EFFECT OF *PARKIA BIGLOBOSA* SEED PROTEIN ISOLATE ON BIOCHEMICAL AND PROTEOMIC PROFILES IN STREPTOZOTOCIN-INDUCED DIABETES MELLITUS IN MALE SPRAGUE-DAWLEY RATS

BY

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF ZULULAND, KWADLANGEZWA, SOUTH AFRICA

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DECLARATION

I Bolajoko Idiat Ogunyinka hereby declare that the experimental work presented in this thesis is a product of my original work conducted in the Department of Biochemistry and Microbiology, University of Zululand (UZ), under the supervision of Dr A. P. Kappo and Prof A. R. Opoku. It has not been submitted before, either wholly or partially, for any degree or examination at any other University.

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Prof Andy Rowland Opoku

This ----------day of----------2015

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“I will instruct you and teach you in the way you should go; I will counsel you with my eye upon you” (Psalm 32:8).

Almighty God hallow be thy name for thy mercy and compassion bestowed unto me made this doctoral degree a reality.

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ABSTRACT

Diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia, the hallmark of diabetic complications and organs damages. Increasing evidence suggests that free radicals play significant role in the beta cell damage. Beta cell destruction in the pancreatic islets results in deficiency and the total loss of insulin secreation. In fact, antioxidant enzymes like catalase and SOD are known to have anti-diabetogenic action. Apparent side effects, huge costs and availability of anti-diabetic medications, coupled with drugs resistance has necessitated the need to search for alternative treatment remedy for this disease. Medicinal plants have attracted a great deal of attention, particularly for their antioxidant activity and presence of bioactive compounds. More so, plant-derived foods which are rich in proteins and antioxidants have been shown to ameliorate various disease conditions and their complications.

Parkia biglobosa (Jacq.) is a legume well known for its nutritional and medicinal values. However, there is paucity of knowledge on the anti-diabetic properties of *Parkia biglobosa*. This study aims to investigate the effect of the protein isolate of *Parkia biglobosa* on biochemical and proteomic profile of streptozotocin-induced diabetic male rats. Protein isolate was obtained from defatted *Parkia biglobosa* through a series of water extraction and centrifugation processes. The proximate, functional, minerals and anti-nutrient composition of fermented, defatted and protein isolate were carried out using standard procedures. Diabetes mellitus was induced on the rats by intraperitoneal administration of Streptozotocin, after which the rats were orally fed (200, 400 mg/bw) the protein isolate for 28 days. Blood glucose was daily monitored. At the end of the feeding experiment the rats were sacrificed and the following parameters determined: serum lipid profile (total cholesterol (TC), total triglyceride (TG), high density lipoprotein cholesterol (HLD-c), very low density lipoprotein cholesterol (VLDL-c) and low density lipoprotein cholesterol (LDL-c). Antioxidant status (superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), total glutathione total (GSH) and the level of lipid perioxidation (thiobabituric acid) was determined. Serum testosterone (sTT) level as well as serum activity of asparate aminotransferase (AST), alanine aminotransferase (ALT) and the concentrations of total protein were also measured using commercial kits. Serum

interleukin-6 (IL-6) was estimated using the enzyme-linked immunosorbent assay kit. Histopathological analysis of the experimental rats' liver was assessed by staining with haematoxylin and eosin (H & E). HPLC analysis of the protein isolate was carried out using Beckman HPLC system, while total protein extracted from rat liver was analyzed on one-dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE) system. The results revealed that protein isolate (PI) was high in ash and protein but low in fat and carbohydrate contents which are desirable anti-diabetic properties. PI significantly ameliorated the low activity of antioxidant enzymes (SOD, CAT, GST and total GSH) and high level of lipid perioxidation in the heart tissues of diabetic induced rats. PI attenuated the level of liver AST, ALT, total protein and interleukin-6 (IL-6) in diabetes rats. PI also reversed the low testosterone status in diabetic rats. Histopathology report confirmed the ameliorative effects of PI. The HPLC fingerprint obtained for PI revealed eleven distinct peaks and five minor peaks; this could provide new leads for future identification of the bioactive components in PI. SwissProt database on a ProteinScape 3.0 workstation positively identified 4 differentially expressed proteins which are known to uphold the normal physiological function of organs and tissues. It is apparent that the PI is good source of protein which normalized the hyperglycemia, dyslipidemia, testerosterone, inflammation, oxidative stress and hepatic damage in the experimental rats. Damaged to vital organs as well as reproductive dysfunction are common complications of diabetes. Therefore, these properties of PI could be considered in the treatment and management of diabetic complications.

DEDICATION

This thesis is dedicated to my mother-in-law, Mrs Mercy Idowu Ogunyinka, for her labor of love.

LIST OF ABBREVIATIONS

AA	Amino acid
AAS	Amino acid score
AGE	Advanced glycation endproduct
ALT	Alanine transaminase
AST	Aspartate amino transaminase
ATP	Adenosine triphosphate
BWT	Body weight
BV	Biological value
CAT	Catalase
CHD	Coronary heart disease
CRP	C-reactive protein
DAG	Diacylglycerol
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPB	Defatted Parkia biglobosa
EAAI	Essential amino acid index
eNOS	Endothelia nitric oxide synthase
FA	Fatty acid
FC	Food conversion
FER	Food efficient ratio
FPB	Fermented Parkia biglobosa
GABA	Gamma amino butyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Reduced glutathione

GLUT 4	Glucose transporter
HDL	High density lipoprotein
H_2O_2	Hydrogen per oxide
HPLC	High-performance liquid chromatography
IDF	International Diabetes Federation
iNOS	Inducible nitric oxide synthase
IL-6	Interleukin-6
IL-10	Interleukin-10
LAA	Limitation amino acid
LDL	Low density liprotein
MDA	Malondialdehyde
MUFA	Mono saturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAA	Non-essential amino acid
NFκB	Necrosis factor kappa B
NI	Nutrition index
$^{\cdot}O^{-}_{2}$	Super oxide ion
PB	Parkia biglobosa
PER	Protein efficient ratio
PI	Protein isolate
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acid
RAGE	Receptor for advanced glycation
ROS	Reactive oxygen specie
RNS	Reactive nitrogen specie

SFA	Saturated fatty acid
SOD	Super oxide dismutase
STT	Serum testosterone
STZ	Streptozotocin
TAA	Total amino acid
TAAA	Total acidic amino acid
TAC	Total antioxidant capacity
TArAA	Total aromatic amino acid
TBAA	Total basic amino acid
TBARS	Thiobabituric acid reactive substances
TC	Total cholestrol
TCA	Tricarboxylic acid
TEAA	Total essential amino acid
TG	Total glyceride
TGFβ	Transforming growth factor beta
TGSH	Total glutathion
TNAA	Total neutral amino acid
TNF	Tumor necrosis factor
TSAA	Total sulphur amino acid
TSM	Technicon Sequential Multi sample Amino Acid Analyzer

CONTRIBUTIONS TO KNOWLEDGE

Published articles

- **Ogunyinka,** I.B, Oyinloye, B.E., Adenowo, A.F., Kappo, A.P. (2015). Potentials of some plant-derived foods in the management of diabetes and **associated** Complications. African Journal of Traditional, Complementary and Alternative Medicine 12(6): 12 – 20.

Submitted Articles under review

- **Bolajoko Idiat Ogunyinka**, Babatunji Emmanuel Oyinloye, Foluso Oluwagbemiga Osunsanmi, Abidemi Paul Kappo and Andrew Rowland Opoku. Comparative study on proximate functional and anti-nutrient composition of fermented, defatted and protein isolates of *Parkia biglobosa* seed. Journal of Food Science & Nutrition.
- **Bolajoko Idiat Ogunyinka**, Foluso Olungbemiga Osunsanmi, Babatunji Emmanuel Oyinloye, Abidemi Paul Kappo and Andrew Rowland Opoku. Comparative studies of amino acids composition of fermented, defatted and protein isolates of *Parkia biglobosa* (locus beans) seeds. Nutrition Research and Practice.
- **Bolajoko Idiat Ogunyinka**, Foluso Olungbemiga Osunsanmi, Babatunji Emmanuel Oyinloye, Abidemi Paul Kappo and Andrew Rowland Opoku. Hypoglycemic, hypolipidemic and cardioprotective potential of protein isolate from *Parkia biglobosa* in streptozotozin induced diabetic rats. Iranian Journal of Pharmaceutical Sciences.
- **Bolajoko Idiat Ogunyinka**, Babatunji Emmanuel Oyinloye, Foluso Oluwagbemiga Osunsanmi, Abidemi Paul Kappo and Andrew Rowland Opoku. Modulatory influence of *Parkia biglobosa* protein isolate on testosterone and biomarker of oxidative stress in brain and testes of streptozotocin-induced diabetic male rat. International Journal of Physiology, Pathophysiology and Pharmacology.
- **Bolajoko Idiat Ogunyinka**, Babatunji Emmanuel Oyinloye, Foluso Oluwagbemiga Osunsanmi, Abidemi Paul Kappo and Andrew Rowland Opoku. Protective effects of *Parkia biglobosa* protein isolate on

streptozotocin-induced hepatic damage and oxidative stress in diabetic male rats. Journal of Pharmaceutical Science Journal.

- **Bolajoko Idiat Ogunyinka**, Babatunji Emmanuel Oyinloye, Folusho Oluwagbemiga Osunsanmi, Abidemi Paul Kappo and Andrew Rowland Opoku. Proteomic analysis differentially expressed proteins in Streptozotocin-induced diabetics rats treated with Parkia biglobosa protein isolate. Acta Biochimica Polonica

Conference attended

- **Ogunyinka B.I**., Oyinloye B.E., Osunsanmi F.O., Kappo A.P. and Opoku A.R. Comparative study on proximate functional and anti-nutrient composition of fermented, defatted and protein isolatesof *Parkia biglobosa* seed. 42nd annual conference of SAAB on plant Biodiversity, past, present and the future 10-13 January 2016. Bloemfontein, South Africa.

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Chapter One

1.0 Introduction

Diabetes, once regarded as a disease of affluence is now becoming rampant in developing countries (Siegel and Narayan, 2008) due to increasingly sedentary life-styles, nutrition transition, rapid urbanization and genetics. Studies have shown that, in 2010, over 12 million people in the sub-Sahara Africa had diabetes and 330,000 people were going to die from diabetes-related complications over the next 20 years (Mbanya *et al.,* 2010). Diabetes is the fifth leading cause of death globally, with over 200,000 deaths occurring each year from diabetes-related complications (Faleyimu *et al.,* 2010). It was also predicted that, by the year 2030, the estimated numbers will have risen to 23.9 million (Mbanya, 2009; IDF, 2009).

Diabetes mellitus usually occurs due to a low level of insulin production or an inability to use insulin properly, leading to several acute complications like ketoacidosis, hyperosmolar non-ketoacidosis coma, and chronic complications like cataracts retinopathy, neuropathy, nephropathy and cardiovascular diseases. It is known to involve changes in lipid metabolism and oxidative stress (Scoppola *et al.,* 2001). These changes are linked to microvascular and macrovascular complications, which are implicated in the morbidity and mortality of diabetic individuals (El-Ghaffar and El-Said, 2006; American Diabetes Association, 2010).

Diabetes is a continual, incapacitating metabolic disease that required lifelong management and greatly increases the risk of serious complications, which are usually managed or treated with exercise, nutrition and drugs like insulin injection, glibenamide, and metformin. However, the cost of management therapy and accompanied side effects of these drugs remain a major global concern. Therefore, there is a need for an alternative, cost-effective way of managing the disease, especially in the sub-Saharan Africa which is plagued with poverty. The World Health Organization (WHO) report revealed that more than 80% of the population in low and middle income countries depends on traditional treatments such as herbs for their daily needs, especially in countries where access to conventional diabetes medication is not adequate (WHO, 1980). This observation by the global health

watchdog has encouraged studies into traditional medicine practices (Magaji *et al.,* 2010).

Kouadio and co-workers (2000) reported that *Parkia biglobosa* seeds exhibit analgesic and anti-inflammatory properties, while Odetola and colleagues (2006) showed that the seeds from this same plant have anti-lipidemic and possible anti-diabetic potentials. This study is premised on both earlier studies and investigated the anti-diabetic properties of the seeds of *P. biglobosa* by evaluating the biochemical and proteomic profiles of this plant when administered to male Sprague-Dawley rats undergoing streptozotocin-induced diabetes mellitus.

1.1 Structure of the Thesis

This thesis consists of nine chapters and appendices:

Chapter One gives a brief background and motivation for the study.

Chapter Two gives a detailed review of the literature of the study. It also provides the aim and objectives of the study conducted.

Chapter Three is a review of the article titled “Potentials of some plant-derived foods in the management of diabetes and associated complications”.

Chapter Four is the research article titled “Comparative study on proximate, functional and anti-nutrient composition of fermented, defatted and protein isolate of *Parkia biglobosa* seeds”.

Chapter Five is the research article titled “Comparative studies of amino acids composition of fermented, defatted and protein isolates of *Parkia biglobosa* (Locust beans) seeds”.

Chapter Six is the research article titled “Hypoglycemic, hypolipidemic and cardioprotective potentials of protein isolate from *Parkia biglobosa* in streptozotozin induced diabetic rats”.

Chapter Seven is the research article titled “Modulatory influence of *Parkia biglobosa* protein isolate on testosterone and biomarker of oxidative stress in brain and testes of streptozotocin-induced diabetic male rats”.

Chapter Eight is the research article titled “Protective effects of *Parkia biglobosa* seeds protein isolate on streptozotocin-induced hepatic damage and oxidative stress in diabetic male rats”.

Chapter Nine is research article titled “Proteomic analysis of differentially expressed proteins in streptozotocin-induced diabetic rats treated with *Parkia biglobosa* seeds protein isolate”.

Chapter Ten gives a general discussion and conclusion of the study, as well as recommendations for future research endeavours.

Chapter Two

2.0 Diabetes

Diabetes is normally depicted as elevated blood glucose often referred to as hyperglycaemia, which results from a defect in insulin functions; giving rise to impaired metabolism of glucose and other energy-yielding fuels such as proteins and lipids (Schnell and Standl, 2006). The classical symptoms are polydipsia (increased thirst), polyphagia (increased hunger), polyuria (frequent urination), weight loss, ketoacidosis, neuropathies, nephropathies, retinopathies, macrovascular complications and foot ulcers. It is a non-communicable disease, yet the incidence is increasing globally. In 2010, 300 million people had diabetes, mostly of type 2, making up to 6.6% of the adult population and this has been increasing yearly by 7 million (Mbanya, 2009; IDF, 2009). It has been estimated that, by 2030, 438 million people will have diabetes, making up to 7.8% of the adult population: this is a potential increment of 54% in 20 years (Mbanya, 2009).

Further studies in 2010 showed that 10,000 lives were lost daily to diabetes in the sub-Saharan Africa (Mbanya *et al*., 2010) and about 4 million adults globally (IDF, 2009). Apparently, life expectancy is reduced by 5-10 years by diabetes. In Africa, the mortality rates for diagnosed diabetes mellitus vary between 7.6 % and 41 %; the continent having the highest age-specific mortality rate in the world. Diabetes is classified into two types: type 1 diabetes or Insulin-dependent Diabetes Mellitus (IDDM) and type 2 diabetes or Non-insulin Dependent Diabetes Mellitus (NIDDM). Both forms of the disease are characterized by continuous β-cell failure.

2.1 Types of diabetes

2.1.1 Insulin-dependent diabetes mellitus (IDDM)

Insulin-dependent diabetes mellitus is prevalent at any young age but pronounced during childhood or young adulthood (Figure 2.1). This type is less common and constitutes only about 10% of all diabetes. Insulin-dependent diabetes mellitus is usually characterized by an insulin deficiency, which arises from the autoimmune elimination of β-cells in the pancreas (Rother, 2007). This progressive cell necrosis is

caused by the infiltration of the islets of the pancreas by mononuclear cells, which lead to an inflammatory reaction termed ‘insulitis’, resulting in the damaging of β-cells of the pancreas over a period of years (Kloppelg *et al.,* 1985). The cell death caused by insulitis is probably responsible for the direct contact with activated macrophages and T-cells, which enhance the expression of soluble mediators such as cytokines, nitric oxide (NO), and free radicals (Eizirik and Mandup-Poulsen, 2001). Over a prolonged period of time, this functional impairment triggers the death of β cells, which then can be attributed to three related mechanisms: auto-immunity, genetic susceptibility and environmental factors.

The auto-immune response of the islet β-cells usually occurs in persons with certain susceptibility alleles who lack other protective alleles of the major histocompatibility complex (MHC) genes or the human leukocyte antigens (HLA). This leads to the emergence of the islet β-cell auto-reactive T cells, with the resultant destruction of the β-cells (Horwitz *et al.,* 2002; Lampeter *et al.*, 1993; 1992). The T-cells drive the destruction of the autoimmunity of β-cells, supported in the mouse models by a reduction of the CD40 or CD60 populations which prevent the type 1 diabetes, while replacing the population that reverses the disease (Yoon and Jun, 2001; Seewaldt *et al*., 2000). The T-cells produced in the local lymph node infiltrate the pancreatic islets and destroy the β-cell cytokines (CD8T and natural killer cells) or the pro-inflammatory cytokines (CD4T) (van Belle *et al*., 2011). The lymphocytes produced by antibodies are known to enhance the damage of β-cells, but the mode of mechanism of action is under debate (Pescovitz *et al.,* 2009). Endogenous proteins (proinsulin, glutamate acid decarboxylase, protein-tyrosine phosphatase-like protein and the zinc transporter) are attacked by the auto-antibodies produced in the plasma cells that contribute to the development of type 1 diabetes (Wenzlau *et al.,* 2007). However, apart from the β-cells and T-cells, some other immune cells are involved in the development of the type 1 diabetes, such as, the natural killer cells, dendrites and macrophages. The elimination of natural killer cells in mice models prevents diabetes symptoms although the mode of action is poorly understood (Cardell, 2006).

Environmental impact in the development of the disease has given rise to the “hygiene hypothesis”: the poor management of western lifestyle diseases led to

hyper-immune defenses, which develop into auto-reactivity. This hypothesis indicates that a range of infections poorly managed develop into diseases due to hyperactivity of the immune response (van Belle *et al.,* 2011; Pozzilli *et al.,* 1993). Viral infections contribute to the development of type 1 diabetes, through the auto reactivity of T-cells (Jun and Yoon, 2003). Diet plays a crucial role in the development of type 1 diabetes. A gluten-rich diet and milk albumin are known to contribute to the development of immune imbalance side effects (Antovorskov *et al.,* 2012). The relationship between gluten and type 1 diabetes is supported by the frequent development of celiac diseases in type 1 diabetes patients (Hansen *et al.,* 2006).

2.1.1.1 Insulin-dependent diabetes mellitus and pancreatic β-cell activity

Pancreatic β-cells are activated by changes in the exogenous levels of glucose. This triggers the production and secretion of insulin. The glucose 2 (GLUT2) transporters takes up glucose and activates glycolytic pathways, which produce adenosine triphosphate (ATP) and lead to the closure of ATP-dependent potassium channels. This reduces the action potential of the plasma membrane and opens up the calcium gate, increasing the intracellular calcium level (Jansson *et al.,* 2003). The high level of calcium stimulates the secretory vesicle to release insulin that has already been produced. Glucose triggers other metabolic pathways within the pancreas β-cells, which enhances the synthesis of insulin for later use. β-cells hyperactivity can lead to damage due to a decrease in the level of reactive oxygen species enzymes which increases the production of free radicals (Mandrup-Poulsen, 2003). Frequent β-cell damage leads to the onset of type 1 diabetes, characterized by conditions associated with an increased insulin demand (Buschard, 1991). Such condition include: high glycemic index, overfeeding and insulin resistance (Fourlanos *et al.,* 2004; Pundziute-Lycka, 2004). Insulin resistance is known to be more prevalent in the young and adolescent because of rapid growth in the individual, which requires more energy (Amiel *et al.,* 1986). In pregnant women, the onset of type 1 diabetes occurs mostly in the 3rd trimester, during which the baby's growth greatly increases. This triggers the mother's β-cells to produce and secrete more insulin to compensate for the needed energy (Buschard *et al.,* 1987).

The closure of ATP-dependent potassium channels during insulin secretion makes the pancreas cells prone to damage from the toxic effects of streptozotocin. This implies that β-cells are more susceptible to damage during resting state than when active (Kullin *et al.,* 2000). Cellular stresses occur during the hyperactivity of β-cells due to a build-up of proteins within the endoplasmic reticulum. This activates unfolded proteins that lead to endoplasmic reticulum stress, oxidative stress and triggers apoptotic pathways (Mizock *et al.,* 1995; Eloui *et al*., 2007). β-cell antigens are produced during the hyperactivity of pancreatic cells. This up-regulates the expression of insulin, glutamatic acid decarboxylase (GAD), GLUT2 transporter, sulfatide, heat shock protein (HSP65), IAPP (Islet amyloid polypeptide) and monotonal autoantibody (IC2) isotypes that enhance the incidence of type 1 diabetes (Hou *et al.,* 1999; Pedersen *et al.,* 1992).

2.1.2 Non-insulin dependent diabetes mellitus (NIDDM)

Type 2 diabetes (Figure 2.2), also known as non-insulin dependent diabetes mellitus is common in adults. It accounts for about 90 % of all the diabetic complications. It is usually characterized by progressive decline in β-cell function or impaired pancreatic insulin secretion and chronic insulin resistance or impaired insulin action, which is associated with impaired fibrosis, impaired insulin signaling, and inflammation (DeFonzo, 1988; Kudva and Butler, 1997). Studies have revealed that abnormalities in the key molecules of the insulin-signaling pathways may result in insulin resistance and also enhances the over-expression of phosphatases and activation of protein kinase cascade (Avramoglu *et al.,* 2006). These actions initiate abnormalities in the expression and action of various peptides, cytokines, growth factors, and also result in the over-production of very low-density lipoproteins (VLDL) (Fonseca *et al.,* 2004). More so, insulin resistance may be responsible for the over-production of pro-inflammatory cytokines such as C-reactive protein (CRP), tumor necrosis factor (TNF), interleukin-6 (IL-6) and a relative deficiency of anti-inflammatory cytokines such as adipokines, which arise from adipose tissues due to obesity (Eckel *et al.,* 2005). Adipokines initiate the production of reactive oxygen species (ROS), leading to a process known as oxidative stress.

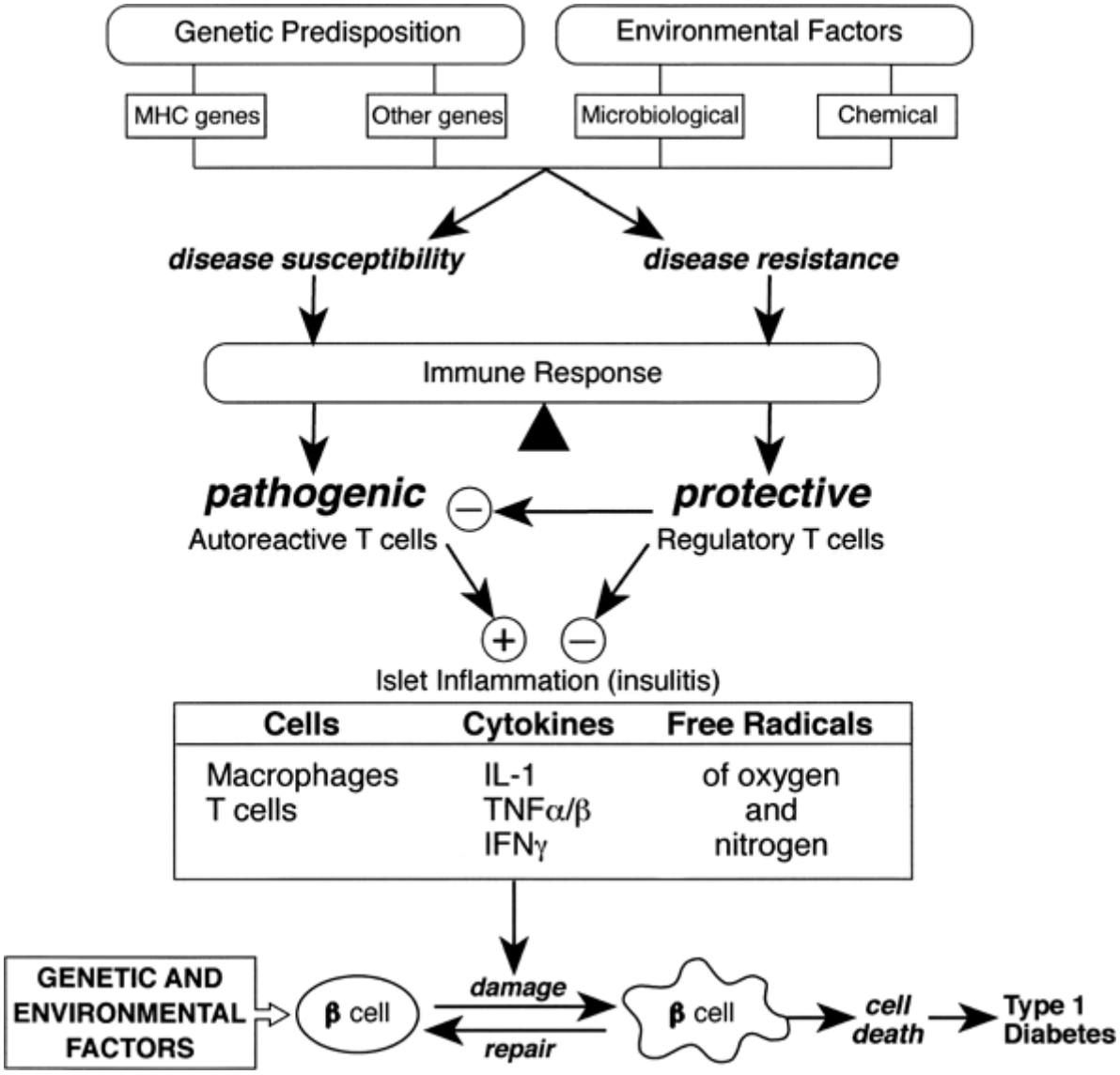

Figure 2.1: Type 1 Diabetes (Figure taken from Rabinovitch, 2004. Diabetes Mellitus: A Fundamental and Clinical Text, 3rd Edition)

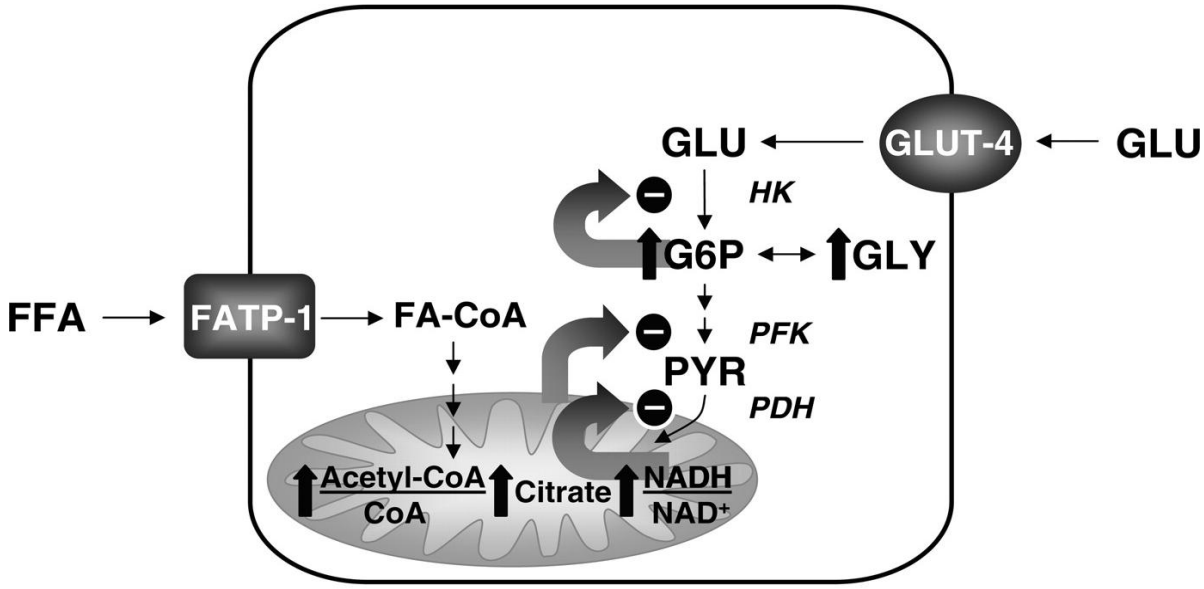

Figure 2.2: Type 2 Diabetes (Figure taken from Roden, 2004. New Physiol. Sci. 19: 92-96)

2.2 Insulin synthesis

Pancreatic β-cells produce insulin from preproinsulin that contain 110 amino acids. The preproinsulins translocate through rough endoplasm reticulum (RES) membranes and cleave off 24 amino acids of the N-terminal of the precursor to produce proinsulin, which contains disulfide bonds and protein fold. The proinsulins will then be converted to insulin in Golgi complex by a proteolysis of the four amino acids and C-peptide forming the A and B peptide bonds of the insulin molecule (Figure 2.3). The zinc bond in proinsulin contributes for ease of conversion to and storage of insulin within the pancreatic β-cells (Davis and Granner, 1996).

