraction of *P. biglobosa* leaves



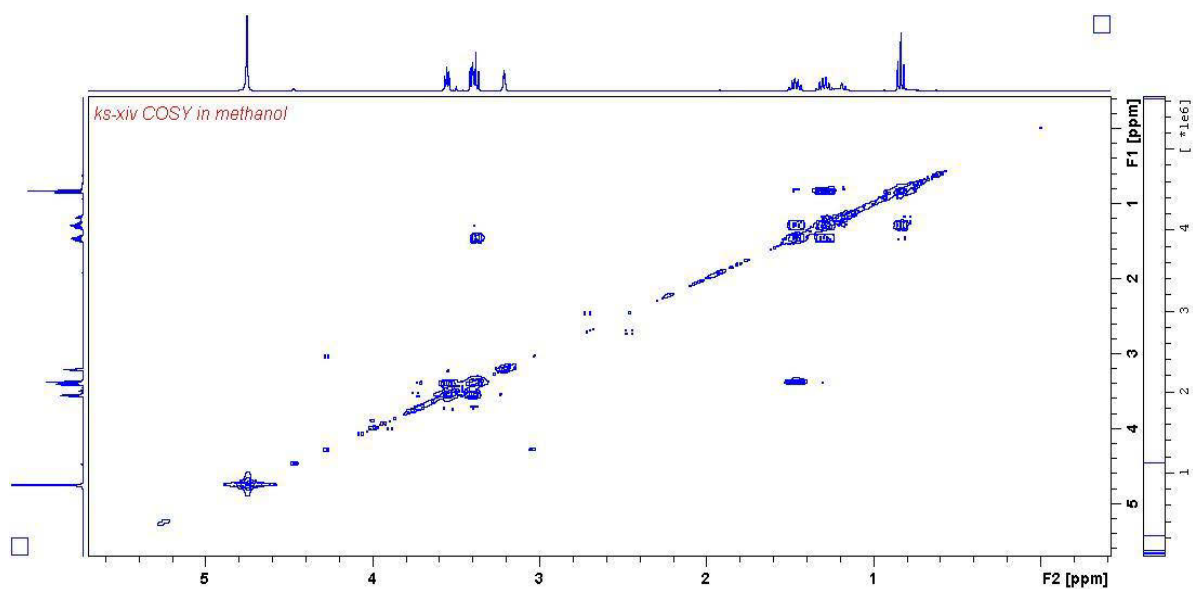
Appendix 5: ^1H NMR spectrum of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



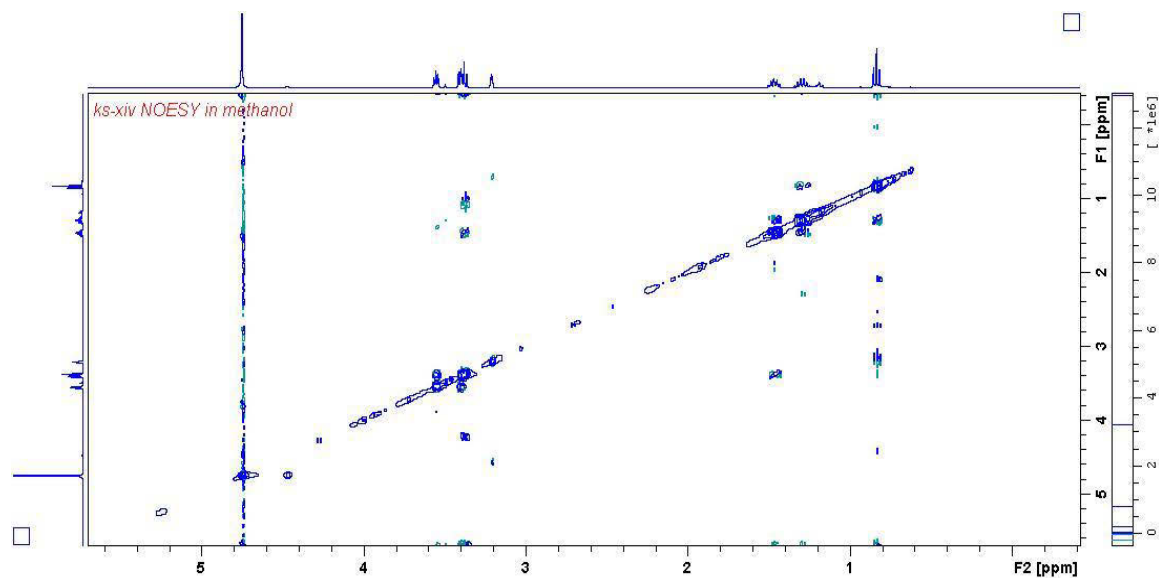
Appendix 6: ^{13}C NMR spectrum of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