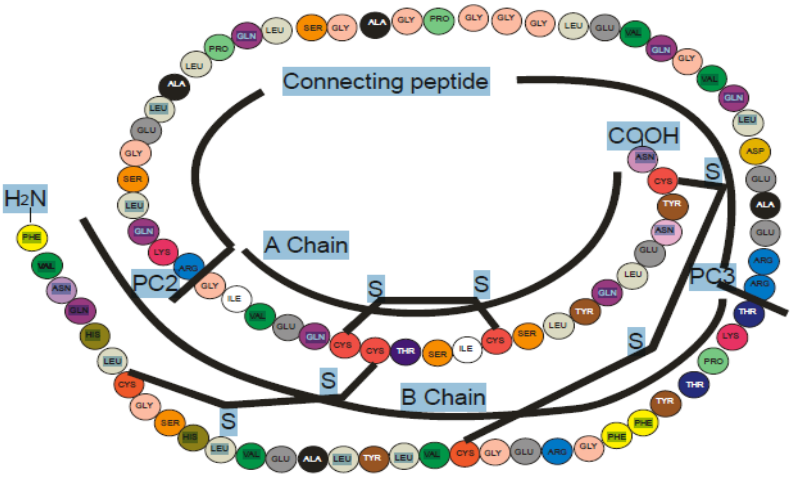

Figure 2.3: Structure of human proinsulin, where insulin is shown as shaded peptide chains A and B (Figure taken from Davis and Granner, 1996).

2.3 Insulin secretion

Hyperglycaemia stimulates an increase in production of the intracellular adenosine triphosphate (ATP) by enhancing glucose metabolism within the pancreatic cell. The excess ATP produced closes the potassium ATP-dependent channels and encourages the opening of the voltage gate calcium channels, increasing the intracellular calcium (Figure 2.4) and leading to the depolarization of the β-cells and insulin secretion (Nolte and Karam, 2001).

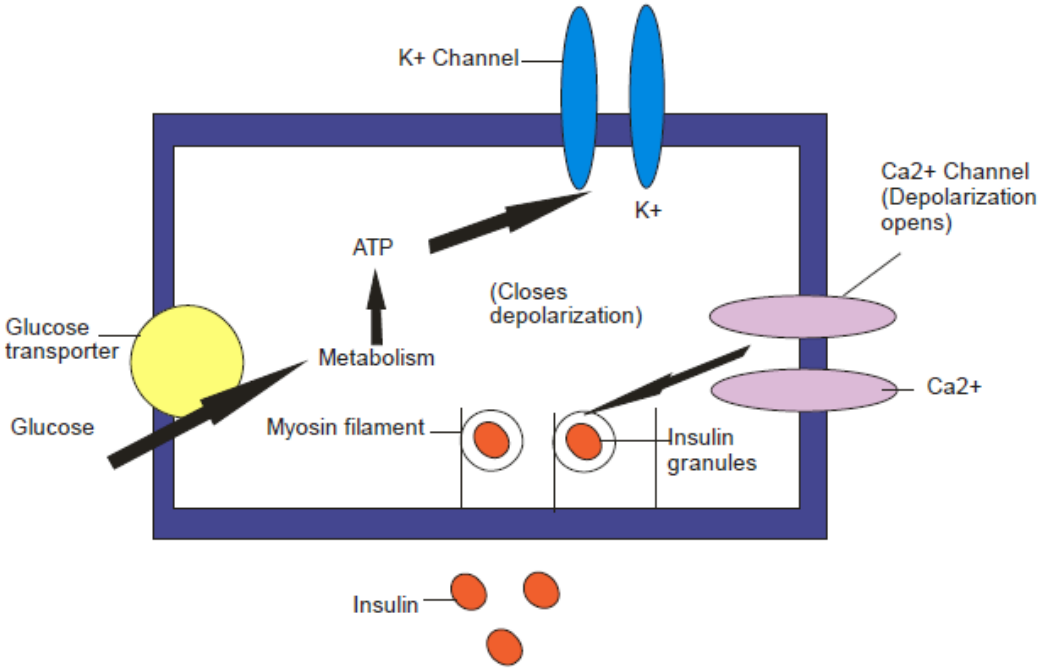

Figure 2.4: Model of control of insulin release from the pancreas β-cell by glucose (Figure taken from Nolte and Karan, 2001).

2.4 Degradation of insulin

Insulin is cleared from circulation after the liver and kidney have effected their actions. Degradation of insulin involves endocytosis of the insulin-receptor complex. The disulfide bond between the A and B insulin chains is hydrolyzed by glutathione insulin hydrogenase and subsequent proteolysis for complete degradation. However, because of portal vein anatomical position, the liver clears about 60% of insulin from the pancrea, while the kidney clears between 35%-40%. In patients under medication with insulin, the reverse is true (Nolte and Karam, 2001).

2.5 Insulin receptors

Insulin in circulation binds to insulin receptors found on the outer membrane of most cells to effect its action. The insulin receptors belong to the family of tyrosine kinase (TK) and are commonly found in the liver, adipose tissues and muscle cells (Chang *et al.,* 1997). The binding of insulin receptors to its ligands induces a structural change within the receptors that triggers an autophosphorylation of the TK domain

within the β chain. This leads to the expression of adaptor proteins such as the protein-tyrosine phosphatase 1B (PTP1B) and the insulin receptor substrate protein (IRS) (Jang *et al.,* 2007). The binding and phosphorylation of IRS1 stimulates migration of the glucose transporter (GLUT4) from the cellular vesicles to the outer membrane of insulin sensitive tissues to initiate glucose uptake into the cells for metabolism (Figure 2.5) (Katsoyannis *et al.,* 1964).

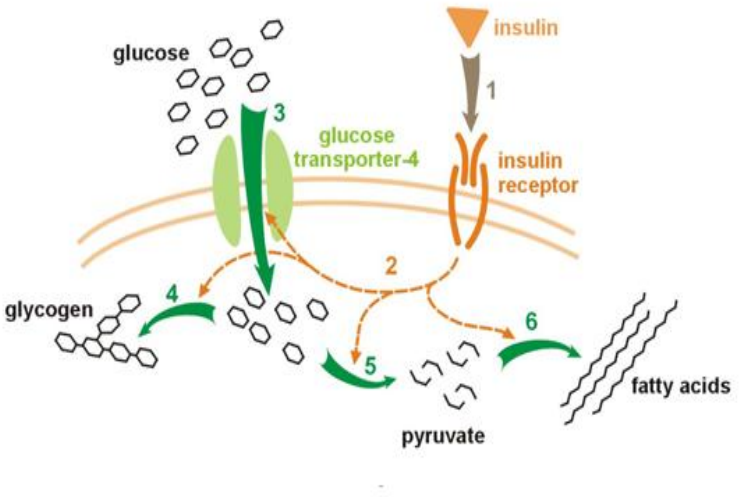

Figure 2.5: Effects of insulin on glucose uptake and metabolism

In some pathological conditions characterized by high levels of insulin, such as obesity and insulin resistance, insulin receptors are reduced and this leads to a loss of insulin sensitivity (Nolte and Karam, 2001).

2.6 Oxidative stress and diabetes

Oxidative stress occurs as a result of an imbalance between the cellular antioxidant defense system and reactive oxygen species (ROS) production. Increased ROS levels gives rise to apoptosis, necrosis and cell damage via the oxidation of lipids, protein and DNA (Elahi *et al.,* 2009), which then triggers the activation of inflammatory cells and endothelial dysfunction (Hulsmans *et al.,* 2012). Studies have revealed that hyperglycaemia-induced generation of the super-oxide ion (O_2) at the mitochondria level initiates a series of oxidative stress cycles in diabetes (Brownlee, 2001; Nishikawa *et al.,* 2000). Hyperglycemia is a major causative factor in the

development of diabetic complications via: (i) the hexosamine pathway; (ii) the polyol pathway; (iii) the formation of advanced glycation end products (AGEs); and (iv) the activation of protein kinase C (PKC) (Figure 2.6) (Ceriello and Testa, 2009).

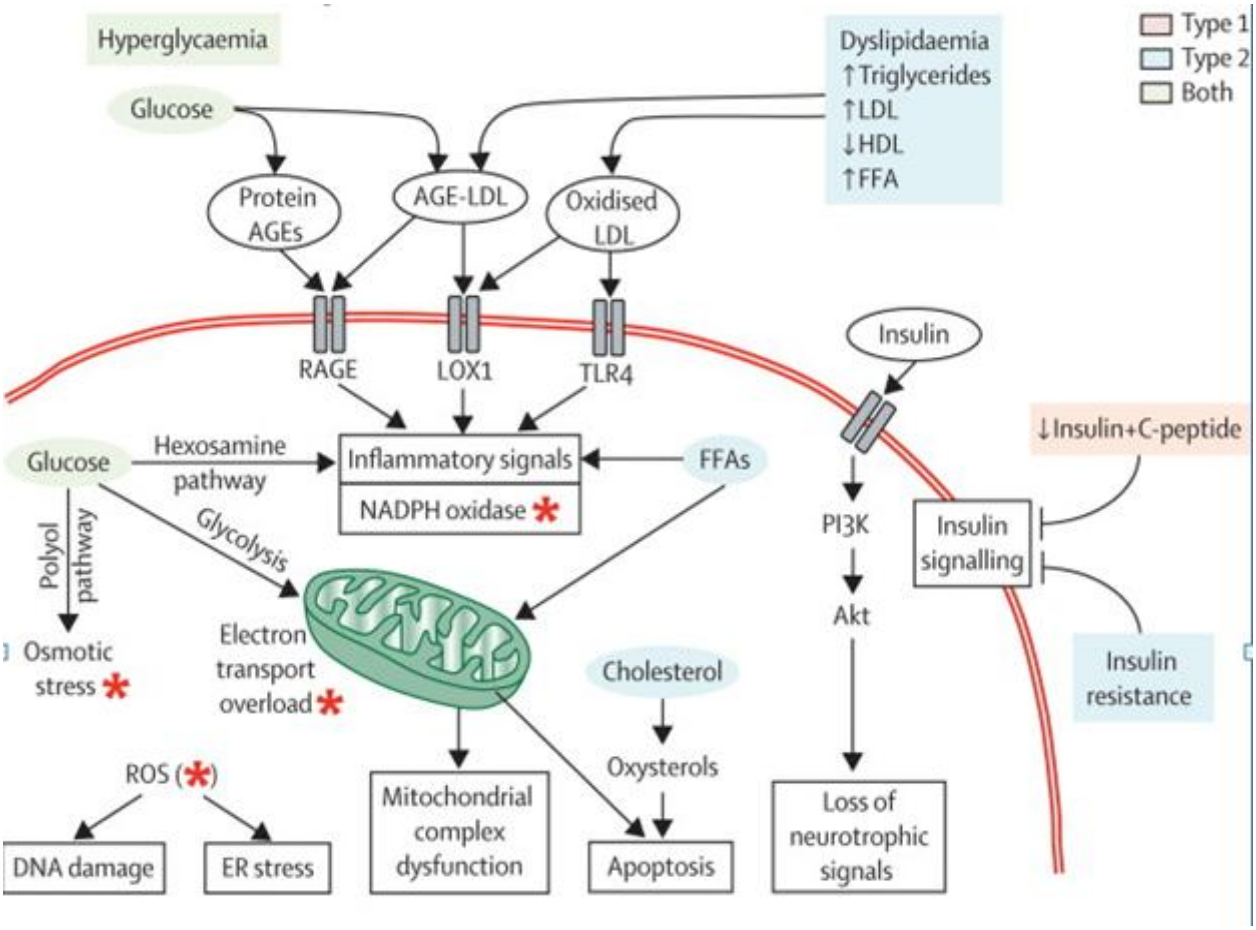

Figure 2.6: Pathophysiology of diabetes mellitus (Figure taken from Callaghan et al., *2012*).

2.6.1 Hexosamine pathway

The diversion of excess glucose during hyperglycaemia into the hexosamine pathway (Figure 2.7), contributes to diabetes complications (Du *et al.,* 2000). This pathway uses fructose-6-phosphate for glycolysis as a substrate for the formation of O-linked glycoprotein that requires UDP-N-glucosamine. Glutamine fructose amidotransferase is the rate-limiting enzyme that up-regulates the expression of tumor necrosis factor (TGF-α, TGF-β1) and plasminogen activation inhibitor-1 (PAI-

1) (Geraldes and King, 2010). This pathway is implicated in fat-induced resistance and hyperglycaemia conditions (Figure 2.7). The activation of this pathway leads to a loss of protein function and DNA modification, resulting in changes in gene expression (Du *et al.,* 2000).

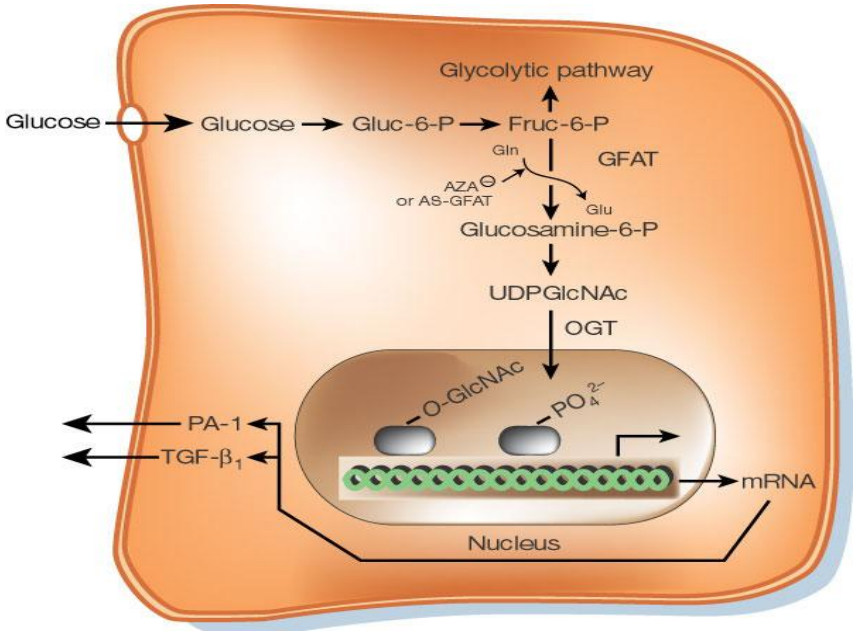

Figure 2.7: Hexoamine pathway (Figure taken from Brownlee, 2001).

2.6.2 Polyol pathway

The main enzyme in the polyol pathway is aldose reductase (Figure 2.8). It is upregulated during hyperglycaemia and it is found to be reduced in non-diabetics due its low affinity to glucose. In non-diabetics, the glucose metabolic rate with this pathway is low. During hyperglycaemia, aldose reductase converts intracellular glucose to polyachol sorbitol by utilizing NADPH. The sorbitol is converted to fructose by sorbitol dehydrogenase, which oxidizes the NAD^+ to NADH (Vikramadithyan *et al.,* 2005).

The utilization of glucose during hyperglycaemia by using polyol pathway was implicated in diabetic complications. The formation of sorbitol in this pathway is

thought to induce a reduction in NADPH, an increase in NADH, osmotic stress and decreased Na^+ and K^+ ATPase activity. The NADPH in the intracellular is greatly reduced due to the utilization of NADPH for the conversion of glucose to sorbitol; thereby affecting the regeneration of NADPH-dependent reduced glutathione (GSH) which regulates intracellular oxidative stress. In mice models, a decrease in the level of GSH has been found and an up-regulation of aldose reductase has been documented (Li *et al.,* 2004).

The oxidation of sorbitol using NAD^+ increases the concentration NADH: NAD ratio. This inhibits the activity of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), enhancing the concentration of triose phosphate. The high concentration of triose phosphate triggers the expression of methylglyoxal, a precursor of diacylglycerol and advance glycation endproducts (AGEs), which contribute to the activation of protein kinase C (Chung *et al.,* 2003). Protein kinase C encourages the production of Na^+ K ATPase inhibitors, prostaglandin E_2 and arachidonate by increasing the activity of cytosolic phosholipase A_2. In the endothelium cells, poly ADP-ribose polymerase (PARP) is consumed instead of NAD^+ for oxidation, due to the enhanced production of reactive oxygen species (Zhang *et al.,* 2000).

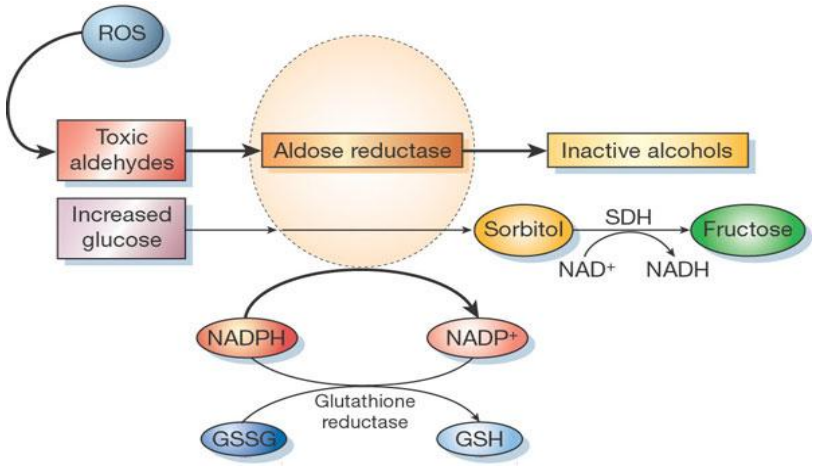

Figure 2.8: Polyol pathway (Figure taken from Brownlee, 2001).

2.6.3 Protein kinase C (PKC)

An intracellular hyperglycaemia increases the synthesis of diacylglycerol (DAG) by diverting it from the glycoltic pathway for dihydroxyacetone phosphate, this is latter metabolised into glycerol-3-phosphate and acylation. The formed DAG then activates the nine isoforms of PKC (Craven *et al.,* 1990).

In the literature, activation of PKC (Figure 2.9) has been implicated in renal and retinal blood flow abnormality by an up-regulation of endothelin-1 activity and down-regulation of nitric oxide production. Protein kinase C also inhibits the messenger RNA responsible for the expression of endothelial nitric oxide synthesis in endothelial cell cultures (Ayo *et al.,* 1991). In smooth muscle, vascular endothelial growth factor (VEGF) expression is enhanced through activation of PKC in hyperglycaemia (Pugliese *et al.,* 1994). Hyperglycemia contributes to abnormality in blood flow and permeability, this leads to the accumulation of intracellular matrix proteins that enhance the expression of fibronectin, collagen and TGF-β1 in glomeruli cells of diabetic rat models and this mesangial culture cells (Craven et al., 1997; Kikkawa *et al.,* 1994). Protein kinase C is also implicated in the expression of PAI-1 (fibrolytic inhibitor), activation of NF-Kb in smooth muscles and endothelial cell, and in the activation of cell membrane associated NADPH dependent oxidase that contributes to diabetic complications (Sayeski and Kudlow, 1996; Kolm-Litty *et al.,* 1998).

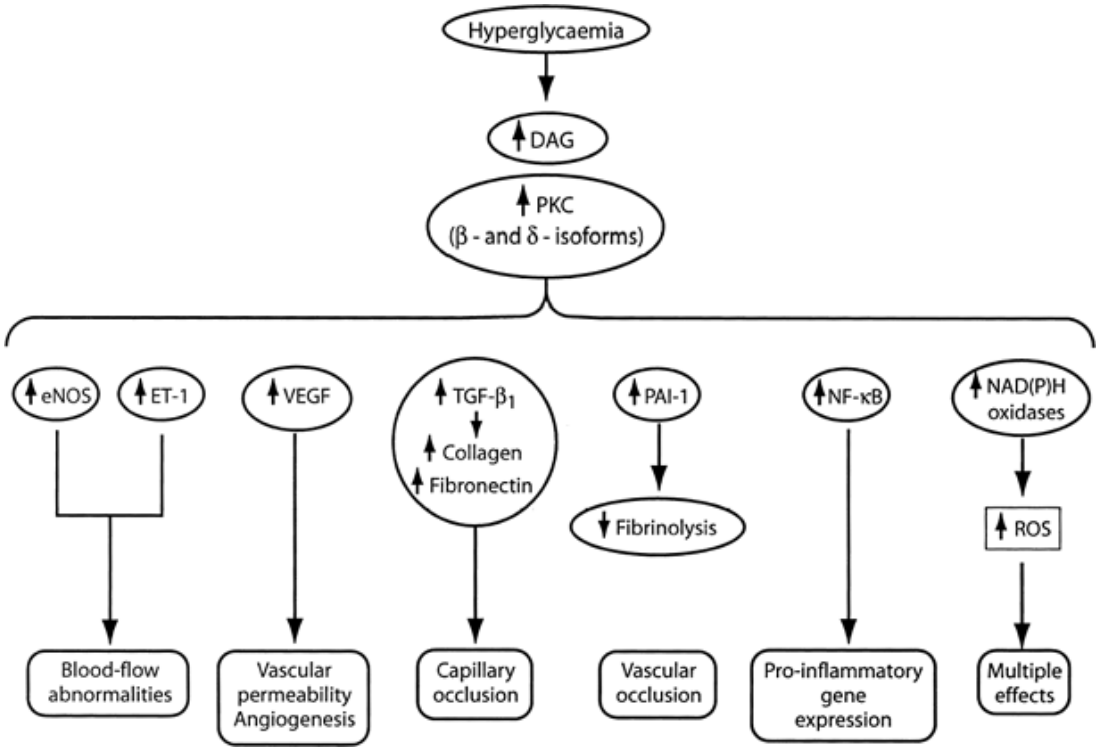

Figure 2.9: Protein kinase C (PKC) (Figure taken from Brownlee, 2001).

2.7 Effects of hyperglycaemia in diabetes

2.7.1 Increment in superoxide anions in mitochondria

Mitochondria are sites of redox reactions in cells. In diabetes, excess glucose increases the rate of redox reaction imbalances, thereby enhancing the production of excess electron donors such as the $NADPH/H^+$ from the tricarboxylic acid cycle (TCA). This involves the transfer of single electrons instead of the normal electron pairs to oxygen, thus producing superoxide anions instead of the usual water end-product (Ceriello, 2000). The superoxide anions produced inhibit the major glycolytic enzyme glyceraldehde-3-phosphate dehydrogenase, leading to the spilling of glycolytic intermediates into the hexosamine and polyol pathways. This process further stimulates other pathways that culminate in protein kinase C (PKC) activation and intracellular advanced glycation end products (AGEs) formation, triggering oxidative stress and inflammatory activities (Ceriello, 2000).

2.7.2 Non-enzymatic glycation of proteins

Non-enzymatic glycation of proteins is enhanced during hyperglycaemia. These proteins accumulate in the plasma, cells and tissues and bind to the receptor-advanced glycation end-products (RAGE) to initiate inflammatory cascades, which activate NADPH oxidases and cause oxidative stress (Ahmed *et al.,* 2009). Advanced glycation end products are produced by a non-enzymatic reaction between aldehyde moieties of glucose with the free amino groups of proteins to form a reversible Schiff base that rearranges itself to form the more stable Amadori products (Figure 2.10). The large amount of accumulated Amadori products undergo further rearrangement and cross-linkage in the presence of molecular oxygen to form a new class of molecule called Maillard products. Through autoxidation, AGEs generate reactive oxygen intermediates (Chappey *et al.,* 1997) which are instrumental in the functional and structural alteration of proteins seen during diabetes and aging (Bousova *et al.,* 2005; Monnier *et al.,* 1986).

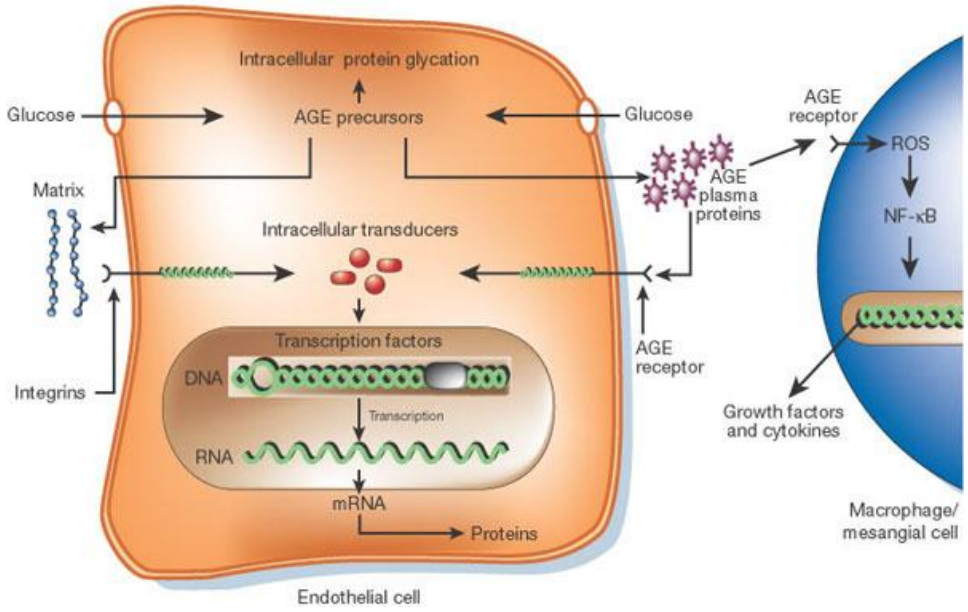

Figure 2.10 Advanced glycation end-products (AGEs) (Figures taken from Brownlee, 2001).

2.7.3 Glucose autoxidation

During hyperglycaemia, excess glucose in its enediol form is oxidized to an enediol radical anion thereby reducing molecular oxygen under physiological conditions to ketoaldehydes and superoxide anion radicals (Wolf *et al.,* 1984). The ketoaldehyde analogues then bind to lysine to form chromophores (McLaughlin *et al*., 1980), which contribute to the non-enzyme protein glycosylation and associated proteins fluorophoric and chromophoric alterations. The superoxide anions produce other radicals such as singlet oxygen, hydrogen peroxide and hydroxyl radicals in the presence of transitional metals. Superoxide anions also react with nitric oxide (NO) to form peroxynitrite radicals (Robertson, 2004). These reactive oxygen species promote oxidative stress, lipid peroxidation, cleavage of DNA and damage of cell membranes, thereby inducing cell destruction. However, an increased concentration of free radicals as well as secondary impaired antioxidant content causes conformational changes of structural proteins and leading to cell damage (Robertson, 2004).

2.7.4 Increment in inflammation mediator levels

In diabetes, hyperglycaemia is induced through a number of pathways such as the aldose, advanced glycation end-products (AGEs), protein kinase (PKC) and reactive oxygen intermediates. These pathways stimulate the release of inflammatory mediators resulting in deleterious effects, both systemically and locally in tissue. The resultant effects include altered gene expression, which contributes to cellular dysfunction and damage due to vascular complications (Dana and David, 2005). Hyperglycemia activates necrosis factor kappaB (NF_kB), upregulates the expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) favouring the formation of peroxynitrites. These peroxynitrites enhance other degradable processes, which later result in lipid peroxidation, DNA damage and oxidation of low-density lipoproteins (LDL) (Ceriello, 2000).

2.8 Diabetes and apoptosis

Apoptosis is a programmed cell death aimed as maintaining and developing healthy tissues by removing unwanted, damaged cells. The activation of necrosis factor kappa B (NF_kB) due to hyperglycaemia also triggers pro-apoptotic factor Bcl-2 homologous antagonist killer (BAK) and Bcl-2-associated X protein (BAX), which initiates apoptosis. The apoptotic process builds up reactive oxygen species (ROS) which cause cell damage by increasing membrane permeability (Ceriello, 2000). More so, apoptosis leads to major complications associated with diabetes like nephropathy, neuropathy, cardiovascular problems, ulcer and retinopathy.

2.9 Proteins and diabetes

Protein intake in different age groups is inconsistent, from young to old. Protein intake is used to replace daily loss of protein, while excess protein is converted to glucose through gluconeogenesis. This implies that 100 grams of protein can produce 50 grams of glucose which is consistent with the statement that half of all protein ingested is converted to glucose and half the protein will have effect of carbohydrates on blood (Ganon and Nuttal, 1999). It has been reported that the ingestion of protein does not alter blood glucose levels after a meal; though a rise in

blood urea nitrogen was reported indicating the utilization of protein (Krezowki *et al.,* 1986). Nuttal and colleagues (1984) reported an experiment in which nine models with type 2 diabetes were administered with 50 g protein, 50 g glucose or 50 g glucose and 50 g protein. The levels of glucose in the blood and insulin response were measured over five hours. The blood glucose levels increased in models injected with glucose, while the models injected with protein experienced static blood glucose levels for two hours before declining. In the models injected with a combination of protein and glucose, the blood glucose levels were similar to the models injected with only glucose. However, the insulin response was the same in both protein and glucose injected models, while in the models injected with a combination of protein and glucose, the insulin response was double (Nuttal *et al.,* 1984).

Several theories have been postulated to answer the question why glucose produced from protein through gluconeogenesis does not appear in blood circulation. The first theory states that only small amounts of glucose are actually produced by protein and they are readily utilized due to an increase in demand for glucose by insulin. The second theory speculates that the glucose production is very slow; it takes over 24 hours and is also disposed of over a long period of time. In the third theory, the glucose from protein is converted to glycogen in the liver and skeletal muscles and then released in low insulin and high glucose conditions without the body indentifying its sources (Conn and Newburgh, 1936).

The majority of the protein is digested and the remaining amino acids are metabolized in the intestinal mucosal barrier and transported to the liver through the portal vein for protein synthesis (Nuttal and Ganon, 1991). The nonessential amino acids are deaminated in the liver where the amino group is reduced to urea for excretion by the kidneys. The carbon skeleton of the non-essential amino acids is used for the synthesis of glucose (Nuttal and Ganon, 1991). The protein synthesized aids in the expression of hormones and cytokines found in the adipose tissue which plays a critical role in the metabolism of lipids and glucose. These include: adiponectin, resistin, cholesteryl ester transfer protein (CETP), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), plasminogen activating inhibitor (PAI-1),

angiotensin converting enzyme, angiotensin ll, angiotensinogen, and leptin (Yamauchi *et al.,* 2001; Chu *et al.,* 2001).

The plasma levels of adipocytokine and other hormones increase in the adipocyte in obesity conditions except for adiponectin, which decreases (Skurk *et al.,* 2007). The ingestion of protein enhances the expression of insulin which stimulates the adipocyte to produce leptin which is also affected by TNF-α, fatty acids, estrogen and growth hormones (Zhang *et al.,* 2000). The leptin increases lipid oxidation in the liver and enhances lipolysis in skeletal muscles and adipose tissues (Long and Zierath, 2006). Deficiency of leptin implicates amenorrhea complication in anorexia nervosa. Also leptin deficient children are not always obese, but exhibit prepubertal symptoms. The expression of leptin has been reported in early or delayed puberty in some children (Nuttal and Ganon, 1991).

Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced in abnormal adipose tissue and has been implicated in insulin resistance with the onset of type 2 diabetes, without an increase in body weight (Wannamethee *et al.,* 2007). Interleukin-6 is reported to up-regulate the production of VEGF in mice. This is in agreement with literature, which reveals that angiogenesis is enhanced during adipose tissue growth (Rega *et al.,* 2007). Interleukin-6 is also involved in the expression of C-reactive proteins and hemostatic enzymes (PAI-1, tissue plasminogen activators and fibrinogen). It contributes to dyslipidemia via the expression of microsomal triglyceride transfer proteins that help in the assembly of apolipoprotein B in the liver (Navasa *et al.,* 1998).

Adiponectin is usually up-regulated in models with low obesity, metabolic syndrome and insulin resistance. Women tend to have more adiponectin than men. The adiponectin exhibits both anti-atherosclerotic effects and improved insulin sensitivities through aiding glucose uptake in muscles, the inhibition of gluconeogenesis in the liver, up-regulation of lipid oxidation in muscle and the liver (Kadowaki and Yamauchi, 2005). In insulin resistant mouse models, it is reported that the injection of adiponectin reversed hyperinsulineamia and hyperglycaemia levels to the minimum (Yamauchi *el al.,* 2001).

Tumour necrosis factors are abundant in large adipose tissues and are produced by macrophages. The adipocyte is enlarged and releases free fatty acids which bind to

macrophage toll-like receptor-4 (TLR-4) resulting in the activation of NF-κB. This further stimulates lipolysis of adipocytes which enhances the expression of more pro-inflammatory (intracellular adhension molecules-1 (ICAM-1), marcophage chemo-attractant protein-1 (MCP-1) and IL-6. This vicious cycle leads to a constant pro-inflammatory state of macrophage and adipocyte (Lee *et al.,* 2001).

In an obese models, the increase of an angiotensin converting enzyme, angiotensinogen and angiotensin ll leads to an elevation in blood pressure (Massiera *et al.,* 2001). Waist circumference decreases and weight loss is associated with a reduction in effectiveness of the angiotensin–aldosterone system, characterized by a 7 mmHg decrease in blood pressure (Andraws and Brown, 2007). The blockage of ROS enhances the expression of adiponectin and increased insulin sensitivity. Treatment with ROS inhibitors increases the insulin sensitivity and decreases the adipocyte size (Furuhashi *et al.,* 2003).

The ingestion of proteins enhances the up-regulation of protective factors (insulin, vascular endothelium growth factor (VEGF), activated protein (APC), platelet derived growth factor (PDGF) and antioxidant enzyme) and a down-regulation of the activity of casual factors (AGE, ROS, Anginostensin 11, NF-κB and PKC) (Figure 2.11). The effective expression of protective factors within a protein diet inhibits the possible effect of casual factors in diabetes complications.

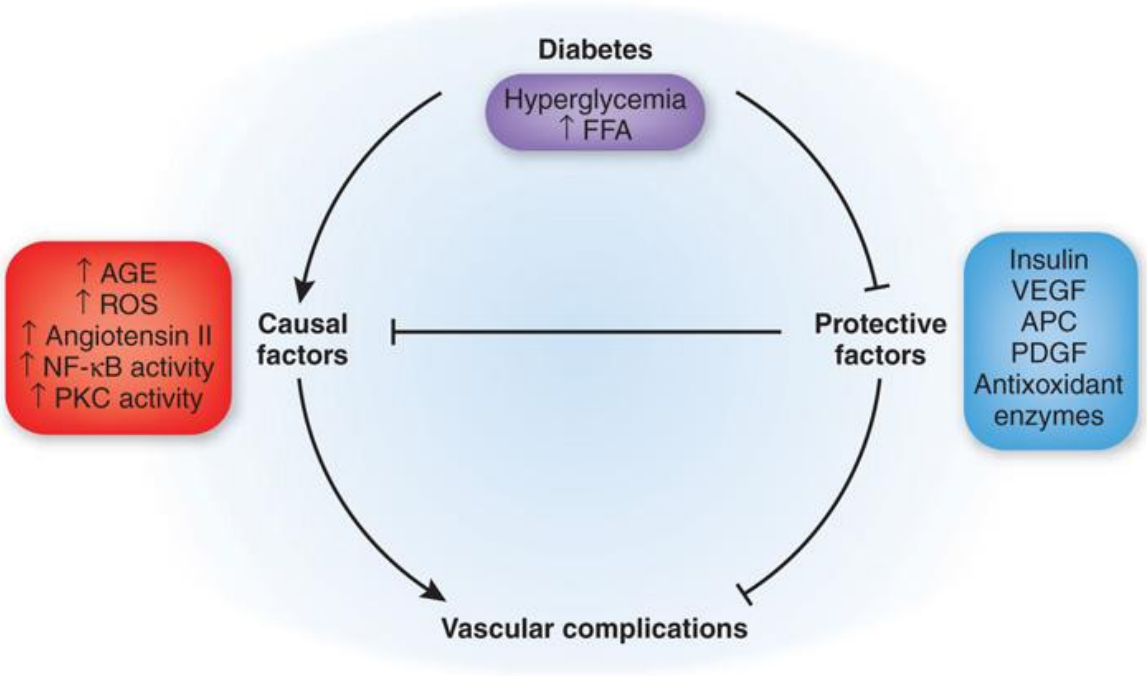

Figure 2.11: Proteins and diabetes (Figure taken from Christian and George, 2010).

2.10 Streptozotocin

In experimental animal models, streptozotocin is one of the agents used to induce diabetes. A concentration of 180 mg/kg dissolved in 0.01 M citrate buffer at pH 4.5 is often administered intraperitoneally. Streptozotocin (STZ; N-nitro derivative of glucosamine *i.e.* glucosamine-nitrosourea) is a naturally occurring broad-spectrum antibiotic, DNA alkylating agent, and cytotoxic chemical, which is toxic to the pancreatic insulin producing β-cells in mammals (Hayashi *et al.,* 2006; Takeshita *et al.,* 2006). Streptozotocin infiltrates the β-cells via the glucose transporter GLUT 2 which causes the alkylation of DNA (Elsner *et al*., 2006). DNA damage then stimulates the activation of poly ADP-ribosylation leading to an exhaustion of cellular ATP and NAD^+. Enhanced ATP dephosphorylation increases the supply of substrate for xanthanine oxidase resulting in the formation of hydroxyl radicals, super-oxide radicals and hydrogen peroxide, which then stimulate rapid and irreversible necrosis of β-cells (Szkudelski, 2001).

2.11 Drugs used in the management of diabetes

Oral medications, other than insulin injections used in the treatment of diabetes, include sulfonylureas, metformin, alpha-glucosidase, thiazolidinediones and meglinides.

Sulfonylureas works by enhancing the pancreas to increase insulin secretion thus reducing blood glucose levels, but is usually accompanied by side effects such as hypoglycaemia, hunger and weight gain.

Metformin reduces blood glucose by monitoring the amount of glucose released by the liver into the bloodstream. Associated side effects include nausea, abdominal discomfort, a metallic taste in the mouth and a loss of appetite.

Alpha-glucosidase inhibitors (starch blockers) reduce the digestion rate of carbohydrates by inhibiting the enzymes responsible for carbohydrate breakdown in the small intestine (acarbose and miglitol), thereby enhancing steady glucose uptake from food, with the resultant regulation of postprandial blood glucose. However, side effects from these inhibitors include abdominal pain, diarrhea and flatulence.

Thiazolidinediones reduce the body's ability to resist insulin, leading to low blood glucose levels. Possible side effects include heart attack, stroke, fluid retention and weight gain.

Meglitinides, on the other hand, excite the pancreas, increasing the release of insulin. Side effects include hypoglycaemia, back pain and joint pain.

On the whole, the costs, availability issues and serious side effects associated with these drugs have led to the clamour for alternative options for treatment using medicinal plants.

2.12 *Parkia biglobosa*

Parkia biglobosa (Jacq.) G. Don is a perennial tree, which belongs to the family *Leguminosae* and sub-family *Mimosoideae*, with a height ranging between 7-20 m. The tree has low branches but a widespread crown and is widely distributed within the savannah belt of West Africa (Sacande and Clethero, 2007). The tree is rated as being among the most valued multipurpose trees of West Africa due to its economic,

nutritional and medicinal uses. The tree has many uses. It is used as timber for making pestles, seats, arrows and hoe-handles (Figure 2.12) (Alabi, 2005). It also habours *Bunaea alcinoe*, an edible insect which provides cheap sources of protein for rural people (Ajayi and Adedire, 2007). The seeds and pulp are mainly used as food and also serve medicinal purposes, while the pods and husks are used for feeding live stock (Akoma *et al.,* 2001; Obiozoba, 1998). Studies have shown *Parkia biglobosa* to have several pharmacological and nutritional properties, depending on which part of the plant is used (Badu *et al.,* 2012; Kouadio *et al.,* 2000). Fermented seeds from *Parkia biglobosa* is known as 'African locust beans' (English); 'dawadawa' in Ghana; 'soumbala' in Burkina Faso; 'kinda' in Sierra Leone; 'neteteous' in Gambia and 'iru' in Togo and Nigeria.

Studies of the nutritional and medicinal values of *Parkia biglobosa* have revealed that its fermented seeds are used to season traditional soups in parts of West Africa (Ajaiyeoba, 2002; Assane *et al.,* 1993). It has further been shown that the chemical and biological potential of the seeds is due to the presence of glycosides, polysaccharides, alkaloids, terpenoids, flavonoids and other bioactive compounds, which have also been implicated in the management of diabetes (Loew and Kaszkin, 2002). Odetola and co-workers (2006) showed that extracts obtained from the seeds have possible anti-diabetic and anti-lipidemic effects, while Kouadio and colleagues (2000) showed that extracts from the plant have anti-inflammatory and analgesic effects. More so, Agunu and colleagues (2005) showed the anti-diarrheal potential of the plant, while further studies have shown that it also has anti-hypertensive properties (Ajaiyeoba, 2002; Assane *et al.,* 1993).

The hypoglycaemic and hypolipidemic activities of the leaves, bark and stem of *Parkia biglobosa* have been documented. Knowledge of the use of protein isolate from the seeds of this plant in the management of diabetes is non-existent, however. this present study aims to investigate the effects of protein isolate from *Parkia biglobosa* seeds on the biochemical and proteomic profiles of streptozotocin-induced diabete male Sprague-Dawley rats.

Figure 2.12: *Parkia biglobosa* plant and seeds (Figure taken from Sacande and Clethro, 2007. Forest and Landscape, Leaflet No 124).

2.13 Problem statement

Diabetes is one of the fastest leading causes of death globally due to its associated disease complications such as stroke, hypertension, atherosclerosis, glaucoma, and coronary heart disease (CHD). Oxidative stress is known to be caused by the hyperglycaemia-induced excessive generation of free radicals, which further aggravate the development and progression of diabetes and its complications (Brownlee, 2001). Although various conventional drugs such as insulin injection, sulfonylureas, metformin, alpha-glucosidase, gliazone and meglitinides have been used in the management of this disease, their side effects, such as abdominal pain, hunger, weight gain, diarrhea, nausea or vomiting, flatulence, back pain, and joint pain, are cause for concern. More so, these drugs are not easily accessible to the poor, especially in sub-Saharan Africa due to high prices and the technicalities involved in their administration. There is therefore a need for alternative medication or a strategy with minimum side effects which will be relatively cheap and easily accessible to the poor.

2.14 Aims and objectives

2.14.1 Aims

This study investigates the potential beneficial effects of administering the defatted protein isolates from *Parkia biglobosa* seeds on some biochemical parameters in streptozotocin-induced diabetes. The extent of hepatic injury to the liver and other relevant organs such as the pancreas was investigated using histological examinations. More so, the study aims to identify and characterize differentially expressed new genes that could be found in the organs of diabetic rats fed with *Parkia biglobosa* seeds protein isolate.

2.14.2 Objectives

The objectives of the study are to:

1. Extract protein isolates from defatted, fermented *P. biglobosa* seeds and investigate their proximate analysis, functional properties, mineral, anti-nutrients and total amino acids composition.
2. Examine the effects of *P. biglobosa* seed protein isolate on the serum lipid index, such as total glycerides (TG), total cholesterol (TC), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).
3. Ascertain the glycemic status of the diabetic rats after being fed with the protein isolate.
4. Examine the oxidative status of the rats using the enzymatic index, such as glutathione peroxidase activity (GPx), glutathione reductase (GR), superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT).
5. Ascertain the level of hepatic injury to the liver and pancreas via histological changes.
6. Measure liver enzymes such as aspartate amino transaminase (AST), gamma-glutamyl transferase (GGT) and alanine amino transaminase (ALT).

7. Measure oxidative stress biomarkers such as the total antioxidant capacity (TAC), total glutathione and conjugated dienes.
8. Investigate possible histological changes due to hepatic injury to the liver, kidney and pancreas after feeding the animals with protein isolates from *Parkia biglobosa* seeds.
9. Investigate differential gene expression in the liver, kidney and pancreas of diabetic rats.

2.14.3 Research questions

1. What are the potential effects of *Parkia biglobosa* seed protein isolate on the serum lipid profile and glycemic status in diabetic rats?
2. What are the potential effects of *Parkia biglobosa* seed protein isolate on the oxidative status in diabetic rats?
3. What are the potential effects of *Parkia biglobosa* seed protein isolate on inflammatory mediators in diabetic rats?
4. What are the effects of *Parkia biglobosa* seeds protein isolate on the liver and kidney function of diabetic rats?
5. What are the histological changes observed in diabetic rats fed on *Parkia biglobosa* seed protein isolate?
6. What potential genes are expressed differentially in response to feeding *Parkia biglobosa* seed protein isolate to diabetic rats?

2.14.4 Research hypothesis

Biochemical investigations of the seeds of *Parkia biglobosa* have been shown to contain substances like flavonoids, glycosides, alkaloids and to be high in proteins, all of which may play an ameliorative effect in the management of diabetes. This study hypothesized that the protein isolates from the seeds of *Parkia biglobosa* have high hypoglycaemic and hypolipidemic properties.

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Chapter Three

3.0 Potentials of some plant-derived foods in the management of diabetes and associated complications

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Abstract

Background and Objective: Diabetes is an insidious and debilitating metabolic disease with a variety of causes. It could lead to severe complications in multiple organs within the body system. There has been no documented scientific evidence of a total cure of this complex and chronic disease; therefore demands for a lifelong management. This has necessitated the recent evaluation of several plant derived foods as cost-effective alternatives in the management of this diseases and its associated complications.

Materials and Methods: This review is based on an integration of information from multiple databases after a comprehensive literature search on the various plant derived foods that are reported to have shown a certain degree of amelioration in the management of diabetes and diabetic complications.

Result and Discussion: Published reports suggest that oxidative stress primarily mediated by uncontrolled hyperglycaemia plays a pivotal role in the pathogenesis of diabetes and its associated complications. However, various plant-derived foods are believed to delay, prevent or manage diabetes and its associated complications using different mechanisms which could be established through their potential to increase insulin sensitivity; scavenging free radical abilities; inhibits the α-amylase and α-glucosidase and also the hypolipidemic, hypoglycaemic, hypocholesterolemic, antioxidative and anti-inflammatory activities.

Conclusion: Based on the evidence presented in this review, plant-derived foods possess various bioactive constituents believed to be rich in antioxidants and proteins which may be responsible for their modes of action. It is proposed that *Cucuma longa* (curcumin), *Garcinia kola* (kolaviron), *Telfairia occidentalis* and *Parkia biglobosa* be explored in the management of diabetes and its associated complications due to their outstanding beneficial properties.

Keywords: Antioxidants, diabetes, diabetic complications, hyperglycaemia, nutraceuticals, plant foods

3.1 Introduction

The global burden of diabetes and its associated complications has increased exponentially in recent years. Sedentary life-styles, an escalation in the consumption of processed foods containing preservatives, obesity, genetics and family history, amongst others, have been implicated in the striking increase observed in the occurrence of this disease. This insidious disease appears to be one of the oldest diseases with severe economic and global health challenges (Hu, 2011; Olokoba *et al*., 2012). The incidence of diabetes varies from one geographical location to the next, as it is affected by environmental and lifestyle associated factors. Recently, the International Diabetes Federation (IDF) reported that over four million people die from diabetes annually (Oputa and Chinenye, 2012). A literature search in 2011 revealed that an estimated 366 million people had diabetes globally and this value was likely to increase to about 552 million by 2030 (Olokoba *et al*., 2012; Siegel and Narayan, 2008).

Diabetes is a complex chronic disease with a variety of causes. It was thought to be a disease of the affluent, but is unfortunately now increasing in prevalence in developing countries where poverty levels are very high (Kharkar *et al*., 2013). It is a life-threatening as well as lifestyle-modifying metabolic disease, characterized by abnormalities in carbohydrate, protein and fat metabolism, and associated with a deficiency in insulin secretion, insulin action, or both, resulting in hyperglycaemia (American Diabetes Association, 2014). Documented evidence suggests that chronic hyperglycaemia tends to be a primary cause of a long-term dysfunction, damage and failure of various organs, particularly the kidneys, eyes, blood vessels, nerves, and heart (International Diabetes Federation, 2011).

Diabetes can be grouped into two major types: insulin-dependent diabetes mellitus, commonly called type 1 diabetes, and non-insulin-dependent diabetes mellitus, commonly called type 2 diabetes (Khan *et al.,* 2012; Modak *et al.,* 2007). Type 1 diabetes can occur at any age, even though higher rates of incidence in children and young adults are documented in literature. Primarily, type 1 diabetes occurs due to autoimmune destruction of pancreatic beta cells which results in a deficiency of insulin production within the body and subsequent insulin insufficiency in the body (Atkinson, 2012; Cooke and Plotnick, 2008). Type 2 diabetes accounts for

approximately 90 % to 95 % of all diabetic cases globally (International Diabetes Federation, 2011; Wang *et al.,* 2013; Lin and Sun, 2010). Various metabolic irregularities leading to the ineffective control of hyperglycaemia associated with the gradual dysfunction of pancreatic beta cells, distressed incretin physiology, compromised regulation of glucagon secretion and insulin resistance are the major predisposing factors implicated in type 2 diabetic conditions (Meece, 2007, Tikoo and Gupta, 2012; Cade, 2008).

Nephropathy, retinopathy and neuropathy among others are major specific complications associated with diabetes (Modak *et al.,* 2007). The role of free radicals in the pathogenesis of diabetes and its associated complications is abundantly documented in literature (Ramachandran *et al.,* 2011; Folli *et al.,* 2011). Intense hunger, increased sugar level in the blood, extraordinary thirst concomitant with frequent urination and weight loss, blurred vision, mood swings, occasional nausea and vomiting, severe tiredness and weakness are common symptoms in both type 1 and type 2 diabetic conditions (Modak *et al.,* 2007). Amelioration in individuals suffering from type 1 diabetes is achieved by absolute dependence on exogenous sources of insulin, whereas type 2 diabetic conditions can be ameliorated with dietary changes, exercise and medication (Joseph and Jini, 2011).

Over the years, various conventional drugs and synthetic chemical agents have been employed in the treatment and management of diabetes and diabetic complications; but up to date, none has been reported to have provided absolute recovery from diabetes and its complications (Inzucchi *et al.,* 2012). Added to this, the cost of medication therapy management, the amount of medication and doses per day, the development of resistance, as well as a lack of responsiveness in some patients to administered drugs and the accompanying side effects of these drugs is of major global concern (Inzucchi *et al.,* 2012; Rwegerera, 2014; Zammitt and Frier, 2005).