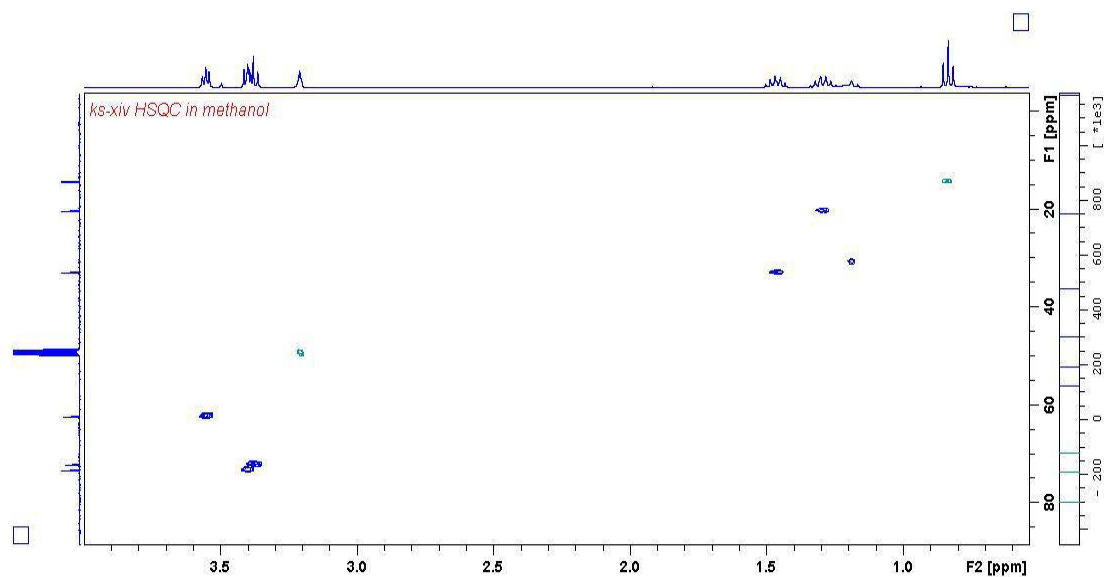
Appendix 7: DEPT spectra of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



Appendix 8: COSY spectrum of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



Appendix 9: NOESY spectrum of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



Appendix 10: HSQC correlation of bicyclo [2.2.0]hexane-2,3,5-triol isolated from the butanol fraction of *K. senegalensis* root



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05 December 2011

Reference: 022/12/Animal

Mr. AMI Ibrahim
Biomedical Research Lab
BGM
Westville Campus
UKZN

Dear Mr. Ibrahim

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011/12 on the following project:

Anti-diabetic effects of some fractionated extracts of medicinal plants in a type 2 diabetes model of rats.

Yours sincerely

**Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee**

Cc Registrar, Prof. Jane Meyerowitz
Research Office, Mr N. Moodley
Supervisor,
Head of School,
BRU, Dr.S.Singh



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Appendix 12: The ethical clearance letter obtained from the University animal ethics sub-committee to conduct the *in vivo* intervention trial