This has necessitated the search for and evaluation of numerous plant-derived foods as cost-effective alternatives in the management of diabetes and its associated complications. Various plant-derived foods that have been employed in the management of diabetes and its associated complications worldwide possess bioactive constituents believed to be rich in antioxidants and proteins, giving plant-

derived foods prominence. This review therefore focuses on the various plant-derived foods that have been reported to show a certain degree of amelioration in the management of diabetes and its associated complications.

3.2 Role of oxidative stress

Oxidative stress, primarily mediated by hyperglycaemia, has been implicated in the pathogenesis of diabetes. The generation of free radicals, especially reactive oxygen species (ROS), as a result of various metabolic abnormalities within the body has been identified as a possible mode of action by which oxidative stress comes into play, eliciting obvious deleterious effects associated with the conditions of the disease (Tiwari *et al.,* 2013; Giacco and Brownlee, 2010; Johansen *et al.,* 2005). Oxidative stress promotes pancreatic β-cell dysfunction and death, because they are predominantly susceptible to it, leading to a relative or absolute lack of insulin and insulin resistance as a result of gradual pancreatic β-cell dysfunction, which is responsible for insulin synthesis and secretion (Jaganjac *et al.,* 2013).

Oxidative stress takes place when there is an overwhelming effect on the endogenous antioxidant system by free radicals generated as a result of the overproduction and insufficient scavenging of these free radicals (Rahman, 2007). This state of imbalance (oxidative stress) plays a fundamental role in the manifestation of diabetes and its complications. Five key mechanisms have been identified as responsible for hyperglycaemia induced diabetic complications, these are: the increased flux of glucose and other sugars through the polyol pathway; the increased formation of advanced glycation end products (AGEs); increased expression of the receptor for AGEs and its activating ligands; the activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (Giacco and Brownlee, 2010). Overproduction of ROS contributes greatly, directly or indirectly, to the perturbation of each of the pathways above, thereby enhancing the progression of diabetes and its associated complications.

3.3 Challenges with current anti-diabetic drugs

People affected by diabetes, irrespective of the type are expected to manage their blood glucose level with medication. Several studies reveal that the side effects of diabetic medications and diabetes-associated complications, combined, are accountable for approximately 3.2 million deaths annually (Chawla *et al.,* 2013). The literature evidence reveals about five categories of oral drugs employed in the treatment of diabetes. This includes: biguanides, sulfonylureas, thiazolidinediones, meglitinides and alpha-glucosidase inhibitors (Inzucchi *et al.,* 2012). Treatment with some of these drugs results in hypoglycaemia and other severe side effects ranging from liver or renal function impairment to heart failure. Gastro-intestinal upset, fluid retention, edema or weight gain as well as nausea and diarrhea are negligible side effects (Brunetti and Kalabalik, 2012).

Added to this, poor health facilities and the cost of assess to good health facilities in developing countries, where this disease is on the increase, makes it wearisome and frustrating for people with diabetes. For instance, the majority of people with type 1 diabetes do not receive insulin injections as and when they are due and most of the drugs available are very expensive and not readily available (Irons and Minze, 2014). This has contributed in no small way to non-adherence to therapies among diabetic patients (Blackburn *et al.,* 2013). The evaluation of plant-derived foods in the management of diabetes and its complications is based on the aforementioned challenges.

3.4 Comparison between nutraceuticals and conventional drugs

Naturally occurring bioactive components abundantly available in foods, spices, vegetables, herbal products and fruits which have the potential to delay, defend, control or even prevent the pathogenesis and progression of diseases, formed and which possess health-promoting abilities and medicinal properties are generally referred to as nutraceuticals. The nutraceutical approach is mainly based on the utilization of nutrition for alleviating ill-health or for general body fitness and wellness (Pandey *et al.,* 2011). Conventional drugs are usually referred to as pharmaceuticals, made up of chemically active substances ingested for pre-

protection or used mainly to treat disease or illness. The majority of conventional drugs have their drawbacks, ranging from serious to minor side effects. Nutraceuticals have been documented in literature to have competitive costs with few and transient side effects, making it an attractive alternative in the management of human health and disease (Mishra, 2012).

Plant-derived foods and nutraceuticals in the management of diabetes and associated complications

Various studies have established the potential of plant-derived foods (natural products) and nutraceuticals in the management of diabetes and its associated complications (Table 1) in conjunction with lifestyle modification and adjustment of dietary intake (Omar *et al.,* 2010). The majority of nutraceuticals presently in use have been reported to delay or prevent the pathogenesis of diabetes and its associated complications (Figure1). These nutraceuticals also regulate some of the biochemical and clinical outcomes of diabetes (Bahadoran *et al*., 2013; Davi *et al.,* 2010). Studies have shown that the ability of a food substance to be able to retard the rate of absorption of glucose from ingested carbohydrate meals makes the food a potential candidate as a nutraceutical for the prevention of diabetes. A good example of a nutraceutical presently approved and commercially available is glucomannan®, which is a natural fibre with the ability to lower down the glycemic index of meals if it is taken as a drink or mixed with food. Fibre in general is reputed to have the ability to lower the glycemic index of carbohydrate meals. Beans have also been reported to have a low glycemic index because of the presence of α-amylase inhibitors (McCarty, 2005).

A major polyphenolic compound chlorogenic acid, present in coffee, is suggested to be responsible for the documented reports that heavy consumption of coffee lowers an individuals' risk of having diabetes. Chlorogenic acid is suggested to have the ability to slow down carbohydrate uptake by hindering intestinal glucose transportation (McCarty, 2005; Johnston *et al.,* 2003). Chlorogenic acid is also reported to inhibit the cellular activity of the enzyme glucose-6-phosphate translocase (Hemmerle *et al.,* 1997). A nutraceutical made up of green coffee extract with 55% chlorogenic acid is already available in the market.

Barley malt extracts are also available in the market; their mechanisms of action are reported to be similar to that of metformin in that, they possess the ability to activate AMP kinase (McCarty, 2005).

Biotin, a nutraceutical agent, has also been demonstrated, by means of animal and clinical studies, to be capable of assisting in glycemic control of diabetic individuals if taken at a high dosage of 9-15 mg per day (Zhang *et al.,* 1996; Zhang *et al.,* 1997; Maebashi *et al.,* 1993). Various animal studies and a few clinical studies have been established the effect of supplementary magnesium on the preservation of adipocyte insulin sensitivity (McCarty, 2005). High magnesium administration was also shown to be useful in preventing the onset of diabetes in obese, diabetes-prone experimental rodents (Lima *et al.,* 1996).

It has been suggested that an ample intake of calcium and vitamin D may be helpful in reducing the risk of diabetes by preserving insulin sensitivity, even though further research is still needed to be done to confirm this (McCarty, 2005).

Table 3.1: Various plant-derived foods in the management of diabetes and its associated complications

S/N	Plant Name	Main active ingredients	Effects/Activities	Model Used	References
1	Mangnifera indica: Mango	Mangniferin	Antioxidative, Anti-inflammatory, Hypoglycaemic	Rat, Mice	Shah et al., 2010; Aderibigbe et al., 2001; Muruganandan et al., 2002; Muruganandan et al., 2005
2	Carica papaya: Pawpaw	Carotene, Riboflavin	Antioxidative, Hypoglycaemic, Hypolipidemic, Wound healing associated with diabetes complications	Rat	Collard and Roy, 2010; Juárez-Rojop et al., 2012
3	Tetracarpidium conophorum: African walnut	Tocopherol, Ascorbic acid	Antioxidative, Antihyperglycaemic	Rat	Onwuli et al., 2014; Abam et al., 2013
4	***Garcina kola***: Bitter kola	Kolaviron	Hypolipidemic, Hypoglycaemic	Rat	Adaramoye and Adeyemi, 2006

5	Telfairia occidentalis: Fluted pumpkin leaf	Ascorbic acid, Riboflavin, Thiamine	Inhibition of α-amylase and α-glucosidase activities	Rat	Kayode and Kayode, 2011; Oboh et al., 2012
6	Vernonia amygdalina: Bitter leaf	Tannins, Phytosterols Flavonoids	Anti-hyperlipidemia, Hypoglycaemic, Alpha-glucosidase inhibitor	Rat	Adaramoye et al., 2008; Yeap et al., 2010
7	Allium sativum: Garlic	Water-soluble sulphur compounds	Hypocholesterolemic	Human, Rat	Yeh and Liu, 2001; Eidi et al., 2006
8	Cinnamomum verum: Cinnamon	Polyphenols	Increases insulin sensitivity, Hypolipidemic, Hypoglycaemic	Human, Rat, Cultured cells	Anderson, 2008; Cao et al., 2010
9	Cucuma longa: Turmeric	Curcumin	Hypolipidemic, Hypoglycaemic	Rat, Mice	Kuroda et al., 2005; Masry, 2012

10	Ocimum gratissimum: Scent leaf	Tannins, Saponins, Flavonoids	Hypolipidemic, Hypoglycaemic	Rat	Mohammed et al., 2007; Oguanobi et al., 2012
11	***Parkia biglobosa***: African locust bean	Phenols	Hypolipidaemic, Hypoglycaemic, Inhibition of Alpha-Amylase	Rat	Adi et al., 2013; Odetola et al., 2006; Fred-Jaiyesimi and Abo, 2009
12	Zingiber officinale: Ginger	Shogaols, Gingerols, Zingerone	Hypolipidaemic, Hypocholesterolemic	Rat	Nwozo et al., 2014; Nammi et al., 2009; Li et al., 2012
13	***Piper guineense***: African black pepper	Protein, Alkaloids	Anti-hyperglycaemic, Anti-hyperlipidemic	Rat	Udofia et al., 2014; Nwozo et al., 2012; Ekoh et al., 2014

meals if it is taken as a drink or mixed with food. Fibers generally are reputed to have ability to lower the glycemic index of carbohydrate meals. Beans have been reported to have a low glycemic index because of the presence of α-amylase inhibitors (McCarty, 2005).

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3.5 Plant-derived foods and diabetes management

3.5.1 Fruits

3.5.1.1 Mangnifera indica: Mango

Mango is a very important tropical fruit loved by most people for its juicy sweet pulp when ripe. The fruit is reputed to be a very good source of vitamin C. various parts of

this tropical plant are used among traditional folk for the treatment of various ailments and diseases such as hypertension. The occurrence of phytochemicals such as flavonoids, mangniferin, polyphenolics, triterpenoids and some others in this plant accounts for its reported anti-inflammatory, analgesic and hypoglycaemic activities (Shah *et al.,* 2010). Administration of the leaf extracts in experimental studies resulted in a decrease in the blood glucose level in glucose-induced hyperglycaemic mice, indicating that the plant could possess active constituents capable of decreasing blood glucose levels (Aderibigbe *et al.,* 2001). Administration of mangniferin, an active constituent extracted from *M. indica* intra-peritoneally, to diabetic rats showed some antioxidative potentials as well as the attenuation of glycosylated heamoglobin levels (Muruganandan *et al.,* 2002). Twenty-eight days chronic administration (10 and 20 mg/kg) of mangniferin also exhibited a reduction in the diabetic state of experimental rats (Muruganandan *et al.,* 2005).

3.5.1.2 *Carica papaya*: Pawpaw

Carica papaya is a tropical fruit commonly consumed in many parts of the world. It has a low calorific value (32 kcal/100g of ripe fruit) and contains various natural vitamins and minerals like folate, thiamine, riboflavin, calcium, niacin, potassium and iron. It is one of the best natural sources of vitamin A and vitamin C. It has a high content of carotene necessary for the prevention of damage due to free radicals (Krishna *et al.,* 2008).

Fermented *C. papaya* fruit serves as a good antioxidant and is thus a promising nutraceutical. Oral administration of the preparation at 9 g/day elicited an improvement in the antioxidant defence of elderly human subjects (Marotta et al., 2006). The fermented *C. papaya* fruit preparation was also demonstrated in animal studies to be beneficial in the healing of wounds associated with diabetes complications by influencing wound-site macrophages and angiogenic responses (Collard and Roy, 2010). Various experimental studies established the efficacy of *C. papaya* to improve the various metabolic disorders associated with diabetes. Juarez-Rojop and co-workers (2012) reported that the administration of an aqueous extract of *C. papaya* leaves, extracted at varying concentrations, in drinking water (0.75 g/100 mL, 1.5 g/100 mL and 3 g/100 mL) elicited a significant decrease in blood glucose levels as well as a reduction in plasma triacylglycerol in streptozotozin-

induced diabetic rats. The extract also showed a remarkable antioxidant activity and improvement in the lipid profile as well as in the liver and pancreas integrity of the rats (Juárez-Rojop *et al.,* 2012).

3.5.2 Nuts

3.5.2.1 Tetracarpidium conophorum: African walnut

The African walnut, propagated for the production of nuts eaten as a delicacy, is found to possess abundant bioactive constituents rich in antioxidants and essential nutrients. Different parts of this plant have been documented to have antioxidant, chelating, antimicrobial and anti-diabetic potential (Onwuli *et al.,* 2014; Abam *et al.,* 2013). Onwuli and co-workers (2014) concluded, in a study where rats were fed with varying doses of *T. conophorum* (21.3 g, 42.6 g, and 85.2 g), that *T. conophorum* nuts have hypoglycaemic effects with the ability to ameliorate and decrease hyperglycaemia, increase haemoglobin levels, as well as preventing diabetes mellitus-associated renal damages (Onwuli *et al.,* 2014).

3.5.2.2 *Garcina kola*: Bitter kola

Garcina kola, also known as bitter kola because of its taste, contains various pharmacologically bioactive compounds (Ikpesu *et al.,* 2014). In order to explain its hypolipidemic and hypoglycaemic potentials, Adaramoye and Adeyemi (2006) administered kolaviron (100 mg kg^{-1} body weight), a biflavonoid complex from *Garcinia kola* to STZ-induced diabetic rats and concluded that *G. kola* has potent anti-diabetic agents which could also prevent the complications associated with diabetes with great potential for effectively scavenging the excessive free radicals generated (Adaramoye and Adeyemi, 2006).

3.5.3 Vegetables

3.5.3.1 *Telfairia occidentalis*: Fluted pumpkin leaf

Telfairia occidentalis is a common vegetable in Central and West Africa especially in Nigeria, Cameroon and Benin. The green leaves of the plant, which are greatly cherished as a staple vegetable have high vitamin and iron content. Various experimental studies established that the leaves contain minerals such as phosphorus, calcium, potassium, magnesium, iron and sodium, and are rich in

antioxidants and vitamins like riboflavin, nicotinamide, ascorbic acid and thiamine (Kayode and Kayode, 2011). They also contain essential phytochemicals like phenols, tannins, saponins and alkaloids. *In vitro* and *in vivo* experiments have shown the antioxidant ability of extracts of the plant by elevating the antioxidative enzymes such as catalase and superoxide dismutase, thus preventing free radical production as well as scavenging free radicals (Oboh *et al.,* 2006). *T. occidentalis* leaf was also shown to inhibit the activities of α-amylase and α-glucosidase (500 µL and 50 µL respectively) which are key enzymes associated with type-2 diabetes (Oboh *et al.,* 2012).

3.5.3.2 *Vernonia amygdalina*: Bitter leaf

Vernonia amygdalina is a shrub of tropical Africa that grows wild or cultivated in the different countries of sub-Saharan Africa. The leaves are dark green in colour with a peculiar odour and bitter taste (Oboh and Masodje, 2009). Adaramoye and co-workers (2008) established the anti-hyperlipidemia effect of *V. amygdalina* by administering of 200 mg/kg of body weight of the extract to rats fed with a high cholesterol diet. This resulted in a significant reduction in both plasma and post-mitochondrial fraction total cholesterol levels of the rats (Adaramoye *et al.,* 2008). Various studies established the anti-diabetic effect of both the ethanolic and aqueous leaf extracts of the plant. The hypoglycaemic activity of the plant extracts may be due to the presence of tannins, phytosterols and flavonoids, which may act as alpha-glucosidase inhibitors (Yeap *et al.,* 2010).

3.5.4 Spices

3.5.4.1 *Allium sativum*: Garlic

Garlic is traditionally used as a spice for cooking various delicious dishes. Experimental studies have shown its anti-parasitic, antifungal, antiviral, antibacterial, anti-carcinogenic activities as well as anti-hypertensive properties (Corzo-Martínez *et al.*, 2007). Garlic is made up of distinctive organosulphur compounds, which are responsible for its biological activities, flavour and unpleasant odour. A garlic preparation in the form of a supplement referred to as ‘aged garlic extract’ has been studied extensively for its bioactivity both *in vitro* and *in vivo*.

Aged garlic extract has anti-oxidative properties and the ability to scavenge ROS by promoting cellular enzymes such as glutathione peroxidase, superoxide dimutase, and catalase (Borek, 2001). Human and animal studies have successfully established the hypocholesterolemic activity of aged garlic extracts, due to the presence of water-soluble sulphur compounds capable of inhibiting hepatic cholesterol synthesis (Yeh and Liu, 2001). Aged garlic extracts have also been studied and shown to be capable of reducing the risk of cardiovascular diseases by selectively inhibiting platelet aggregation and adhesion (Steiner and Li, 2001). Ethanolic extracts of garlic administered at 0.1, 0.25 or 0.5 g/kg of body weight to streptozotocin-induced diabetic rats showed that garlic is an excellent candidate for study in the management of diabetes (Eidi *et al.,* 2006).

3.5.4.2 *Cinnamomum verum*: Cinnamon

The addition of a water-soluble cinnamon compound in meals could be effective in reducing the risks associated with cardiovascular diseases and diabetes (Roussel et al., 2009). *In vitro* studies in animals and humans showed that the polyphenols in cinnamon increase insulin sensitivity. Daily intake of 1 – 6 g of cinnamon by type-2 diabetic models led to a reduction in fasting blood glucose, total cholesterol, low-density lipoproteins cholesterol and triacylglycerol (Anderson, 2008). Experimental studies on gene expressions in cultured mouse adipocytes established that phenolic extracts of cinnamon have an insulin-like action and an insulin independent action on the control of gene expression in adipocyte tissues (Cao *et al*., 2010).

3.5.4.3 *Cucuma longa*: Turmeric

Turmeric is a rhizome plant that is traditionally used in various parts of the world as a spice, a food preservative and colourant. It is a vital home remedy for coughs, colds, rheumatism, diabetic wounds, anorexia, and hepatic disorders, amongst others (Chattopadhyay *et al*., 2004). Turmeric contains essential and fatty oils, and a small amount of carbohydrates, proteins and omega-3 fatty acids. It contains 2 – 9% of cucuminoids, an important polyphenol, depending on the variety. The whole turmeric preparation or its extract has been reported to have effect on cardiovascular disease, inflammation and cancer (Krishnaswamy, 2008).

Kuroda and co-workers (2005) have shown the ability of turmeric extract to significantly suppress an elevation in blood glucose in type-2 diabetic mice and

suggested that curcumin in turmeric could be useful in attenuating diabetes in humans if prepared as a functional food (Kuroda *et al*., 2005). Curcumin, the principal bioactive constituent in turmeric, when administered in aqueous suspension (80 mg/kg body weight) to streptozotocin-induced diabetic rats, instigated a significant reduction in oxidative stress parameters measured in the rats, especially ameliorating the extent of lipid peroxidation (El-Masry, 2012).

3.5.4.4 *Ocimum gratissimum*: Scent leaf

Ocimum gratissimum is a herbaceous plant usually grown in tropical and subtropical regions for its medicinal and cooking (food spice) uses (Nweze and Eze, 2009). Aqueous extracts of the leaf, administered orally in a study, show that the leaf exhibits significant hypoglycaemic effects in streptozocin-induced diabetic rats. Notably, 500 mg/kg of body weight of the extract was identified as the effective antidiabetic dose which significantly lowered blood glucose levels of the diabetic rats by 81.3% after 24 hours of administration of the extract. They concluded that *O. gratissimum* possesses antidiabetic properties, indicating the presence of biologically active components, which may be worth further investigation and elucidation (Mohammed *et al*., 2007). Hypoglycaemic activity of *O. gratissimum* (100, 200 and 300mg/kg body weight of the aqueous extract) has also been reported in type 2 streptozotocin-induced diabetic rat models (Oguanobi *et al.,* 2012).

3.5.4.5 *Parkia biglobosa*: African locust bean

Parkia biglobosa is an important household spice commonly used in Nigeria and many other West African countries in the preparation of various foods as well as a seasoning agent in soups (Odetola *et al.,* 2006). Many reports have justified and documented the use of several medicinal plants such as *P. biglobosa* in the treatment of various cardiovascular diseases such as hypertension, cardiac failure and cardiac disturbances. Medicinal and pharmacological investigations of *P. biglobosa* have shown that the fermented seeds had hepatoprotective, hypolipidaemic, antimicrobial and anti-inflammatory potential (Adi *et al*., 2013). A documented report reveals that both aqueous and methanolic extracts of *P. biglobosa* (6 g/kg body weight) had hypoglycaemic effects in alloxan-induced diabetic models (Odetola *et al*., 2006; Fred-Jaiyesimi and Abo, 2009). Taken together, the aqueous extract demonstrated better therapeutic effects in managing

diabetes and some of the complications associated with diabetes (Odetola *et al*., 2006).

3.5.4.6 Zingiber officinale: Ginger

Globally, *Zingiber officinale* is one of the most commonly consumed spices and functional foods. Its involvement in the treatment of various ailments has been known for ages. Its hepatoprotective, anti-cancer, anti-inflammatory, analgesic and anti-clotting as well as anti-arthritic, anti-nausea, hypolipidaemic and hypocholesterolemic properties have been reported in recent literature (Nwozo *et al*., 2014; Al-Amin *et al.,* 2006). *Z. officinale* contains essential volatile oils and non-volatile pungent compounds as its major chemical constituents. Terpenoids are the major components of the volatile oil while the non-volatile compounds abundant in *Z. officinale* are shogaols, gingerols, zingerone and paradols. In fresh and dried *Z. officinale*, gingerols and shogaols were identified as the major bioactive constituents (Nammi *et al.,* 2009). In order to justify the traditional use of *Z. officinale* in the management of metabolic diseases, especially diabetes, scientific evidence has been documented and most authors conclude that ethanolic extracts of *Z. officinale* (100 mg/kg, 200 mg/kg and 400 mg/kg of body weight) show obvious protective abilities in the control and treatment of diabetes and its associated complications in animal models (Nammi *et al*., 2009; Li *et al*., 2012).

3.5.4.7 *Piper guineense*: African black pepper

Piper guineense is an important spice in West Africa, especially Nigeria, with both nutritional and medicinal values. Its high protein, alkaloid and iodine content makes it unique among other commonly used household spices (Umoh *et al.,* 2013). An evaluation of *P. guineense* revealed that it is a promising candidate in the formulation of novel therapeutic drugs that would have significant effects in the management of inflammation and other related chronic diseases (Udofia *et al.,* 2014; Nwozo *et al.,* 2012). Recently, Ekoh and co-workers (2014) reported the anti-hyperglycaemic and anti-hyperlipidemic effects of the aqueous extract of *P. guineense* (500 mg/kg of body weight) in alloxan-induced diabetic rats. They concluded that the extract had some diabetic management potentials and that it reduces the risk of cardiovascular complications associated with diabetes (Ekoh *et al*., 2014).

3.6 Conclusion

As a result of the exponential increase in the global prevalence of diabetes and its associated complications, and the drawbacks associated with the current anti-diabetic drugs available in the market (ranging from the high cost of drugs to adverse side effects, unavailability and obvious non-adherence to therapy among patients) it is highly imperative to explore plant-derived foods with proven pharmacologically-bioactive constituents as alternatives to the current anti-diabetic drugs in the management of diabetes and its associated complications.

Based on recently documented reports on the mechanisms of action of various plant-derived foods reviewed in this paper, it is proposed that *Cucuma longa* (curcumin), *Garcinia kola* (kolaviron), *Telfairia occidentalis* and *Parkia biglobosa* could be excellent candidates for use on a broader base in humans to treat/prevent diabetes and its associated complications.

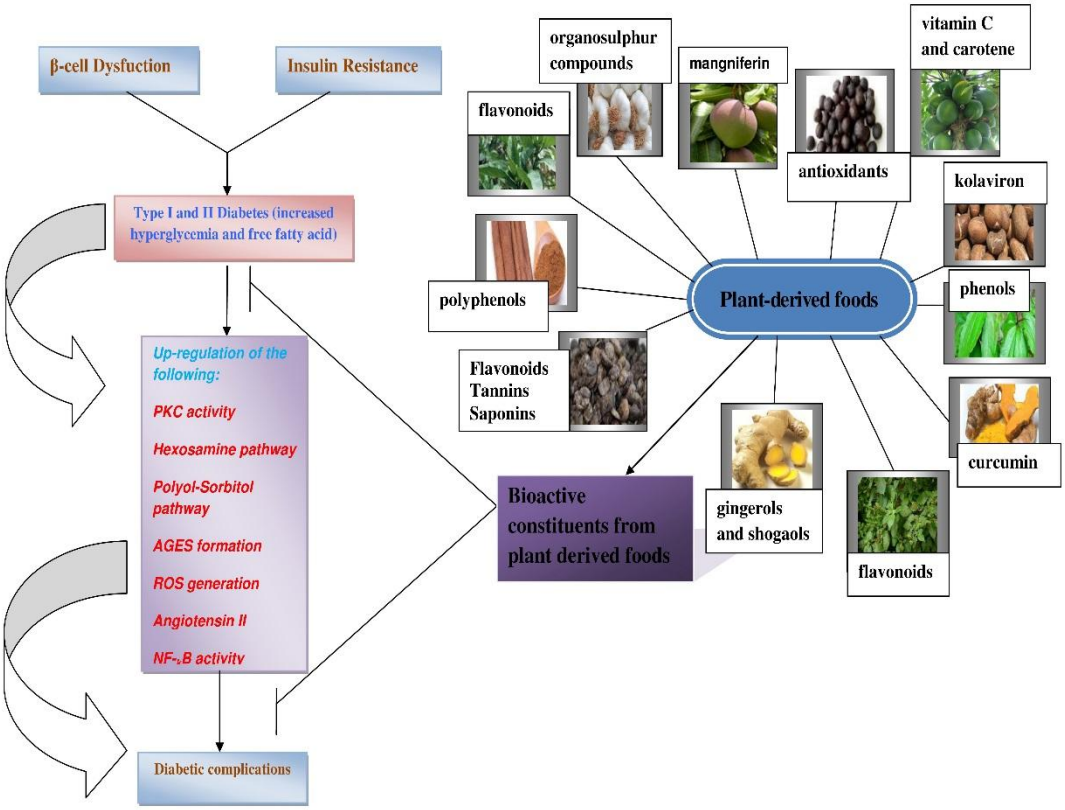

Figure 3.1. Proposed model of plant-derived foods in the amelioration of diabetes and its associated complications. The autoimmune destruction of pancreatic beta cells and insulin resistance play a crucial role in the pathogenesis of type I and type II diabetes which is associated with increased hyperglycaemia and free fatty acid. Up-regulation of PKC activity, hexosamine pathway, polyol-sorbitol pathway, AGES formation, ROS generation, angiotensin II and NF-$_k$B activity (contributory factors) lead to vascular complications. Plant-derived foods possess the protective ability to delay or prevent the pathogenesis of diabetes and its associated complications by inhibiting the up-regulation of some of these contributory factors or by acting as survival mechanisms directly on vascular cells.

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Chapter Four

4.0 Comparative study on proximate, functional, mineral and anti-nutrient composition of fermented, defatted and protein isolates of *Parkia biglobosa* seed

Abstract

The use of plant-derived foods and their associated physico-chemical properties in the prevention, treatment or management of metabolic diseases especially diabetes has gained prominence. This study was conducted to compare the proximate, functional, minerals and anti-nutrient composition of the fermented, defatted and protein isolates of *Parkia biglobosa* seed. The results showed that the fermented, defatted and protein isolates varied in composition within the parameters studied. The proximate analysis revealed that the protein isolate had the highest ash (6.0 %) and protein (59.4 %) as well as the lowest fat (5.7 %) and moisture (5.1 %) content when compared to the fermented and defatted samples. In a similar manner, the functional properties of the protein isolate were relatively better than those of the fermented and defatted samples; with an oil absorption capacity of 4.2 % and emulsion capacity of 82 %. The mineral contents in the protein isolate were also significantly higher in magnesium and zinc content compared with the fermented and defatted samples, while a negligible amount of anti-nutrient was present in all samples, with the protein isolate having the lowest value. The overall data suggests that the protein isolate had better proximate, mineral, functional and anti-nutrient properties when compared with the fermented and defatted sample. Therefore, the synergetic effect of all the components present in the protein isolate from *Parkia biglobosa* seeds, in association with its low carbohydrate and high protein/ash content, could play a vital role in the management of diabetes and its associated complications.

Keywords: Anti-nutrient, functional properties, *Parkia biglobosa*, protein isolate, proximate analysis.

4.1 Introduction

Parkia biglobosa is one of the indigenous medicinal plants commonly found in Nigeria and many other West African countries. The fermented seeds of *P. biglobosa* are believed to possess nutritional and medicinal values. They are used extensively as a household spice in the preparation of various delicious foods (Ogunyinka *et al*., 2015; Ajaiyeoba, 2002). *P. biglobosa* belongs to the family *Leguminosae* and the sub-family *Mimosoideae*. They are known as African locust beans (English); 'dawadawa' in Ghana; 'soumbala' in Burkina Faso; and 'iru' in Benin and Nigeria (Pelig-Ba, 2009).

In recent times, several pharmacological and nutritional benefits of *P. biglobosa* have been reported (Olabinri *et al.,* 2013; Badu *et al*., 2012). Consequently, considerable research interest has been directed towards exploring *Parkia biglobosa* in the prevention, treatment and management of a number of metabolic diseases, one of which is diabetes mellitus. The pharmacological and nutritional benefits of *P. biglobosa* has been associated with its physico-chemical properties, perceived to act independently or in synergy, thus playing a pivotal role in the maintenance of normoglycemia by activating diverse protective mechanisms (Ogunyinka *et al*., 2015; Fred-Jaiyesimi, 2009).

Diabetes is a multifactorial disease connected with numerous complications and consequently requires a multiple therapeutic approach (Modak *et al.,* 2007). *P. biglobosa* has been identified as one of the candidates with promising therapeutic potentials. As part of the exploration into the protective and antidiabetic mechanisms of *P. biglobosa*, this study was carried out to compare the proximate, functional, mineral and anti-nutrient composition of the fermented, defatted and protein isolates of the *P. biglobosa* seed.

4.2 Materials and Methods

4.2.1 Plant material, authentication and extract preparation

After obtaining an import permit (P0060156) from the Department of Agriculture, Forestry and Fisheries (DAFF; Republic of South Africa), the raw and fermented *Parkia biglobosa* seeds used in this study were purchased from a local market in Ijebu-Ode, Ogun state, Nigeria. The seeds were identified and authenticated by the Chief botanist of the Department of Botany, University of Zululand and a voucher specimen (B07) was deposited at the university herbarium. Prior to extraction of the protein isolate, the fermented seeds were oven-dried at 50 °C. The dried seeds were then grounded into a uniform powder using an electric blender. One kilogram of powdered seeds was defatted with hexane to obtain the defatted extract.

The defatted extract was air dried and extracted (1: 10 w/v) with an acidic butanol to remove any possible anti-nutrients. Protein isolate was extracted from the defatted extract using the method described by Nkosi and colleagues (Nkosi *et al.,* 2005). The dry defatted extract was re-suspended in distilled water at pH 10. The resultant suspension was filtered to remove debris and the filtrate adjusted to pH 5, followed by centrifugation at 5000 rpm for 15 minutes at 4 °C. The supernatant from this centrifugation step was discarded, while the pellet containing the protein isolate was retained and freeze-dried to yield a brown extract. The lyophilized extract was then kept dry until needed.

4.2.2 Proximate analysis

A proximate analysis was carried out on fermented, defatted and protein isolates of *Parkia biglobosa* to ascertain the differential composition of the various nutritional components and their quantitative amounts. Nutritional parameters, such as ash content, moisture content, mineral composition, crude fat, carbohydrates, crude fiber, and total protein, were determined. The official method of AOAC (2005) was used to determine the moisture content of the samples. The Tecator Digestion System and Kjeltec Auto 1030 Analyzer (Tecator AB, Sweden) were used to determine the protein content. The Soxtec System HT method (Tecator Soxtec System HT 1043 Extraction Unit, Tecator AB, Sweden) was used to determine the fat content. The

official AOAC (2005) methods were used to determine ash content. The crude fibre content was determined in accordance with the AOAC Official Method of Analysis (2005).

The value of 5.95 was used as a protein conversion factor during calculations. Protein yields were calculated as follows:

$$\textbf{Yeild (\%)} = \frac{\textbf{weight (g) of PBPI x protein content (\%) of PBPI}}{\textbf{10 g (weight of DPB) x Protein content (\%) of DPB}} \times 100$$

The carbohydrate content was determined by adding all the moisture, fat, crude protein, ash and crude fibre percentages together and subtracting them from 100 %. The result provided the amount of nitrogen free extract, otherwise known as carbohydrate.

Carbohydrate (%) = 100% – [protein (%) + Moisture (%) + Ash (%) + Fibre (%) + Fat (%)].

The caloric value of the samples in kilojoules per 100 g (kJ/100g) was determined using the “Atwater factors”, by multiplying the values of crude protein, lipids and carbohydrates by 37, 17 and 17 respectively (Eknayake *et al.,* 1999).

4.2.3 Mineral Analysis

The mineral analysis was carried out on fermented, defatted and protein isolates to determine the amount of inorganic elements like sodium, potassium, calcium, manganese, copper, phosphorus, zinc, magnesium and iron present in these samples. The official method of the AOAC (2005) was adopted. The samples were previously ashed in a furnace for five hours at 600 °C, then boiled with 10 cm^3 of 20 % hydrochloric acid which was filtered into a 100 cm^3 standard flask. The filtrate was then made up to the mark with deionized water. Sodium (Na) and Potasium (K) levels of the sample were ascertained using a flame emission photometer with NaCl and KCl as standards. All other metal ions were determined by atomic absorption spectrometry (AAS).

4.2.4 Anti-nutritional Composition

The anti-nutrient contents of the fermented, defatted and protein isolates of the samples were determined, whereby Phytate was determined by the method described by Ola and Oboh (2000), while the oxalate and nitrate levels were ascertained using the methods described by Krishna and Ranjahan (1980).

4.2.5 Functional Properties

The functional properties of the fermented, defatted and protein isolates of *Parkia biglobosa* were evaluated as follows:

4.2.5.1 Seed Density

The seed density of the fermented seeds of *Parkia biglobosa* was determined using a selection of one hundred seeds. The seeds were weighed and then transferred to a half-filled 100 cm^3 measuring cylinder of tap water for calibration and allowed to soak for 10 minutes. The water volume displacement was noted (Akinyele *et al.,* 1986) and the seed density calculated as follows:

Density = mass / volume.

4.2.5.2 Bulk density

A previously marked 10 cm^3 measuring cylinder was filled with 2 g of sample. The measuring cylinder base was gently tapped on the laboratory table to a constant volume, and the bulk density (g/cm^3) was calculated with the method of Akpapunam and Markakis (1981), with the following formula:

Bulk density = Weight of sample / volume of sample after tapping (g/cm^3).

4.2.5.3 Swelling Index

The swelling index was evaluated with the method described by Akinyele *et al*., 1986. One gram of the sample was weighed and dispensed into a test tube, leveled and the height noted. Distilled water (10 cm^3) was added and allowed to stand for 1 hour. The height was then recorded and the swelling index calculated with the formula:

Swelling index (S) = Initial height (H^1) / Final height (H^2)

4.2.5.4 Water absorption capacity

The water absorption capacity was determined by measuring 1 g of sample and mixed with 10 cm^3 of distilled water in a weighed 20 cm^3 centrifuge tube. The suspension was vigorously mixed with a vortex mixer for 2 minutes, after which it was rested for 30 minutes. The suspension was then centrifuged at 3250 rpm for 25 minutes. The supernatant was discarded and the tube air-dried. Bound water was calculated from the increase in weight of each sample (Sathe and Salunkhe, 1981). The water absorption capacity (WAC) was then calculated using the formula:

WAC = Initial volume (V_1) – Final volume (V_2) × 1.0 / 100.

4.2.5.5 Oil absorption capacity

Five hundred milligrams (0.5 g) of sample was added to 3.0 cm^3 of corn oil in a 15 cm^3 graduated conical centrifuge tube. The emulsion was mixed with a vortex mixer for 2 minutes, incubated at room temperature for 30 minutes, and later centrifuged at 3200 rpm for 25 minutes. The supernatant was decanted and the volume of separated oil was noted (Sathe and Salunkhe 1881). The oil absorption capacity (OAC) was evaluated by difference and using the formula:

OAC = V_2 – V_1 / 100 × 1.0

4.2.5.6 Foam capacity and stability

The foam capacity and foam stability of the protein isolate was determined using a modified method of Lin and co-workers (1974). Two grams (2 g) of sample were dissolved in 50 cm^3 of distilled water. A food mixer at a speed set for fast beating was used to enhance adequate mixing. The initial and final volumes, before and after whipping in a 100 cm^3 graduated cylinder, were recorded. The percentage increase in volume due to whipping was calculated according to the method of Lawhhon and co-workers (1972). For foam stability, the foam volumes in the standing cylinder after whipping were recorded at ½, 1, 10 and 120 min (Sosulski, 1962).

4.2.5.7 Emulsion Capacity

The sample emulsion capacity was determined using the method described by Aluko and Yada (1995). One gram of sample was blended in 50 cm^3 of distilled water for 30 seconds using a blender at high speed. 2.5 cm^3 of vegetable oil was added continuously while blending until properly mixed. This mixture was set aside for 30 minutes. The emulsion capacity was evaluated as the amount of oil emulsified by 1g of sample.

Emulsion capacity = Emulsion height x 100 / water height x 1

4.2.5.8 Wettability

The wettability of each sample was measured as the time in seconds required by 1 g of the flour sample to be completely wet on the surface by water under laboratory conditions. This was determined using the methods described by Aluko and Yada, (1995), whereby a clean glass beaker (600 cm^3 capacity) was filled with 500 cm^3 of distilled water. This was placed on a retort stand with a clean test tube clamped in an inverted position over the water in the beaker, with a distance of 10 cm between the beaker and the mouth of the test tube. The clean position on the test tube and water in the beaker were both marked with masking tape. Subsequently, 1 g of the sample was lowered into a marked test tube and the mouth of the test tube covered with finger's thumb. This was cautiously converted over the water and clamped with the retort stand at the marked spot without removing the thumb. With the stop watch set to read, the thumb was removed and the sample allowed to fall into the water surface as the stop watch was started simultaneously. The flour sample was observed and the stop watch stopped as soon as the last samples got wet.

4.3 Results

Proximate composition of the fermented, defatted and protein isolates of Parkia biglobosa seeds

A detailed proximate analysis of the fermented, defatted protein isolates of *Parkia biglobosa* seeds are presented in Table 4.1. The results reveal that the protein isolate had the highest ash (6.0 %) and protein (59. 4 %) contents as well as the lowest carbohydrate (8.8 %), fat (5.7 %) and moisture (5.1 %) content when compared with fermented and defatted samples. The fermented sample shows the

highest energy value (470.5 kcal) and fiber content (9.5 %) when compared with protein isolate (381.7 kcal, 1.1 %) and defatted (366.7 kcal, 8.6 %) samples respectively.

Functional properties of fermented, defatted and protein isolates of Parkia biglobosa seeds.

Presented in Table 4.2 are the functional properties of the fermented, defatted and protein isolates of *Parkia biglobosa* seeds. The protein isolate shows the highest oil absorption capacity (4.2 %), emulsion capacity (82 %), and the lowest foaming capacity (10 %), bulk density (0.36 %) foaming stability (12 %), and swelling index (1.77 %) when compared with the fermented (2.0 %, 45.0 %, 18.0 %, 0.48 %, 46.0 %, 3.25 %) and defatted (1.6 %, 54.0 %, 14 %, 33.0 %, 0.38 %, 3.69 %) samples respectively. The defatted sample shows the highest water absorption capacity (8 %) and wettability (26 %), compared with the protein isolate (3.0 %, 14 %) and fermented samples (5.6 %, 6.5 %) respectively.

Mineral Composition of fermented, protein and defatted isolates of Parkia biglobosa seeds

The mineral compositions (in mg/100 g) of the fermented, defatted and protein isolates of *Parkia biglobosa* seeds are presented in Table 4.3. The mineral content in the protein isolate were significantly higher in magnesium and zinc content compared to the fermented and defatted samples.

Anti-nutritional composition (g/100g) of the fermented, defatted and protein isolates of Parkia biglobosa seeds

The results of the anti-nutritional composition of the fermented defatted and protein isolates of *Parkia biglobosa* seeds are presented in Table 4.4. The results showed that the presence of phytate and oxalate more negligible.

4.4 Discussion

The nutritional and protective potential of *Parkia biglobosa* were assessed in this study through a comparative evaluation of the proximate, functional, mineral and

anti-nutrient composition of the fermented, defatted and protein isolates of this plant and as part of an exploration into their anti-diabetic mechanisms. Proximate composition is an important criterion in determining the nutritional value and quality of food (Qayyum *et al.,* 2012). The findings of this present study showed that the protein isolate contained higher protein content than the fermented and defatted samples. This is in agreement with similar reports from other studies (Olawuni *et al.,* 2012; Al-Numair and Ahmed, 2008). The high protein content of the protein isolate sample could be attributed to its production processes which increase its protein availability. Dietary proteins play an important role in the natural synthesis and maintenance of body tissues, enzymes and hormones as well as other substances required for healthy functioning (Hayat *et al.,* 2014).

Similarly, the protein isolate also contained higher ash content when compared to its fermented and defatted counterparts. Generally, the high ash content of the protein isolate is an indication that it contains abundant mineral content (Iqbal *et al.,* 2012), and this also totally agrees with the findings of Dwiani *et al.,* (2014). The moisture value of the protein isolate was lower when compared to the fermented and defatted samples. This finding is in accordance with the investigation reported by Olawuni *et al.,* (2012). The low moisture content of the protein isolate is desirable because high moisture content usually encourages the growth of bacteria and mould, which could reduce stability and shelf life.

Excess consumption of fat has been implicated in certain cardiovascular diseases such as stroke, atherosclerosis and heart attack, (Antia *et al.,* 2006). The fermented and defatted samples had higher fat content compared to the protein isolate. The low fat content of the protein isolate is therefore desirable because it could be consumed instead of the animal protein that contains high fat content (Adeyeye *et al.,* 2013). Apart from low fat content, the protein isolate also contained a low fiber content, which could be attributed to the processing method in which other nutrients had been removed. The poor fiber content of protein isolates has been reported previously (Ojukwu *et al.*, 2012). On the other hand, the protein isolate contains low carbohydrate content. This could also be attributed to the processing method in which the isoelectric precipitate removed soluble carbohydrate. This agrees with the report of Dwiani *et al*., (2014).

Functional properties are important in determining the appropriateness of nutrients, especially in growing children (Omueti *et al.,* 2009). Functional properties have also been reported to be improved through processing methods such as fermentation, spouting, cooking and soaking (Jirapa *et al.,* 2001; Yagoub and Abdalla, 2007). Findings from this study revealed that the protein isolate possessed better water and oil absorption capacity than the fermented and defatted samples. A similar result was also previously published in respect of the protein isolate from *Cajanus cajan* (Olawunmi *et al.,* 2012). The high water and oil absorption capacity suggested that the protein isolate comprised high polar amino acid content and high hydrophobic residue number on the protein molecules respectively. The protein isolate could therefore be used in some food formulations such as baked products, sausages, cheese, meat extender and soups (Olaofe *et al.,* 1998).

Foamability is essential in maintaining the constituency, texture and appearance of food that requires leavening and aeration properties (Onimawo and Asugo, 2004). The protein isolate possessed high foaming capacity and stability. This could be attributed to its high protein and low fat content. High protein content increases viscosity, which encourages the formation of cohesion protein layers. Fat is widely used as a foam inhibitor that destroys air-water surface and thereby making water insoluble (Zayas, 1997). Interestingly, the protein isolate had a high emulsion capacity when compared to the fermented and defatted samples. This implied that the protein isolate contained both polar and non polar amino acids which increase the interaction with both oil and water molecules. Protein can function as an emulsifier as it contains both hydrophobic and hydrophilic properties that can interact with oil and water in a food system (Mao and Hua, 2012).

Bulk density explains the packaging capacity of food material. The low bulk density of a protein isolate implied that an increased quantity of this isolates could be packaged in a constant volume, thereby ensuring economical packaging. Furthermore, low bulk density also promotes easy digestion of food materials and therefore the protein isolate would be easily digested (Osundahunsi and Aworh, 2002). The protein isolate exhibited a higher swelling index than the other samples. This is consistent with protein isolate from *Phaseolus lunatus*, which has previously been reported as having a high swelling index (Ojukwu *et al.,* 2012). Swelling index

is determined by the amount of water that may be absorbed and the degree of swelling within a stipulated time. Swelling index is influenced by temperature, water availability, carbohydrates and protein (Ezema, 1989). The high swelling index of the protein sample could be as a result of its high protein content. Equally, the high wettabilty of the defatted sample, compared to the fermented and protein isolate, could be attributed to the processing methods. This implies that the defatted sample gets wet before other two samples.

An analysis of the mineral content of *P. biglobosa* revealed that the protein isolate was a richer source of mineral elements when compared to the other samples. This is based on the values obtained in this study for copper, iron, manganese, magnesium and zinc. The appreciable copper content of the protein isolate implied that it is a good source of copper. Copper plays a fundamental role in cellular metabolism as it serves as a cofactor for the proper functioning of various important enzymes. Copper has also been identified as playing an essential role in the formation of haemoglobin, myelin and melanin. In addition, copper may act as an antioxidant or a pro-oxidant. As an antioxidant, it prevents free radical damage by scavenging or neutralizing excess free radicals (Osredkar and Sustar, 2011). For example, it is essential for the catalytic role of superoxide dismutase (SOD) in cellular defence against superoxide radicals (Khan and Awan, 2014). However, an abnormal copper metabolism has been implicated in the pathogenesis of several chronic diseases, such as diabetes and its associated complications. These pathogenic conditions appear to be common in copper deficiency as well as copper overload patiences, and therefore a tightly coordinated regulation is required (Zheng *et al*., 2008).

The low Na/K ratio of the protein isolate is an indication that it may reduce the incident of hypertension as it would not induce high blood pressure, which is the major cause of cardiovascular diseases (Du *et al.,* 2014). The high calcium content in the protein isolate also implies that it could prevent bone diseases such as rickets, osteroporosis and osteomalacia. Notably, calcium also enhances the effective usage of iron in the system (Adeyeye, 2013). Iron is another essential mineral present in the protein isolate. Athough its overload may lead to toxicity (Liu *et al*., 2009), iron is required for a number of biological functions including the proper functioning of the

immune system, electron transfer reactions, gene regulation, cell growth and differentiation as well as the binding and transport of oxygen (Siddiqui *et al.,* 2014).

Manganese, though in trace amounts, was present in the protein isolate. Manganese plays an important role as a cofactor in several antioxidant enzymes and in the metabolism of carbohydrates, proteins and fats. Added to this, it also plays an essential role in insulin synthesis and secretion. Alteration in the metabolism of manganese has been implicated in the pathogenic conditions associated with diabetes development (Khan and Awan, 2014). el-Yazigi and co-workers (1991) reported that manganese levels were lower in the white blood cells of diabetics as compared to the non-diabetic controls (el-Yazigi *et al.,* 1991).

Our results have revealed the presence of magnesium in the protein isolate. Magnesium is required for the action of over 300 enzymes in the body, where it participates in several significant physiological functions in the maintenance of good health and glucose homeostasis. Added to this, it has been identified as playing a significant role in the release of insulin and in the maintenance of pancreatic β-cells. Its deficiency has been implicated in insulin resistance and carbohydrate intolerance as well as in diabetes complications and dislipidaemia (Khan and Awan, 2014; Akhuemokhan *et al.,* 2013; Piero *et al.,* 2012).

Zinc was also appreciably detected in the protein isolate. Zinc plays a crucial role in antioxidant defence in type 2 diabetic patients where it enhances reduction and neutralization of free radicals, and acts as a cofactor of SOD, by modulating glutathione metabolism and metallothionein expression. Its deficiency has been implicated in a number of metabolic abnormalities such as impaired glucose tolerance, decreased pancreatic insulin content as well as insulin degradation (Cruz *et al.,* 2015; Piero *et al*., 2012).

The results of this study revealed the presence of low anti-nutrient values in the protein isolate when compared with other two samples. The processing methods may account for the result obtained in this case. This is in agreement with what was reported previously on the Africa locust bean (Ijarotimi and Keshinro, 2012). Anti-nutrients are natural compounds that interfere with the bioavailability of nutrients by reducing their absorption in the gastrointestinal tract. Phytate and oxalate are examples of anti-nutrients components that interfere with some mineral components

such as calcium, iron, zinc and magnesium through the formation of insoluble complexes (Ijarotimi and Keshinro, 2012).

4.5 Conclusion

In conclusion, this study has shown that the proximate, functional, mineral and anti-nutrient compositions of the protein isolate from *Parkia biglobosa* is a balanced and rich source of macro- and micronutrients. The most remarkable finding of this study is that the protein isolate possessed high protein content associated with low fat, carbohydrate and anti-nutrient content. Therefore, the protein isolate of *Parkia bilogbosa* can be considered or recommended as a good source of protein supplement which could be developed as a nutraceuticals for the prevention, treatment and management of chronic metabolic diseases such as diabetes and its associated complications.

4.6 Acknowledgements

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4.7 Conflict of Interest

There is no conflict of interest.

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Table 4.1. Proximate composition of the fermented, defatted and protein isolate of *Parkia biglobosa* seeds

Parameters	Fermented *Parkia biglobosa* (%)	Defatted *Parkia biglobosa* (%)	Protein isolate (%)
Moisture	7.3 ± 0.30[b]	10.2 ± 0.11[a]	5.1 ± 0.11[c]
Ash	4.3 ± 0.30[b]	2.9 ± 0.12[c]	6.0 ± 0.13[a]
Fibre	9.5 ± 0.32[a]	8.6 ± 0.14[b]	1.1 ± 0.15[c]
Protein	32.4 ± 0.21[c]	46.6 ± 0.15[b]	73.3 ± 0.17[a]
Carbohydrate	21.4 ± 0.12[b]	22.7 ± 0.21[a]	8.8 ± 0.13[b]
Fat	25.1 ± 0.11[a]	9.0 ± 0.32[b]	5.7 ± 0.21[c]
Energy (kJ)	470.5[a]	366.3[c]	381.7[b]

Data were expressed as mean ± SE. Means with different superscript along the row differs significantly ($p<0.05$).

Table 4.2. Functional properties of fermented, defatted and protein isolate of *Parkia biglobosa* seeds.

Parameters	Fermented Parkia biglobosa	Defatted Parkia biglobosa	Protein isolate
Water absorption capacity	3.0 ± 0.21[c]	5.6 ± 0.22[b]	8.0 ± 0.21[a]
Oil absorption capacity	1.6 ± 0.12[c]	2.0 ± 0.25[b]	4.2 ± 0.15[a]
Foaming capacity	10 ± 0.208[c]	14 ± 0.15[b]	18.0 ± 0.15[a]
Foaming stability	12.0± 0.153[c]	33.0±0.15[b]	46.0 ± 0.25[a]
Emulsion capacity	45.0 ± 0.252[c]	54.0±0.12[b]	82.5 ± 0.15[a]
Wettabilty	6.5 ± 0.21[c]	26.0±0.23[a]	14 ± 0.2[b]
Swelling index	3.25 ± 0.15[a]	3.69±0.15[a]	1.77 ± 0.153[b]
Bulk density	0.48 ± 0.01[a]	0.38 ± 0.1[b]	0.36 ± 0.01[b]

Data were expressed as mean ± SE. Means with different superscript along the row differs significantly ($p<0.05$).

Table 4.3. Mineral composition of fermented, protein isolate and defatted Parkia biglobosa seed

Parameter	Fermented Parka biglobosa(%)	Deffated Parkia biglobosa (%)	Protein isolate (%)
Calcium	1.97 ± 0.34 [c]	9.15 ±0.45[b]	10.70 ±0.07[a]
Copper	0.02 ±0.01 [b]	0.04 ±0.02 [a]	0.07 ±0.10 [a]
Iron	0.22 ±0.10 [b]	0.24 ±0.09 [a]	0.28 ± 0.12 [a]
Potassium	2.17 ±0.02 [b]	4.24 ±0.34 [a]	4.03 ± 0.11 [a]
Magnesium	2.30 ±0.11 [c]	4.54 ±0.66 [b]	4.98 ± 0.34 [a]
Manganese	0.05 ± 0.09 [c]	0.27 ±0.02 [b]	0.33 ±0.03 [a]
Sodium	49.78 ±0.01 [a]	19.46 ±0.02 [b]	19.49 ±0.34 [c]
Zinc	0.05 ±0.02 [c]	0.15 ±0.03 [a]	0.18 ±0.01 [a]
Na/K	25.26 ± 0.03 [a]	2.13 ±0.48 [b]	1.82 ±0.22 [c]
K/Na	0.04 ±0.10 [c]	0.47 ± 0.03 [b]	0.55 ±0.01 [a]
K/(Ca+ Mg)	0.86 ±0.02[a]	2.02 ±0.05 [a]	0.26 ±0.05 [c]

Data were expressed as mean ± SE. Means with different superscript along the row differs significantly (p<0.05).

Table 4.4. Anti-nutritional composition (g/100 g) of fermented, defatted and protein isolates of *Parkia biglobosa* seeds

Parameter	Fermented Parkia biglobosa	Defatted Parkia biglobosa	Protein isolate
Phytate	0.174±0.02[a]	0.36 ±0.03[b]	0.14 ±0.03[c]
Oxalate	0.189 ±0.01[a]	0.31± 0.04 [b]	0.02 ±0.01[c]

Data were expressed as mean ± SE. Means with different superscript along the row differs significantly (p<0.05).

Chapter Five

5.0 Comparative studies of amino and fatty acid composition of the fermented, defatted and protein isolates of *Parkia biglobosa* (locust beans) seeds.

Running title: Amino acids and acid composition of *Parkia biglobosa* seeds.

Abstract

The present study was designed to compare the amino and fatty acid composition of the fermented (FPB), defatted (DPB) and protein isolates (PI) of *Parkia biglobosa* seeds using standard methods. The PI showed the highest total non-essential amino acids, essential amino acids and conditional essential amino acids when compared to FPB and DPB. In addition, the PI showed higher total neutral amino acids, basic amino acids, total aromatic amino acid contents and a lower percentage cystine ratio of total sulphur amino acids and total amino acids in comparison to FPB and DPB. The PI also showed a better Predicted Protein Efficient Ratio, Essential Amino Acid Index, Biological Value, protein content and nutritional index than the FPB and DPB. Furthermore, the PI amino acid composition score compared favorably with the reference score for whole hen's egg, preschool child and provisional scoring pattern. The PI value for the arginine and histidine samples was higher than the recommendation for a pre-school child. The FPB (42.29 %) showed the highest polyunsaturated fatty acid levels when compared to the DPB (41.94 %) and PI (26.7 %). The dominant saturated, monounsaturated and polyunsaturated fatty acids were stearic acid (15.1 DPB), linoleic acid (42.29 DPB) and oleic acid (14.05 PI). The DPB (1.84) showed a better polyunsaturated to saturated (P: S) ratio than the FPB (1.74) and PI (0.91). The results also revealed that the PI processing method improved the relative amino acid composition whereas the fatty acid composition was improved in DPB. Overall, the samples possessed good nutritional quality and could serve as alternatives to the challenging animal foods in the formulation of complementary foods.

Keywords: amino acids, fatty acids, *Parkia biglobosa*, protein isolate, fermented, defatted

5.1 Introduction

The main dietary proteins are either from animal or plant origin. Animal proteins are known to possess the full spectrum of essential amino acids whereas proteins of plant origin are deficient in one or more essential amino acids. Despite animals' protein providing a complete protein and numerous vitamins and minerals, there are still health concerns about its consumption due to the presence of saturated fatty acid composition (1). There is therefore an urgent need to search for a highly nutritive source of protein that is of plant origin with low saturated fatty acid content. Proteins are a source of essential amino acids, which play a major role in tissue growth and the production of hormones, hemoglobin, enzymes and energy (2). A deficiency in any of the essential amino acids could lead to disease. Fatty acids are good source of energy (3). While polyunsaturated fatty acids such as linolenic, linoleic and arachidonic acids can improve the normal physiology of the body system, saturated fatty acids have been implicated in cancer and some other metabolic diseases such as cardiovascular diseases and diabetes mellitus (4).

Legumes are a good, cheap source of plant protein in place of animal protein in the developing world (5). It has also been reported that about 18 – 35 % of legumes is protein, which acts as a good compliment to cereals in supplementing the needed minerals and B vitamin complex (6). The nutritive value and functional properties of some legumes, such as soyabean and cowpea seed, have been evaluated (7). *Parkia biglobosa* (Jacq.), commonly known as African locust bean is widely distributed within the savannah belt of West Africa (8). As a legume it has both nutritional and medicinal uses. Its fermented seeds are widely used as a spice for meals (9, 10). Currently, there is little or no literature on the amino acid and fatty acid composition of *P. biglobosa*. The present study aims to investigate the amino acid and fatty acid compositions of the fermented, defatted and protein isolates from *P. biglobosa.*

5.2 Materials and methods

5.2.1 Plant material

The fermented seeds of *Parkia biglobosa* (Jacq.) (African locust beans) were purchased in a local market in Ijebu-Ode, Ogun state, Nigeria. The seeds (voucher IB3) were authenticated at the Department of Botany, University of Zululand and deposited at the University's Herbarium.

5.2.2 Method for protein extraction

The fermented seeds were thoroughly rinsed and oven-dried at 50 ^{o}C. The dried seeds were milled into a fine powder and stored in a dry place prior to use. A portion of fine powder was defatted with hexane to produce the defatted *Parkia biglobosa* samples. Portions of the residue from defatted samples were air dried and treated with acidic butanol (1:10 w/v) to remove anti-nutrients. Protein was then isolated from the treated sample using the method described by Nkosi and colleagues (11). The treated sample was re-suspended in distilled water at pH 10. The resultant suspension was filtered and the filtrate adjusted to pH 5. The filtrate was then centrifuged (5000 rpm) for 15 minutes at 4 ^{o}C. The supernatant was discarded and pellets containing the protein isolate were lyophilized and kept dry until needed.

5.2.3 Amino acid analysis

The amino acid composition of the samples was determined using the ion-exchange chromatography method (12). A Technicon Sequential Multi-sample Amino Acid Analyzer (TSM) was usedin the study. The reported amino acid values are the mean of the two determinations, expressed as a percentage of total protein. Tryptophan was not determined.

5.2.4 Determination of the quality parameters

Dietary protein quality was determined using the essential amino acid scoring pattern (13) method. The amino acid scores for essential and non-essential amino acids were determined based on a whole hen's egg amino acid profile (14). The essential amino acid score was also based on the suggested pre-school child requirement (15). The essential amino acid score was also determined on the basis of a provisional amino acid scoring pattern with the following formula:

Amino acid score (AAS) = amount of amino acid per test protein in [mg/g] / amount of amino acid per protein in reference protein [mg/g].

5.2.5 Determination of the predicted protein efficiency ratio (P-PER)

The predicted protein efficiency ratio (P-PER) of the samples was determined from their amino acid compositions using the equation of Alsmeyer *et al.* (16).

$P\text{-}PER_1 = -0.684 + 0.456\ (Leu) - 0.047\ (Pro)$

$P\text{-}PER_2 = -0.468 + 0.454\ (Leu) - 0.105\ (Tyr)$

5.2.6 Biological values

Biological values of the samples were estimated using the suggested equation of Oser (17).

$BV = 49.09 + 10.53\ (PER_2)$

BV = 1.09 x essential amino acid index (EAAI) -11.7

5.2.7 Essential amino acid index (EAAI)

The essential amino acid index was estimated (EAAI) with the equation from Labuda *et al.,* (18):

EAAI = where: [lysine, tryptophan, isoleucine, valine, threonine, leucine, phenylalanine, histidine and methionine] a in test sample and [lysine, tryptophan, isoleucine, valine, threonine, leucine, phenylalanine, histidine and the sum of methionine and cystine] b content of the same amino acids in standard protein (%) (egg/ or casein), respectively.

The nutritional index of the sample was also determined using Crisan & Sands (19) method with the following formula:

Nutritional index [%] = EAAI x % protein / 100

5.2.8 Determination of other protein quality parameters

The ratio of total essential amino acids (TEAA) to the total amino acids (TAA), i.e. (TEAA/TAA), total sulphur amino acid (TSAA), percentage cystine in TSAA (% Cys/TSAA), total aromatic amino acid (TArAA), total basic amino acid (TBAA), total acidic amino acid (TAAA), total neutral amino acid (TNAA) and the Leu/Ile ratios were calculated.

5.2.9 Fatty acid determination

Determination fatty acid of the samples and their methyl ether were estimated by Gas Chromatography, adopting the modified standard method of AOAC 965.49. The separation of methyl ether was conducted using the HP 6890 Gas Chromatography analyzer powered by HP Chem Station Rev A 09.11(1206).

5.3 Results

The amino acid composition of fermented (FPB), defatted (DPB) and protein isolates (PI) of *Parkia biglobosa* is presented in Table 5.1. The protein isolate (35.37 mg/100g) sample showed the highest total non-essential amino acids when compared with the defatted (34.49 mg/100g) and fermented (23.62 mg/100g) samples. Glutamic acid has the highest value of the non-essential amino acids (17.3 mg/100g PI), whereas serine has the lowest value (2.8 mg/100g FPB). The protein isolate (19.59 mg/100g) also showed better total conditionally essential amino acid composition in comparison to the DPB (19.41 mg/100g) and FPB (14.74 mg/100g) sample. Tyrosine (6.02 mg/100g DPB) showed the highest value in total conditionally essential amino acids, while cystine (0.19 mg/100g FPB) showed the lowest value. The protein isolate (40.89 mg/100g) sample exhibited the higher total essential amino acid composition in comparision with DPB (34.93 mg/100g) and FPB (28.36 mg/100g) samples. In comparing lysine and methionine, lysine (8.44 mg/100g PI)

dominated the essential amino acid composition, whereas the concentration of methionine (0.50 mg/100g FPB) was lower.

The nutritional quality of the fermented, defatted and protein isolates of *Parkia biglobosa* seeds is presented in Table 5.2. The PI (94.79 %) sample has the highest total amino acids when compared with the DPB (89.89 %) and FPB (66.72 %) samples. The DPB (56.21 %) sample has the highest total percentage of non-essential amino acids (% TNEAA) when compared with PI (52.29 %) and FPB (44.77 %) samples. The total essential amino acid percentage (% TEAA) values with histidine were 47.74 %, 47.71 %, 43.79 % for FPB, PI, DPB respectively, whereas the values for total essential amino acid percentage (% TEAA) with no histidine were 44.76 %, 43.80 %, 41.27 % respectively. In comparison, PI (51.21 %) has the highest total neutral amino acid (TNAA), compared with DPB (50.77 %) and FPB (37.99 %) samples. The DPB (26.68 %) sample has better total essential amino acid (TAAA) than PI (26.46 %) and FPB (17.90 %) samples. The basic amino acid values (TBAA) were 13.84, 12.44, and 10.83 for the PI, DPB and FPB samples respectively. The DPB (1.33 %) sample for total sulphur amino acid (TSAA) was higher in comparison with the PI (0.94 %) and FPB (0.69 %) samples. The DPB (37.59 %) sample has the highest percentage cystine ratio of total sulphur amino acid (% Cys/ TSAA) when compared with FPB (27.54 %) and PI (26.60 %) samples. The highest total aromatic amino acid (TArAA) was recorded in PI (12.18 %) when compared with DPB (10.32 %) and FPB (9.27 %). The predicted protein efficient ratio (P-PER_1 and P-PER_2) of the samples was 1.67 %, 2.23 %, 2.58 % to 1.79 %, 2.40 %, and 2.73 % for FPB, DPB and PI respectively. The values for the Leu/Ile ratio were 1.94 %, 1.86 %, 1.63 % for FPB, PI to DPB respectively. The PI (3.74 %) sample for the Leu/Ile ratio (difference) was highest when compared with FPB (2.78 %) and DPB (2.75 %). The PI (82.15 %) sample for the essential amino acid index (EAAI) has a higher value in comparison to the DPB (78.95 %) and FPB (73.06 %) samples. The PI (77.84 %) sample has the higher Biological Value (BV) when compared with the BV of DPB (74.46 %) and FPB (67.94 %) samples. The nutritional index of the PI (48.80 %) sample has the higher value when compared with DPB (36.79 %) and FPB (28.06 %) samples. The PI (59.4 %) sample has a higher protein content when compared with DPB (46.6 %) and FPB (38.4 %) samples.

The amino acid scoring pattern of the samples based on a whole hen's egg is presented in Table 5.3. The PI sample has the highest number of amino acid (Tyr > His > Lys > Glu > Gly > Phe > Pro) scores greater than one. The DPB sample also showed amino acid (Tyr > Gly > Glu > Pro > Phe) scores greater than one, whereas FPB has two amino acid scores greater than one (Tyr > Gly). Methionine is the limiting amino acid for all the samples. The co-effient variation (CV %) ranged between 6.0 and 50.0. Cystine has the highest CV % value.

The essential amino acid scores of the samples based on the preschool child requirement are presented in Table 5.4. The following amino acid (Val > Pe + Tyr > His > Ile > Thr) values of the PI sample for EAAS were greater than those with Met + Cys (0.53) as the limiting amino acids (LAA). Similarly, the DPB amino acid (His > Phe + Tyr > Val > Ile > Lys > Leu) values were greater than one with Met + Cys (0.38) as the LAA. FPB amino acids (Val > His > Lys > Phe + Tys > Ile > Leu) are also greater than 1 with Met + Cys (0.28) as the LAA. Histidine (34.5) was recorded as having the highest CV %, while Phe + Tyr have the lowest percentage.

The essential amino acid scores (EAAS) of the samples based on the provisional scoring pattern are presented in Tables 5.5. The amino acid (Phe + Tyr > Val > Ile > Lys > Leu) values of the PI sample for EASS are greater than one, with Met + Cys (0.38) as the LAA. The DPB amino acid values (Phe + Tyr > Lys > Val > Leu > Ile) are greater than one while Met + Cys (0.38) served as the LAA. Phe + Tyr were the only amino acids with values greater than one for FPB and Met + Cys (0.20) served as its LAA. A high CV % of 32.6 was recorded for Ile amino acid.

The fatty acid compositions of each sample are presented in Table 6. The results revealed that linoleic acid dominated the fatty acid composition in FPB (42.29 %), DPB (41.94 %) and PI (26.7 %), while lauric acid showed the least value. Stearic acid dominated the saturated fatty acid (SFA) composition in FPB (15.1 %), DFB (14.41 %) and PI (14.78 %), while myristic acid showed to be the least amino acid in all the samples. FPB (43.1 %) showed to be the highest total poly unsaturated acid (PUFA) when compared to DPB (42.68 %) and PI (27.13 %). Linoleic acid dominated the polyunsaturated fatty acid composition in the samples. Oleic acid showed to be having the highest concentration in monounsaturated fatty acids, with PI (14.05 %),

FPB (13.67 %) and DPB (13.41 %). FPB (1.84) showed to be having the highest polyunsaturated/saturated (p:s) ratio, while PI (0.91) showed to be having the lowest.

5.4 Discussion

The nutritional quality of plant proteins is based on their amino acid and fatty acid profile (20). In this study, the protein isolate from *Parkia biglobosa* exhibited a wider spectrum of amino acid contents when compared to the fermented and defatted samples. The processing of food products has been demonstrated to improve their functional properties and nutritive values (21). Our findings revealed that glutamic acid and aspartic acid are the most abundant amino acids in the PI sample. This finding was in accordance with the report of Audu *et al.,* (23) on black turtle beans, legumes containing a high level of glutamic and aspartic acids. Similarily, protein isolate from red kidney beans have also been reported to contain a high level of glutamic and aspartic acids (24-25). Glutamic acid plays key role in cellular metabolism and brain function (26). Glutamic acid is also a precursor of gamma aminobutyric acid (GABA), which is found in abundance in the pancreas and cerellebrum. During neurological syndromes, such as stiff-man syndrome, there is a decrease in the synthesis of GABA, triggering an autoimmune destruction of the pancreas that results in diabetes mellitus in the patient (27). The detoxification and cardio protective potential of glutamic acid has also been reported (28).

Aspartic acid also plays a major role in brain function, gluconeogenesis and the stimulation of growth hormones (29). The PI sample could therefore be useful in ameliorating mental retardation, cardiovascular diseases and other diabetic complications.

Lysine improves body mass and lower anxiety, glucose and osteoporosis (30). Protein isolates from grains such as corn and wheat are reported to be lower in lysine. The Lysine value for the PI sample was observed to be within the range of the egg reference protein (6.3 g/100g) (31). The value (3.31 – 3.69 g/ 100g) of lysine in the PI sample was better when compared to the value (5.36 – 8.44 mg/g) previously reported in respect of the *Moringa oleifera* tree (32). This implies that the samples could serve as a good source of cereal fortification in weaning foods. Leucine has been reported to enhance protein synthesis, which plays a vital role in muscle

building (33). The value of leucine in the PI sample was observed to favorably compare with other legumes such as pigeon peas (8.40 g/100g), Africa yam beans (7.45 g/100g) and lima beans (34).

Histidine plays an important role in child growth, while arginine has been reported to increase insulin sensitivity and vasodilatation of coronary arteries (35, 36). Arginine and histidine values in the PI sample were observed to be higher than those recommended for pre-school children (2 – 5 years old) and adults (31). Therefore, the sample could serve as a remedy for metabolic complications in child growth. Cystine values were observed to be the least concentrated amino acids in all the samples. This supports the previous report that cystine is the limiting amino acid in legumes (37).

The nutritive value of the protein source depends on its essential amino acid composition and ability to meet nitrogen demand (38). The values of %TEAA with histidine for PI sample were similar to some non-convectional animal sources, such as *Archatina archatina*, *Archachatina marginata* and *Phaseolus vulgaris* L. (38, 39). The PI %TEAA value was within the recommended standard for infants (26%) and adults (11%) (15). High content of essential amino acids in the PI sample implies that they could be used in the complimentary treatment of protein malnutrition.

Aromatic amino acids are the precursors of thyroxine and epinephrine which are important in many biological functions (36). The TArAA values of the PI sample were within the recommended ranges (6.8 -11.8 g) for infant protein (15). The PI value for TArAA was also higher than the value (3.9 – 5.3 g/ 100 g) previously reported by Audu *et al.,* (40) on *Phaseolus vulgaris L* but similar to the values (9.9 – 21.5) reported by Aremu et al (25). The PI samples were basic in nature because they had lower total amino acids (TAAA) and total sulphuric acids, but with higher basic amino acids. Most plant protein has a higher level of cystine /TSAA ratio whereas in animal protein the ratio is lowers (41). The cystine /TSAA ratio value (37.59 g/ 100 g) for the PI sample was observed to fall within the animal protein range. This finding was contrary however to the previous report of Adeyeye (42) on fermented parkia *biglobosa* seeds (44.4 g/ 100 g).

The predicted protein efficient ratio (PER) is used in protein evaluation by measuring the effective quality of protein through growth (12). The PER value for the PI sample

(Table 2) was observed to be higher than for some reported legumes, such as cowpea (1.21) and millet (1.62) but similar to pigeon pea (1.82) and casein (2.7) (43). This could imply that the sample is a good source of protein.

The efficiency of protein utilization in the body is determined by biological values (BV). Animal protein BV values are usually higher than those from plant sources due to the presence of limiting amino acids (43). Our finding showed that BV values for the PI sample (Table 5.2) compared favorably well with some BV reference standards such as: wheat gluten (64), soy protein (74) and casein (77) (43).

The essential amino acid index (EAAI) is a useful tool in ascertaining food formulations for protein quality (44). In spite of that, it does not account for the difference in protein due to the various processing methods. The EAAI values for the PI sample (Table 5.2) compared favorably well with some standard references like soybeans, (72.8), beef (74.3), fish (76.0) and milk (84.5) (45, 46).

The Leu/Ile ratio of the samples (Table 5.2) was observed to be lower in comparison with the recommended value (2.36) (12). The high level of leucine in the diet impaired niacin and tryptophan absorption in the gastrointestinal tract, which lead to pellagra (36). The PI sample in this study could serve as an added advantage to amino acid balance in complementing cereals that are higher in leucine but low in ileucine and trytophan.

The amino acid scores (AAS) for the samples compared favorably with whole hen's eggs profiles, preschool child requirements and provisional scoring patterns (15). Met + Cys (TSAA) were the limiting amino acids (LAA) for all the samples, and this was in accordance with the previous reports (22, 25). This implies that Met + Cys (TSAA) could be supplemented with the studied samples to meet standard daily requirements.

The essential neutral fatty acids, stearic acid and oleic acid, dominated the saturated fatty acids and monounsaturated fatty acids of the samples respectively. Oleic acid has previously been reported to be in the monounsaturated fatty acids dominant in some plant foods (48). Oleic acids are also found predominately in olive, macadamia nut, peanut and canola oils (49). Essential neutral fatty acids have been demonstrated to have no effect on cholesterol. Abnormal cholesterol syntheses have been associated with cardiovascular diseases (50). The polyunsaturated fatty acid

showed the highest values when compared with saturated and monounsaturated fatty acids in the fatty acid composition. Plant food, such as avocados were reported to be rich in polyunsaturated fatty acids (48).

Linoleic acid is dominant in polyunsaturated fatty acids, and this was validated previously in a report of Ijarotimi and Keshinro, (48). Adequate intake of essential fatty acids, such as linoleic acid, could prevent cardiovascular diseases and diabetes (32). Due to the high rate of potential health hazards attributed to the consumption of saturated fatty acids, emphasis is now being laid on the consumption of foods which have a high polyunsaturated to saturated fatty acid ratios (48).

5.5 Conclusion

This present study revealed that the PI sample possesses good nutritional value using the selected nutritional parameters. It could thus serve as a complementary food in the management of metabolic diseases in both infants and adults where animal protein is expensive. For future studies, the hypoglycaemic and hypolipidemic potentials of this studied sample need to be ascertained.

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Table 5.1: Amino acid compositions (mg/100g protein) of fermented, defatted and protein isolates of *Parkia biglobosa* seeds

Amino acids in mg/100g	Fermented *Parkia biglobosa*	Defatted *Parkia biglobosa*	Protein isolate	Mean	SD	CV%
Non-essential amino acids						
Alanine	2.94	4.32	4.81	4.02	0.97	24.13
Aspartic acid	6.90	9.41	10.40	8.90	1.80	20.22
Serine	2.78	3.71	3.88	3.46	0.59	17.05
Glutamic acid	11.0	16.06	17.27	14.78	3.33	22.53
Total	23.62	34.49	35.37	31.16	6.54	20.98
Conditionally essential amino acids						
Proline	3.22	4.84	4.86	4.17	0.84	20.14
Glycine	3.38	3.69	4.81	3.96	0.75	18.94
Arginine	3.49	4.43	4.59	4.17	0.59	14.15
Cystine	0.19	0.25	0.50	0.31	0.16	51.61
Tyrosine	4.46	5.01	6.02	5.16	0.79	15.31
Total	14.74	19.41	19.59	31.16	6.54	20.98
Essential amino acids						
Lysine	5.36	5.75	8.44	6.52	1.68	25.77
Threonine	2.31	2.86	3.44	2.87	0.57	19.86
Valine	4.68	5.89	6.57	5.71	0.96	16.81
Methionine	0.50	0.69	0.83	0.67	0.17	25.37
Isoleucine	2.97	4.35	4.37	3.90	0.80	20.51
Leucine	5.75	7.10	8.11	6.99	1.18	16.88
Phenylalanine	4.81	5.31	6.16	5.43	0.68	12.52
Histidine	1.98	2.26	3.69	2.64	0.92	34.85
Trptophan	ND	ND	ND	ND	ND	ND
Total	28.36	34.93	40.89	34.73	6.27	18.05

Table 5.2: Concentration of Essential, Non-essential, Acidic, Neutral, Sulphur, Aromatic acid (mg/100mg) Protein efficient ratio (PER), Biological value (BV), Iso-electric point (Pi), Leu/Ile ratio Leu/Ile difference of fermented, defatted and protein isolates *Parkia biglobosa* seeds

Amino acids	Fermented parkia biglobosa	Defatted parkia Biglobosa	Protein isolate	Mean	SD	CV %
Total amino acid (TAA)	66.72	89.89	94.79	83.64	14.82	17.7
Total non-essential amino acid (TNEAA)	34.87	50.53	49.31	44.90	8.71	19.4
Total essential amino acid (TEAA) with His	31.85	39.36	45.21	38.74	6.60	17.0
Total essential amino acid (TEAA) with no His	29.87	37.10	41.31	36.09	5.77	15.9
% TNEAA	44.77	56.21	52.29	51.09	5.81	11.4
% TEAA with His	47.74	43.79	47.71	46.41	2.27	4.9
% TEAA with no His	44.76	41.27	43.80	43.28	1.80	4.2
Total neutral amino acid (TNAA)	37.99	50.77	51.21	46.66	7.51	16.1
% TNAA	53.94	54.30	56.48	55.91	1.41	2.52
Total acidic amino acid (TAAA)	17.90	26.68	20.46	24.21	5.51	22.8
% TAAA	26.82	29.68	28.06	28.19	1.43	5.1
Total basic amino acid (TBAA)	10.83	12.44	13.84	12.37	1.51	12.2
% TBAA	14.68	13.84	16.23	14.92	1.21	8.11
Total sulphur amino acid (TSAA)	0.94	1.33	0.69	0.99	0.32	32.3
% TSAA	1.03	1.48	1.00	1.17	0.27	15.8
% Cys/ TSAA	27.54	37.59	26.60	30.58	6.09	19.9
Total aromatic amino acid (TArAA)	9.27	10.32	12.18	10.59	1.47	13.9
% TArAA	13.89	11.48	12.91	12.76	1.21	9.48
P-PER1	1.67	2.23	2.58	2.16	0.46	21.30
Leu/Ile ratio	1.94	1.63	1.86	1.81	0.16	8.84
Leu/Ile ratio (difference)	2.78	2.75	3.74	3.09	0.56	14.36
% Leu/Ile (difference)						
EAAI	73.06	78.95	82.15	78.05	4.61	5.91
BV	67.94	74.46	77.84	73.38	5.02	6.84
P-PER2	1.79	2.40	2.73	2.41	0.48	20.80
NI	28.06	36.79	48.80	38.88	10.41	26.83
Protein	38.4	46.6	59.4	48.13	10.58	21.98

Table 5.3: Amino acid scores with respect to whole hen's eggs (amino acids value were in mg/100g)

Amino acids	Hen eggs	FPB	DPB	PI	FPB	DPB	PI	Mean	SD	CV%
His	2.40	1.98	2.26	3.69	0.83	0.94	1.54	1.10	0.38	34.6
Ser	7.90	2.78	3.88	3.71	0.35	0.49	0.49	0.44	0.08	18.2
Arg	6.10	3.49	4.43	4.59	0.57	0.73	0.75	0.68	0.09	13.2
Gly	3.00	3.38	4.81	3.69	1.26	1.60	1.23	1.36	0.21	15.4
Asp	10.7	6.90	9.41	10.40	0.23	0.64	0.88	0.97	0.83	20.5
Glu	12.0	11.0	17.27	16.06	0.92	1.44	1.33	1.23	0.27	22.0
Thre	5.10	2.31	3.44	2.86	0.45	0.67	0.56	0.56	0.11	19.6
Ala	5.40	2.94	4.81	4.32	0.54	0.89	0.80	0.74	0.18	24.3
Pro	3.80	3.22	4.84	4.46	0.85	1.27	1.17	1.10	0.22	20.0
Cys	1.80	0.19	0.50	0.25	0.11	0.28	0.14	0.18	0.09	50.0
Lys	6.20	5.36	5.75	8.44	0.86	0.93	1.36	1.05	0.27	25.7
Met	3.20	0.50	0.83	0.69	0.07	0.11	0.09	0.09	0.02	22.2
Val	7.50	4.68	5.89	6.57	0.62	0.78	0.88	0.83	0.05	6.0
Ile	5.60	2.97	4.35	4.37	0.53	0.78	0.78	0.70	0.14	20.0
Leu	8.30	5.75	7.10	8.11	0.69	0.86	0.98	0.84	0.15	17.9
Phe	5.10	4.81	5.31	6.16	0.94	1.04	1.20	1.06	0.13	12.3
Tyr	4.00	4.46	5.01	6.02	1.39	1.61	1.88	1.61	0.25	15.5

FPB= fermented parkia biglobosa, PI=protein isolate, DPB= defatted parkia biglobosa

Table 5.4: Amino acids scores with respect to pre-school children requirements (amino acids value were in mg/100g)

Amino acids	Pre-school	Fermented parkia biglobosa	Defatted Parkia biglobosa	Protein isolate	Mean	SD	CV%
Leu	6.60	0.87	1.23	1.08	1.06	0.18	17.0
Ile	2.80	1.06	1.56	1.55	1.39	0.28	20.1
Met+ Cys	2.50	0.28	0.38	0.53	0.40	0.13	32.5
Phe+ Tyr	6.30	1.47	1.93	1.64	1.68	0.23	13.7
Thr	3.40	0.68	0.84	1.01	0.17	0.16	94.1
Val	3.50	1.34	1.88	1.68	1.63	0.27	16.7
His	1.90	1.04	1.94	1.19	1.39	0.48	34.5
Trp	1.10	ND	ND	ND	ND	ND	ND
Lys	5.80	0.92	1.46	0.99	1.12	0.29	25.9

Table 5.5: Amino acids scores with respect to provisional amino acid scoring pattern of FAO (amino acids values were in mg/100g)

Amino acids	Scoring value	Fermented parkia biglobosa	Defatted parkia biglobosa	Protein isolate	Mean	SD	CV%
Ile	4.00	0.57	1.09	1.09	0.92	0.30	32.6
Leu	7.00	0.82	1.01	1.16	0.99	0.17	17.2
Lys	5.50	0.97	1.05	1.53	1.18	0.30	25.4
Met + Cys (TSAA)	3.50	0.20	0.27	0.38	0.28	0.09	32.1
Phe + Tyr	6.00	1.55	1.72	2.03	1.77	0.24	13.6
Thr	4.00	0.58	0.86	0.72	0.77	0.14	18.2
Val	5.00	0.94	1.18	1.31	1.14	0.19	16.7
Trp	1.00	ND	ND	ND	ND	ND	ND

Table 5.6: Fatty acids composition

Fatty Acids		Fermented Parkia biglobosa	Deffated Parkia biglobosa	Protein isolate	Mean	SD	CV%
Lauric acid	$C_{12.0}$	0.01	0.00	0.11	0.04	0.068	170
Mystic acids	$C_{14.0}$	0.02	0.02	0.09	0.043	0.040	93
Palmitic acid	C_{16}	9.5	8.76	14.64	10.97	3.202	29.2
Palmitoleic acid	$C_{16.1}$	0.05	0.03	0.28	0.12	0.139	115.8
Stearic acid	$C_{18.0}$	15.1	14.41	14.78	14.76	0.345	2.3
Oleic acid	$C_{18.1}$	13.67	13.41	14.05	13.71	0.322	2.3
Linoleic acid	$C_{18.2}$	42.29	41.94	26.7	36.98	8.902	24
Linolenic acid	$C_{18.3}$	0.78	0.71	0.18	0.56	0.328	58.6
Arachidic acid	$C_{20.0}$	11.33	12.05	9.37	10.92	1.387	12.7
Behenic acid	$C_{22.0}$	0.08	0.03	0.27	0.13	0.127	97.7
Lignocenic acid	$C_{24.0}$	0.05	0.04	0.39	0.16	0.199	124.4
Saturated Fatty Acid (SFA)							
Myristic acid	$C_{14.0}$	0.02	0.02	0.09	0.043	0.040	93
Palmitic acid	$C_{16.0}$	9.5	8.76	14.64	10.97	3.202	29.2
Stearic acid	$C_{18.0}$	15.1	14.41	14.78	14.76	0.345	2.3
Behenic acid	$C_{22.0}$	0.08	0.03	0.27	0.13	0.127	97.7
Total		24.7	23.22	29.78	25.9	3.44	13.3
Polyunsaturated Fatty Acid (PUFA)							
Linoleic acid	$C_{18.2}$	42.29	41.94	26.7	36.98	8.902	24.1
Linolenic acid	$C_{18.3}$	0.78	0.71	0.18	0.56	0.328	58.6
Arachidonic acid	$C_{20.4}$	0.03	0.03	0.25	0.10	0.13	1.3
Docohexanoic acid	$C_{22.6}$						
Total		43.1	42.68	27.13	36.64	9.10	24.8
Monosaturated Fatty Acid (MUFA)							
Palmitoleic acid	$C_{16.1}$	0.05	0.03	0.28	0.12	0.139	115.8
Oleic acid	$C_{18.1}$	13.67	13.41	14.05	13.71	0.322	2.3
Total		13.72	13.44	14.33	13.83	0.46	3.3
P:S		1.74	1.84	0.91			

Chapter Six

6.0 Hypoglycemic, hypolipidemic and cardioprotective potentials of protein isolate from *Parkia biglobosa* in streptozotocin induced diabetic rats

Running title: Protective potential of *Parkia biglobosa* protein isolate in STZ-induced diabetes.

Abstract

This study was designed to investigate the hypoglycaemia, hypolipidemia and cardioprotective properties of a protein isolate (PI) from *Parkia biglobosa* in streptozotocin-induced diabetic rats. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg, i.p.). Rats having a fasting blood glucose (FBG) of >250 mg/dL 3 days after diabetes-induction were then selected for the study and treated with the PI (200 or 400 mg/kg bw) or insulin (5 U/kg, i.p.) for 28 days with the fasting blood glucose (FBG) determined on weekly basis. Serum lipoprotein (triglyceride, cholesterol, low density lipoprotein (LDL-c), very low density lipoprotein (VLDL-c) and high density lipoprotein (HDL-c)) profiles were then monitored while the extent of lipid perioxdation as well as the antioxidant parameters (superoxide dismustase (SOD), catalase (CAT), glutathione-S-transferases (GST), and total glutathione (total GSH)) were determined in the heart homogenate of the diabetic rats. From our finding the PI showed a significant hypoglycaemic activity that was comparable with insulin. The PI at the tested dosage lowered the FBG, the serum triglyceride, the cholesterol, the LDL-c and the VLDL-c while concurrently increasing HDL-c. PI also reversed the elevations witnessed in LPO levels and further restored the enzymatic and non-enzymatic antioxidant status in the hearts of diabetic rats. Our results thus demonstrated that the *Parkia biglobosa* protein isolate (PI) does display the hypoglycaemic, hypolipidemic and the cardioprotective effects in the experimental diabetic models. It can therefore be concluded that the PI could serve as a potential cardio-protective supplement in the management of diabetes mellitus.

Keywords: Cardioprotective, diabetic rats, hypoglycaemia, hypolipidemia, *Parkia biglobosa*.

6.1 Introduction

Diabetes mellitus is a metabolic disorder characterized by a defect in insulin action, secretion or both, resulting in a disturbance of the carbohydrate, lipid and protein metabolism (Upasani *et al*., 2013). Chronic hyperglycaemia is the landmark of diabetes mellitus which is associated with long term complications, such as nephropathy, neuropathy and retinopathy (Hanssen *et al.,* 1992). Diabetic mellitus patients are more prone to the risk of cardiovascular diseases the than non-diabetic patients. About 80 % of mortality in diabetics is attributed to cardiovascular diseases (Sharma and Pan, 2005). In diabetes mellitus, there is an alteration in the serum lipoprotein profile, such as an increase in low density lipoprotein (LDL), triglycerides, cholesterol, very dense low lipoprotein level (VLDL) and a decrease in high density lipoprotein (HDL) that increases the risk of arteriosclerosis, the onset of coronary heart disease (CHD) (Saravanan and Pan, 2005). Hyperglycaemia in diabetes mellitus also generates free radicals due to glucose oxidation, the glycation of protein and the degradation of glycated protein. These factors lead to a declined in antioxidant activity, in turn leading to tissue damage, lipid perioxidation and insulin resistance (Arora, 2010; Kangralkar *et al.,* 2010).

Despite the improved knowledge in drug formulation, the prevalence of diabetes mellitus and its complications is still increasing unabated (Odetola *et al.,* 2006). Most of the current diabetic drugs such as insulin, metformin, glucosidase inhibitors and sulfonylureas have undesirable side effects and limited efficacy (Yin *et al.,* 2011). There is therefore a need to exploit medicinal plants which are less toxic than synthetic drugs in this regards the uses of hypoglycaemic agents of plant origin are currently receiving continous attention (WHO, 1980). Some anti-diabetic properties of medical plants such as ginger (*Zingiber officinale*) (Al-Amin *et al.,* 2006), pippali (*Piper. nigrum and P. longum*) (Loew and Kaszkin, 2002), turmeric (*Curcuma longa*) (Kim et al., 2006) and onion (*Allium cepa*) (Chu-Won *et al*., 2009) have been previously reported.

Parkia biglobosa, commonly known as the “African locust bean”, belongs to the family of Mimosaceace. It is widely distributed in the Guinea and Sudan savannahs (Hopkins, 1983). The fermented seed of P. biglobosa is used as a traditional seasoning for food in Nigeria and other countries on the west coast of Africa (Ajaiye-

Oba, 2002). The traditional use of *P. biglobosa* as food and medicine is gaining popularity: Traditional healers in Senegal and the Southwest of Nigeria use it for the treatment of diabetes mellitus (Dieye *et al*., 2008; Abo *et al.,* 2008). It is also used in Northern Nigeria for the treatment of diarrheal in infants (Abdulkarim *et al.,* 2005). Chemical evaluation has shown that *P. biglobosa* contains alkaloids, cardiac glycoside, high amino acid and protein contents (Odetola *et al.,* 2006; Ajaiye-Oba, 2005). Antimicrobial and termiticidal previously activities of some crude extracts from *P. biglobosa* have also been reported (Millogo-Kone *et al.,* 2006; Femi-Ola *et al*., 2008). Other studies have also demonstrated the anti diabetic potentials of a methanol extract from *P. biglobosa* seed in alloxan induced diabetes rats (Odetola *et al.,* 2006). The antidiabetic and anti-hyperlipidaemic effects of the extract of fermented *P. biglobosa* seeds have also been reported previously (Odetola *et al*., 2006). However, there is very little or no information on the hypolipedemic, hypoglycaemic and antioxidant effects of the protein isolate from *P. biglobosa* seeds. Proteins comprise the amino acid cysteine, which enhances the glutathione levels. Glutathione is a good source of antioxidants that alleviates the oxidative stresses related to diabetic complications (Ha and Zemel, 2003). Proteins have also been demonstrated to reduce the glucose levels in diabetic patients by stimulating the release of insulin from the pancreas (Ganon *et al.*, 2001). Therefore, the present study aims at investigating the hypoglycaemic, hypolipidemic and cardioprotective properties of a protein isolate from *Parkia biglobosa* in streptozotocin induced diabetic rats.

6.2 Materials and methods

6.2.1 Chemicals

All the reagents and solvents used in this study were of analytical grade.

6.2.2 Plant collection

The seeds of *Parkia biglobosa* (Jacq) Benth., Mimosaceae were purchased from a local market in Ijebu-Ode, Ogun state, Nigeria. The Permit for the importation (P0060156) into South Africa was obtained from the Department of Agriculture, Forestry and Fisheries (DAFF; Republic of South Africa). The seeds were then

identified and authenticated in the Department of Botany, University of Zululand by a Chief botanist. A voucher specimen (B07) was deposited at the university herbarium.

6.2.3 Extraction and preparation of the plant extract

The modified method described by Nkosi and colleagues (2005) was adopted in the extraction of the protein isolate. Briefly, the cleaned dried fermented seeds of *Parkia biglobosa* were grounded into a uniform powder using an electric blender. The powdered sample (1 kg) was defatted with hexane and air dried. The defatted powder was then suspended in distilled water at pH 10 and the resultant suspension filtered using a whatman filter paper. The filtrate was adjusted to pH 5 and later centrifuged (5000 rpm, 15 minutes at 4°C). The supernatant was discarded while the pellet containing the protein isolate was retained and freeze-dried to yield a brown extract. This was lyophilized and stored until needed.

6.2.4 Experimental animals

Ethical clearance (UZREC 171110-030-RA level 02 Dept. 2014/74) for the use of animals was obtained from the Research Ethics Committee (RAEC) of the University of Zululand, South Africa. The guidelines for the proper care of animals and conducting of animal experiments were followed. Adult male Sprague-Dawley rats (250 – 290 g) were obtained from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were kept in a standard room under a controlled temperature of 20-24 ^{o}C, a 12 hour light/dark cycle and humidity at 55 ± 5 %. The animals had access to standard laboratory rat chows and water *ad libitum.*

6.2.5 Experimental design

The rats were divided into 7 groups of 10 rats each, and as follows:

1. Control = untreated non-diabetic rats receiving chows
2. PI 200 = non-diabetic rats treated with protein isolate (200 mg/kg).
3. PI 400 = non-diabetic rats treated with protein isolate (400 mg/kg)

4. STZ = untreated diabetic rats receiving chows
5. STZ I = diabetic rats treated with insulin (600 µg/kg body weight)
6. STZ PI 200 = diabetic rats treated with protein isolate (200 mg/kg)
7. STZ PI 400 = diabetic rats treated with protein isolate (400 mg/kg)

6.2.6 Induction of diabetes

The experimental animals were subjected to an overnight fasting for 18 hrs prior to diabetes induction (Hue *et al.,* 2011). A freshly prepared streptozotocin (Merck, South Africa) (in an ice-cold citrate buffer, pH 4.5) solution was administered intraperitoneally at 60 mg/kg body weight. After 3 days post administration, a glucometer was used to measure the blood glucose levels of each animal. Animals with a fasting blood glucose level of more than 250 mg/dl were used for the diabetes study. Treatment commenced on the fourth day and continued for 28 days.

A daily dose of the treatment was administered orally to the rats for 28 days and, 4 hours after the last treatment. The treated rats were sacrificed by cervical dislocation. Blood samples were collected immediately by cardiac puncture. The blood was centrifuged (2000 rpm, for 2 min at 4 °C) to obtained serum which was stored at -80 °C and until required for biochemical analyses. Subsequently, the visceral organs (heart) were harvested, blotted dry, weighed and washed immediately with normal saline and homogenized. They were then centrifuged (1000 rpm, 4 °C 10 min) to obtained supernatant that was stored at -80 °C, and until required for the antioxidant studies

6.2.7 Food and water intake and body weight determination

The daily food and water intake were measured by giving a predetermined quantity of rat chow and water daily, and measuring the left over quantity at the same time the following day. The difference in quantity was noted as food and water intake (Nna *et al*., 2013). Body weight was determined by taking the initial body weight at the inception of the experiments and weekly thereafter until the end of the experiment. Body weight was recorded and changes in body weight were statistically analyzed. The following formulae were used:

food conversion (FC) = food intake in grams/wt. gain; and

food efficiency ratio (FER) = wt. gain/food intake.

6.2.8 Serum biochemical analysis

The glucose concentration of serum, the total cholesterol (TC), the total triglyceride (TG), and the high density lipoprotein cholesterol (HLD-c) were estimated using a commercial kit (Roche Diagnostics, Mannheim, Germany), and based on the manufacturer procedures. The very low density lipoprotein cholesterol (VLDL-c) and low density lipoprotein cholesterol (LDL-c) were calculated using the Friedewald's equation as follows: **vldl-c = TG / 5; ldl = tc–hdl-c –vld-c** (Friedewald *et al*., 1972).

6.2.9 Antioxidant assay

The superoxide dismutase (SOD), the catalase (CAT), the total glutathione (GHSt) and the glutathione S-transferases (GST) stimulatory activity of the protein isolate were determined on normal control and diabetic rats using a commercial kit (Sciencell Research Laboratories, USA) and based on the manufacturer' procedures.

The extent of lipid peroxidation in the homogenized cell tissues of the experimental animals was determined spectrophotometrically by measuring the formation of thiobabituric acid (TBA) reaction, and according to the method described by Varshney and Kale (1990). This was expressed in terms of malondialdehyde (MDA), which is the end product of the reaction.

6.2.10 Statistical analysis

All the statistical analyses were expressed as the mean ± SD. A one-way analysis of variance (ANOVA) was used for data analysis using the Duncan's multiple ranges. Significant difference was considered as $P< 0.05$.

6.3 Results

The effect of food conversion (FC) and food efficiency ratio (FER) on the experimental animals with *Parkia biglobosa* treatment are presented in Table 6.1 below. The Diabetes induced rats showed a significant ($P < 0.05$) increase and decrease in the food conversion and food efficiency ratios respectively, and in comparison with the normal control. The treatment of diabetic rats with the PI (STZ PI 200 and STZ PI 400) was observed to show a dose-dependent significant increase in FER and a decrease in FC respectively, when compared to the diabetic rats. The STZ PI 400 showed a better activity than insulin in the positive controled diabetic rats. Treatment of the non-diabetic rats with the PI 200 showed no significant ($P < 0.05$) differences in comparison with the control, whereas the PI 400 exhibited a significant change.

Figure 6.1 shows the effect of water consumption on experimental rats treated with PI. The diabetes induced rats presented a significantly ($P < 0.05$) high water consumption rate than the normal controls. Treatment with the PI was observed to reverse the high water consumption rate in diabetic rats in a dose-dependent manner. Insulin also showed a similar trend in comparison with the PI treated group. However, treatment with PI in the non-diabetic group showed no significant change when compared with the control.

The weekly changes in the fasting blood glucose levels of the experimental rats treated with the *Parkia biglobosa* protein isolate are presented in Figure 6.2. An administration of the STZ (60 mg/kg) to the rats was observed to significantly ($P < 0.05$) increase the glucose levels in the diabetic control rats throughout the period of treatment an in comparison with the normal controls. The PI treatment significantly attenuated the high blood glucose levels in diabetic rats - this was dose-dependent. (Insulin treatment is also known to ameliorate the high glucose levels observed in diabetics.) Treatment of the non-diabetic rats with (PI 200 ND and PI 400 ND) showed no significant ($P < 0.05$) changes when compared to the normal controls.

The effects of the serum lipid profile treated with *Parkia biglobosa* protein isolate are presented in Table 6.2. Diabetes induced rats showed a significant ($P < 0.05$), dose-dependent increase in the levels of cholesterol, low density lipoprotein (LDL-c), very

low density lipoproteins (VDL-C) and a decrease in the high density lipoprotein (HDL-c) in comparison with the normal control. The (STZ PI 200 and STZ PI 400) treatments significantly reversed the serum lipoproteins profile in the diabetic rats. Insulin treatment showed a similar pattern to the PI treatment in the diabetic rats. The protein isolate (PI 200 and PI 400) treatment of non-diabetic groups showed no significant ($P < 0.05$) changes in the serum lipoprotein profiles in comparison with the normal control.

Figure 6.3 shows the effect of the catalase stimulatory activity on experimental rats treated with *Parkia biglobosa* protein isolate. STZ-induced diabetic rats presented significantly lower catalase stimulatory activity in comparison with the control. The treatment of diabetic rats with protein isolate and insulin attenuate significantly deceased catalase stimulatory activity in comparison with diabetic rats. The treatment of non-diabetic rats with protein isolate was observed to lower catalase stimulatory activity, in comparison with the normal control group.

The effects of SOD stimulatory activity on experimental rats treated with PI are presented in Figure 6.4. Diabetic induced rats showed a significant decrease in SOD stimulatory activity when compared with the normal control. Treatment with insulin and protein isolate significantly ameliorated the decreased SOD stimulatory activity in diabetic rats. The protein isolate treatment of non-diabetic rats was observed to show a dose-dependent significant increase ($P > 0.05$) in SOD stimulatory activity in comparison with normal control rats.

Figure 6.5 shows the effect of total glutathione levels on experimental rats treated with PI. Induced diabetic rats showed a significant decease in total glutathione levels in comparison with the normal control. Upon treatment of diabetic rats with protein isolate, there was an appreciable increase in total glutathione stimulatory activity when compared with diabetic rats. Protein isolate also showed a better total glutathione levels than insulin, the positive control in diabetic rats. PI treatment on non-diabetic rats significantly increased the total glutathione activity (this was dos dependent) in comparison to the normal contros.

The effects of GST activity on experimental rats treated with PI are presented in Figure 6.6. GST activity was observed to show a significant dose-dependent decrease in diabetic rats when compared with the normal control. In contrast, the

treatment of diabetic rats with protein isolate significantly increased GST activity in diabetic induced rats. Protein isolate (PI 400 ND) showed a better GST activity than insulin, with diabetes induced rats. The protein isolate (PI 200 ND and PI 400 ND) treatment of non-diabetic rats showed a significant dose-dependent increase in GST stimulatory activity when compared to the normal controls.

Figure 6.7 shows the effects of lipid peroxidation on experimental rats treated with PI. Lipid peroxidation was observed to significantly increase in STZ induced diabetic rats in comparison with the normal controls. Upon treatment of diabetic rats with the protein isolate, there was a beneficial reversal of the level of lipid peroxidation in comparison with the diabetes induced rats. Protein isolates also showed a better lipid peroxidation inhibitory activity than insulin, a drug commonly used on diabetes induced rats. Protein isolate PI 200 ND showed significantly deceased lipid peroxidation on non-diabetic rats when compared with the controls whereas PI 400 ND showed no significant changes.

6.4 Discussion

Diabetes mellitus is an endocrine disorder associated with an increase in risk factors such as hyperglycaemia, hyperlipidaemia, oxidative stress, abnormal platelet aggregation, a decrease in fibrinolytic activity and hypertension (Reusch, 2003). These risk factors are implicated in coronary heart diseases, the leading cause of death in diabetes mellitus patients (Movahedian *et al.,* 2010). Streptozotocin, a widely accepted diabetogenic agent has the ability to selectively destroy pancreatic insulin-secreting β-cells, resulting in poor glucose utilization (Lenzen, 2008). Diabetic states are characterized by polyphagia, polyuria, polydipsia and severe weight loss (Babu *et al.,* 2007). Many plant products have been reported to possess medicinal properties such as antidiabetic, antioxidant (Jadhav and Bhutani, 2002), hypolipidemic (Sivara *et al.,* 2009), hypocholesterolaemic (Gupta *et al.,* 2001) and hypoglycaemic (McKenna *et al.,* 2002). The antihyperglycaemic activity of fermented *Parkia biglobosa* has been demonstrated on alloxan-induced diabetic rats (Odetola *et al.,* 2006). *Parkia bigolobosa* and *Padina boergesenii* have also been reported to exhibit hypoglycaemic properties (Senthilkumar *et al.,* 2012).

In the present study, the *Parkia biglobosa* protein isolate significantly reversed the hyperglycaemic state in STZ diabetes induced rats to normal glucose levels after 28 days of treatment. This validated the hypoglycaemic effect of fermented *Parkia biglobosa* (jacq), as previously reported (Odetola *et al.,* 2006).

Various mechanisms have been postulated to explain the mode of action of a number of medicinal plants that have been used for the treatment of diabetics throughout the world. Such mechanisms include the inhibition of digestion (Odetola *et al.,* 2006), absorption (Hong *et al.,* 2008), mimicking of insulin (Zakir *et al.,* 2004) and stimulation of glycogen synthesis (Kian *et al.,* 2000). Even though there was no attempt to determine the apparent mechanism of the observed hypoglycaemic effect of PI, the high protein content and excellent amino acid profile of the protein isolate could have prevented the loss in body weight by the reduction of protein degradation, replenishing the protein loss in diabetic rats. Roasted Arabic coffee has been reported to reverse the low food efficiency ratio in diabetic rats (Gaafar *et al.,* 2013). *Artemisia amygdalina* has also been demonstrated to ameliorate the food and water consumption in diabetic rats (Ravi *et al.,* 2005).

Apart from glucose metabolism, insulin plays a crucial role in lipid and lipoprotein metabolism (Odetola *et al.,* 2006). Diabetes mellitus patients have an increased risk of premature atherosclerosis, coronary insufficiency and myocardial infarction due to an alteration in serum lipid profile (Ravi *et al.,* 2005). Thus the observed effect of the PI in decreasing the serum lipids and lipoproteins such as cholesterol, triglyceride, VLVL-c, LDL-c and increasing HDL-c (Table 6.2) could be beneficial in the prevention of cardiovascular diseases (Ravi *et al.,* 2005). Although the hypolipidaemic mode of action of the protein isolate is unknown, it could be attributed to the stimulation of insulin secretion or action. Insulin has been reported to possess hypolipidemic effects in STZ induced diabetic rats (Pepato *et al.,* 2005).

Hyperglycaemia in diabetes mellitus induces the generation of reactive oxygen species (ROS) which cause cellular damage by oxidizing nucleic acids, proteins and membrane lipids (Haskins *et al.,* 2003). Uncontrollable ROS impair the activities of endogenous antioxidant defense systems such as SOD, catalase and glutathione reductase, which are known to counterbalance the toxic effects of free radicals

(Matough *et al.,* 2012). Therefore, the treatment of diabetic complications, such as cardiovascular diseases, is aimed at reducing the generation of ROS or oxidative stress signaling pathways (Evans, 2007). Antioxidant enzymes have been demonstrated to improve the endothelial dysfunction in diabetic rats (Mohora *et al*., 2007). The results of this study support the previously indicated antioxidant potential of natural products in STZ diabetes induced rats (Anwar and Meki, 2003). It is possible that the anti-diabetes potential of protein isolate could be explained by their stimulatory effect on antioxidant enzymes.

In conclusion, the present study revealed that protein isolate showed hypoglycaemic, hypolipidemia and antioxidant properties in STZ diabestes induced rats. These properties attenuate cardiovascular complications in diabetes mellitus (Tavafi *et al*., 2011). PI could therefore serve as a potential cardio-protective supplement in diabetes mellitus. For further study, the mechanism of action thus needs to be ascertained.

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Table 6.1: The effect of *Parkia biglobosa* protein isolate treatment on food conversion (FC) and food efficiency ratio (FER) of normal, untreated and treated STZ-Induced Diabetic Rats

Groups	Food conversion	Food efficiency ratio
Control	1.03 ± 0.06	0.97 ± 0.16
PI 200	1.14 ± 0.58	0.85 ± 0.08
PI 400	1.23 ± 0.18^{c}	0.78 ± 0.09^{c}
STZ	6.47 ± 0.80^{a}	0.15 ± 0.05^{a}
STZ I	2.98 ± 0.44^{bc}	0.34 ± 0.19^{bc}
STZ PI 200	3.42 ± 0.09^{bc}	0.32 ± 0.01^{bc}
STZ PI 400	2.05 ± 0.34^{bc}	0.49 ± 0.39^{bc}

Data in the table above are expressed as mean ± standard deviation. ^{a} represents a significant difference in value of the normal control group against STZ treated diabetic rats. ^{b} represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. ^{c} represents a significant difference of the normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

Table 6.2: Effect of *Parkia biglobosa* protein isolate treatment on the serum lipid profile of normal, treated and untreated STZ-Induced Diabetic Rats

Groups	Cholesterol	HDL-c	LDL-c	VDL-c	TG
			mg/dl		
Control	94.38±3.40	44.4±4.5	15.6±0.01	35.1±1.10	121.86±0.12
PI 200	82.38±4.53	43.4±.35	14.7±2.18	37.05±4.50	114.81±8.9
PI 400	92.17±2.54	41.6±4.50	15.6±0.01	34.97±2.54	124.69±5.80
STZ	136.11±1.03[a]	32.3±2.25[a]	32.1±2.25 [a]	73.58±2.59[a]	267.22±11.27[a]
STZ I	97.36±4.92 [b]	40.5±2.25 [b]	24.7±2.25[b]	46.83±3.17[b]	149.59±8.13[bcc]
STZ PI 200	93.36±1.58 [b]	42.9±0.01[b]	13.4±4.34[b]	37.30±1.61[b]	120.74±1.58[b]
STZ PI 400	81.9±8.80 [b]	53.3±2.25[bc]	13.0±2.25[b]	15.56±8.16[bc]	130.41±8.16[bc]

Data in the table above are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of the normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

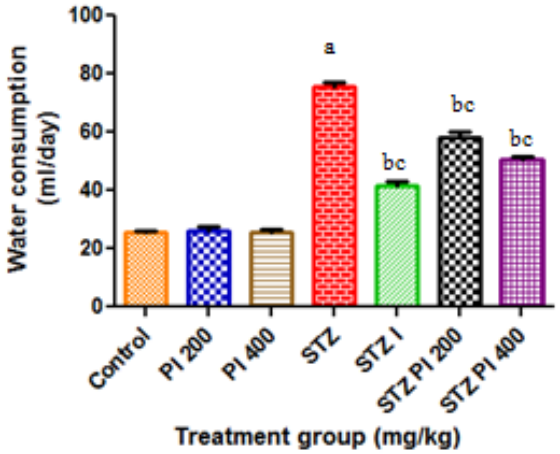

Figure 6.1: Effects of *Parkia biglobosa* protein isolate treatments on water consumption in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

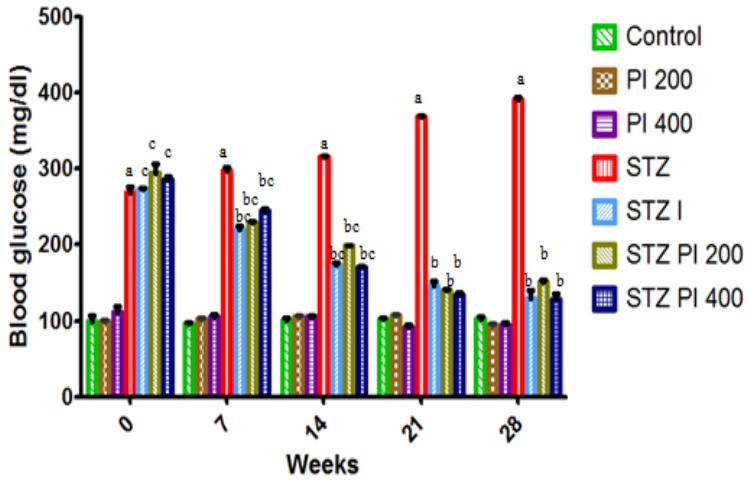

Figure 6.2: Changes in fasting blood glucose levels of diabetic and non-diabetic rats on weekly dose administration treatment with *Parkia biglobosa* protein isolate.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

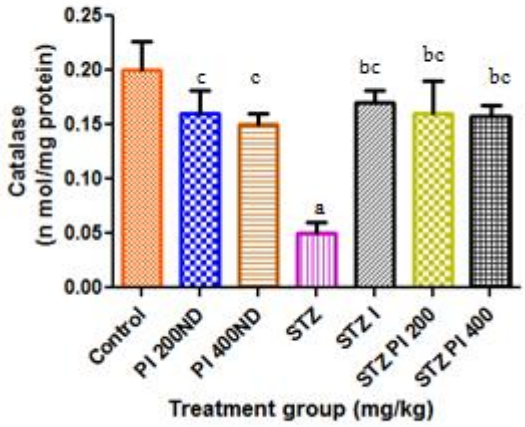

Figure 6.3: Effects of *Parkia biglobosa* protein isolate treatments on catalase in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

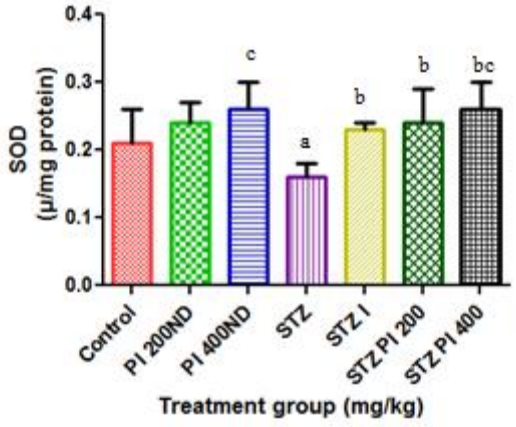

Figure 6.4: Effects of *Parkia biglobosa* protein isolate treatments on SOD in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

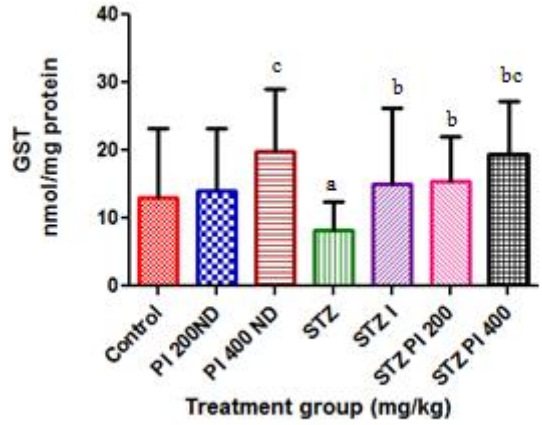

Figure 6.5: Effects of *Parkia biglobosa* protein isolate treatments on GST in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

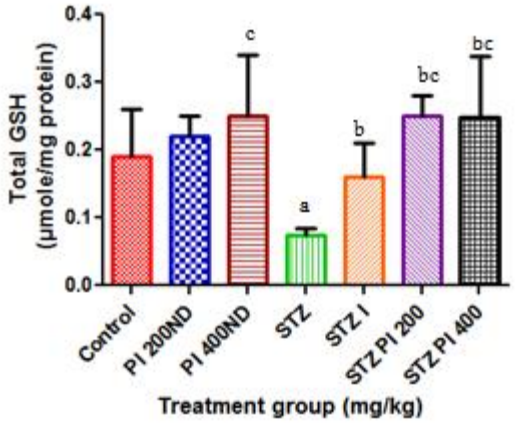

Figure 6.6: Effects of *Parkia biglobosa* protein isolate treatments on total GSH in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

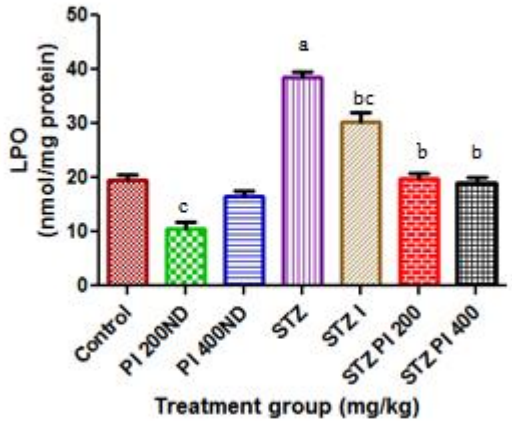

Figure 6.7: Effects of *Parkia biglobosa* protein isolate treatments on LPO in diabetic and non-diabetic rats.

Data are expressed as mean ± standard deviation. [a] represents a significant difference in value of the normal control group against STZ treated diabetic rats. [b] represents a significant difference in value of the diabetic induced rats group against treated diabetic rats. [c] represents a significant difference of a normal control group against all the treated rats (diabetic and non-diabetic rats). The significance level is given at $P < 0.05$.

Chapter Seven

7.0 Modulatory influence of *Parkia biglobosa* protein isolate on testosterone and biomarkers of oxidative stress in brain and testes of streptozotocin-induced diabetic male rat

Running title: Modulatory influence of *P. biglobosa* on brain and testes in STZ-diabetic rats.

Abstract

Parkia biglobosa is believed to possess antioxidant activities that may exert modulatory effects on diabetes and diabetic complications. This study investigated the modulatory potentials of a *Parkia biglobosa* protein isolate (PBPi) on serum testosterone (sTT) levels as well as its influence on biomarkers of oxidative stress in the brains and testes of streptozotocin-induced diabetic rats. Animals were made diabetic with a single intraperitoneal administration of streptozotocin (STZ; 60 mg/kg body weight). (200 or 400 mg/kg body weight) was given orally by gavage or insulin (5 U/kg, i.p.) was administered daily to STZ-induced diabetic rats for 28 days. The results revealed a significant elevation in thiobarbituric acid reactive substance (TBARS) levels in the brains and testes of diabetic rats as aresult of the treatment. This was closely associated with the concomitant reduction in levels of sTT and reduced weight in the testes. This was also closely associated with a noticeable decline in the glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) enzymes as well as total glutathione (total GSH) levels in the brain and testes of diabetic rats. Concievably, treatment with PBPi efficiently prevented the alterations witnessed in the serum sTT and also ameliorated various alterations in the biomarkers of oxidative stress (TBARS, total GSH, GST, SOD and CAT) in the brain and testes of diabetic rats. These results provided evidence that the PBPi could protect the brains and testicular tissues against oxidative stress induced by STZ via a modulation of the serum testosterone concentration and also by an enhancement of their antioxidant defence system of animal in the STZ-diabetic rats.

Keywords: Antioxidant, oxidative stress, *Parkia biglobosa*, STZ-induced diabetes, testosterone.

7.1 Introduction

Diabetes mellitus (DM) has been identified as the fourth leading cause of death globally, it ranks high among the most common non-communicable chronic metabolic disorder affecting people in both developed and developing countries in recent times (Meaney, 2012; Shah and Afzal, 2013; Wu *et al*., 2014). The exponential increase in the prevalence of DM has been linked to obesity and increasing sedentary behaviours, urbanization, modernization, genetics and family history as well as nutritional imbalances associated with the consumption of high energy, fat and cholesterol rich diets among others (Ogunyinka *et al*., 2015; Ramachandran *et al*., 2012). This insidious metabolic disease is characterized by chronic hyperglycaemia (increased blood glucose level) together with an impaired carbohydrate, protein and lipid metabolism due to a deficiency in insulin secretion and or resistance to insulin action (Abdel-Sattar *et al*., 2011; Schmatz *et al*., 2012;).

Recently, documented evidence suggested that a prolonged hyperglycaemia also does promote the upregulation and overproduction of reactive oxygen species in the mitochondria, which ultimately leads to oxidative stress (Giacco and Brownlee, 2010; Matough *et al*., 2012; Tangvarasittichai, 2015). This is believed to occur via well established mechanisms, including: activation of the polyol pathway, increased intracellular formation of advanced glycation end-products, activation of protein kinase C, increased production of superoxide radicals by the mitochondrial electron transport chain and a general decline in endogenous antioxidant capacity (Campos, 2012; Rains and Jain, 2011; Tiwari *et al*., 2013;). To a great extent, experimental evidence have also revealed that a hyperglycaemia-induced oxidative stress enhances the progression of diabetes and its associated complications (Giacco and Brownlee, 2010; Shailey and Basir, 2012).

Oxidative stress is known to arise when there is an imbalance between the free radical production, especially reactive oxygen species (ROS), and the endogenous antioxidant defense system. This shift in balance is associated with an oxidative damage to a wide range of biomolecules including lipids, proteins and nucleic acids, which may eventually impair the normal functioning of the various tissues and organs (Lobo *et al*., 2010; Rahman *et al*., 2012;). Diabetes may have a profound influence

on to the brain chemistry, particularly on brain neurotransmitters and the associated enzymes (Mohamed *et al*., 2007). An excessive ROS production from the auto-oxidation of elevated intracellular glucose levels has been reported as one of the mechanisms underlying the diabetic neuronal injury (El-Akabawy and El-Kholy, 2014). It has also been established that the elevated ROS levels can make the brain susceptible to oxidative stress and damage due to the brain's high oxygen demand as well as its abundant lipid concentration and relatively poor antioxidant defense system compared to the other tissues (Muriach *et al*., 2014; Patil *et al*., 2012; Rahman, 2007).

Similarly, there is also increasing evidence that the male reproductive dysfunction found in experimental animal models and humans with diabetes is one of the consequences of an imbalance between free radical production and the antioxidant defense system, which is associated with diabetic conditions (Ballester *et al*., 2004; Li *et al*., 2013; Roy *et al*., 2013). Diabetes may affect male reproductive functions in various ways, including: a decreased sperm count and motility a decreased testicular testosterone production, testicular morphological changes and reduced libido (Ballester *et al*., 2004; Jain and Jangir, 2014; Ricci *et al*., 2009). The role of medicinal plants in assuaging the devastating effects of hyperglycaemia-induced oxidative stress associated with diabetes in various tissues and organs has been recognized.

Parkia biglobosa is widely consumed in Nigeria and many other West African countries as a spice for the flavouring of foods (Ogunyinka *et al*., 2015). Both the aqueous and methanolic extracts of *P. biglobosa* have been reported to possess some beneficial and pharmacological potentials in experimental animals, including the hepatoprotective, hypoglycaemic, hypolipidaemic, antimicrobial and anti-inflammatory activities (Adi *et al*., 2013; Odetola *et al*., 2006; Ogunyinka *et al*., 2015).

To the best of our knowledge, no study has yet been carried out to explore the modulatory influence of the *Parkia biglobosa* protein isolate (PBPi) on serum testosterone concentrations or the biomarkers of oxidative stress in the brains and testes of STZ-induced diabetic rats. Here we demonstrate that the PBPi, at a dose of 200 or 400 mg/kg bw, could exhibited a significant reversal effect in all biochemical parameters (notably; sTT and TBARS) measured in the serum and tissues (brain

and testes) of STZ-challenged rats. Thus, in the treatment of neurotoxicity and testicular toxicity, PBPi confers protection against the oxidative stress induced by STZ.

7.2 Materials and Methods

7.2.1 Plant Material

Fermented *Parkia biglobosa* seeds were obtained from a local market in Ijebu-Ode, Ogun state, Nigeria. An import permit (P0060156) from the Department of Agriculture, Forestry and Fisheries (DAFF; Republic of South Africa) was obtained. The seed sample was authenticated in the Department of Botany, University of Zululand and a voucher specimen (B07) was deposited at the university herbarium.

7.2.2 Animals

Sprague-Dawley rats, weighing about 250 – 290 g each, were obtained from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were kept for acclimatization for 7 days prior to the commencement of the study; they were maintained at standard conditions of temperature and relative humidity, with a (12 hour light/dark cycle). The animals were provided with standard rat pellets and water *ad libitum*. The animal experimental protocols followed were in accordance with the recommendations of the institutional animal ethical committee (UZREC 171110-030-RA level 02 Dept 2014/74).

7.2.3 Chemicals

All the chemicals used in this study were procured from Sigma Chemical Co. (St. Louis, MO, USA), Merck (South Africa) and Sciencell Research Laboratories (Carlsbad, CA, USA).

7.2.4 Preparation of seed extract of *Parkia biglobosa*

A cup-full of oven-dried (50 °C) seeds of fermented *Parkia biglobosa* were pulverized using an electric blender to obtain a coarse powder. One kilogram of uniformly

powdered seeds was defatted with hexane to obtain the defatted extract. The defatted extract was air dried and then extracted (1:10 w/v) with acidic butanol to remove any possible anti-nutrients. Protein isolate was extracted from the defatted extract using the method described by Nkosi et al. (2005). Briefly, the dry defatted extract was re-suspended in distilled water at pH 10. The resultant suspension was then filtered to remove debris and the filtrate adjusted to pH 5, followed by a centrifugation at 5000 rpm for 15 minutes at 4 °C. The supernatant was discarded while the pellet containing the protein isolate was retained and freeze-dried to yield a brown extract. The lyophilized extract was then kept dry until needed.

7.2.5 Induction of diabetes

Following an overnight fasting, diabetes was induced in the selected rats by a single intraperitoneal injection of a freshly prepared STZ (Sigma-Aldrich Co.) dissolved in 0.1M, pH 4.5 ice cold citrate buffer, at a dosage of 60 mg/kg of body weight (Hule *et al*., 2011). Diabetes was confirmed in rats 72 hours after STZ administration, by measuring the fasting blood glucose (FBG) levels. Rats with a FBG level above 300 mg/dl were considered diabetic and subsequently selected for the study. Actual experimental treatment commenced on the fourth day and continued for a period of 28 days.

7.2.6 Experimental design

Seventy healthy male rats (averaging 12 weeks old) were divided into seven groups of ten animals each and treated as follows: Group 1 (control) was given citrate buffer only. Group 2 (PI 200ND), comprising non-diabetic rats, was given citrate buffer and protein isolate (200 mg/kg of body weight). Group 3 (PI 400ND), comprising non-diabetic rats, was given citrate buffer and protein isolate (400 mg/kg of body weight). STZ-induced diabetic rats were divided into four groups (Groups 4–7). Group 4 (STZ), comprising the diabetic control group. Group 5 (STZ I), comprising the positive control group, was given insulin (5 U/kg, i.p.). Group 6 (STZ PI 200) was diabetic and received the protein isolate (200 mg/kg body weight). Group 7 (STZ PI 400), comprising diabetic rats, received the protein isolate (400 mg/kg body weight). All treatments were given orally for 28 days.

7.2.7 Collection and processing of blood and tissue samples

At the end of the 28 days of treatment, the rats were fasted overnight and then sacrificed under anesthesia. Blood samples were obtained by cardiac puncture in plain tubes without anticoagulants, left for 1 hour to coagulate, and then centrifuged at 3000 rpm for 15 min at 4 °C to obtain the serum. The brains and testes were collected, washed in saline, blotted dry and weighed. Portions of the rats' brains and testes were homogenized in a 56 mM Tris-HCl buffer (pH 7.4) containing 1.15 % KCl, and then centrifuged at 10,000 rpm for 15 minutes to obtain the supernatants that were then stored at −80 °C and until needed for analysis.

7.2.8 Biochemical and enzyme estimation

The extent of lipid peroxidation in the brain and testis homogenates was determined spectrophotometrically by measuring the formation of thiobarbituric acid reactive substances (TBARS), according to the method described by Varshney and Kale (1990). This was expressed in terms of the malondialdehyde (MDA), which is the end product of the reaction. The serum testosterone levels were estimated using an ELISA kit (Cayman Ltd., USA) and according to the manufacturer's instructions, while the brain and testes levels of total glutathione (total GSH), as well as the activities of glutathione-S-transferases (GST) superoxide dismutase (SOD) and catalase (CAT), were determined by using the corresponding assaying kits (ScienCell Research Laboratories, Carlsbad, USA) and according to the manufacturer's instructions.

7.2.9 Statistical analysis

All data obtained presented as the mean ± standard deviation. The data was analyzed by the one-way ANOVA, followed by the Duncan's multiple ranges (SPSS13.0, Inc., Chicago, IL, USA). The differences were considered significant at $P < 0.05$.

7.3 Results

7.3.1 Effects of PBPi on testes weight and serum testosterone levels of STZ-induced diabetic rats

Table 7.1 and Figure 7.1 show the testes weight and serum testosterone levels in the experimental rats. STZ significantly decreased the weight of the testes and the levels of serum testosterone in the diabetic animals compared to the control rats. Conversely, treatment with PBPi (200 or 400 mg/kg bw) for 28 consecutive days caused a significant increase in the testes weight and the levels of the serum testosterone as compared to the diabetic control groups.

7.3.2 Effects of PI on lipid peroxidation levels in the brains and testes of STZ-induced diabetic rats

The extent of lipid peroxidation in the brains and testes of experimental rats are shown in Figure 7.2.

The induction of diabetes led to a significantly increased level of TBARS in the brains and testes of diabetic rats. Interestingly, the increased level of TBARS in the brains was reversed to near normal when PBPi (200 or 400 mg/kg bw) was administered. In a like manner, the PBPi (200 or 400 mg/kg bw) supplementation in the treatment groups considerably decreased the TBARS levels in the testes of diabetic rats.

7.3.3 Effects of PBPi on antioxidant parameters in the brain and testes of STZ-induced diabetic rats

Figure 7.3 represents the various alterations in the antioxidant status of experimental rats.

The induction of diabetes was accompanied by a significant decline in the levels of the total GSH and in the activities of GST, SOD and CAT in the brains and testes of the diabetic rats compared to the control. These altered antioxidant statuses were significantly restored after the administration of PBPi (200 and 400 mg/kg bw).

7.4 Discussion

Brain cell injury or impairment as well as reproductive dysfunction are common complications of diabetes, which may occur through several mechanisms (Ramzy *et al*., 2014; Matough *et al*., 2012). It appears that diabetes, which is characterized by a hyperglycaemia-induced oxidative stress, results in a multi-organ failure and these accounts for most of the common causes of death in people with diabetes (Singh *et al*., 2013).

The present work is one of a series of studies showing the relationship between the STZ-induced free radical damage and the potential of nutraceuticals/medicinal plants in reducing oxidative stress while concurrently, improving the various tissues and organ functions affected by the free radical damage. The results presented in this study demonstrate that the PBPi (200 or 400 mg/kg bw) significantly ameliorate the biochemical indices of the alterations in all biochemical parameters evaluated in the serum and tissue homogenates (brain and testes) of STZ-challenged rats. This complements the protective roles of *P. biglobosa* previously identified in human health and diseases.

A reduction in testis weights and serum testosterone levels was observed in diabetic rats. This is in agreement with similar studies reported in literature (Long *et al*., 2015; Khaki *et al*., 2014). Testicular function is generally controlled by two independent coordinated functions: the biosynthesis of androgens by theLeydig cells and the production of spermatozoa in the epithelium of seminiferous tubules. The reduction in testosterone levels in this study may have arisen due to oxidative damage in the testes, which may in turn have been due to a decrease in the function of both the Leydig cells (testosterone producing cells) and Sertoli cells (supporting cells), and this might have been due to a reduction in insulin secretion (Ballester *et al*., 2004; Ojewale *et al*., 2014).

An enhanced lipid peroxidation plays a significant role in the measurement of toxicity in the STZ-induced diabetes and also serves as one of the most important manifestations of oxidative stress and damage in diabetic complications (Ramesh *et al*., 2012; Subash-Babu *et al*., 2014). The increased TBARS levels in brains and testes homogenates in this study were an indication of damage and dysfunction in

these organs. This finding was consistent with earlier reports (Pari and Murunga, 2007; Ramesh *et al*., 2012; Ramzy *et al*., 2014). A ROS-induced cellular damage is due to chronic hyperglycaemia occurs via lipid peroxidation of unsaturated fatty acids, which in turn alters cellular function (Florence *et al*., 2014). Reversal in the elevated levels of the TBARS, particularly in the brain, suggested a protective influence of PBPi against oxidative stress in the STZ-induced diabetes.

In addition to the increased levels of lipid peroxidation witnessed in the STZ diabetic animals, the endogenous antioxidant defence mechanisms (notably, total GSH, GST, SOD and CAT) in the brains and testes were also altered. Uncontrolled hyperglycaemia, which promotes free radical generation, may be the underlying causative factor (Rolo and Palmeira, 2006). Our observation was also in agreement with the previously documented reports (Aitken and Roman, 2008; Kumar *et al*., 2007; Pari and Latha, 2004; Singh *et al*., 2013). It is widely accepted that antioxidant enzymes provide a first line defence against the ROS in response to oxidative challenges (Robertson and Harmon, 2007). Enzymatic antioxidants play a very important role in protecting cells against the overwhelming deleterious effects of ROS in the hyperglycaemia-induced oxidative stresses (Ullah *et al*., 2015).

Superoxide dismutase and CAT are the two main important radical scavenging enzymes. SOD catalyzes the conversion of superoxide radicals to H_2O_2, a less reactive intermediate, thereby protecting the cells from the deleterious effects of the superoxide radicals. CAT is required to neutralize reactive species (H_2O_2) to water (H_2O) and molecular oxygen (O_2) (Al-Enazi, 2014; Armagan *et al*., 2006; Avelar *et al*., 2015,).

On the other hand, glutathione defends the cells from oxidative damage by reducing disulfide bonds of the cytoplasmic proteins to cysteines. The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione-S-transferases (Liou and Storz, 2010). The decreased activities of these antioxidants in diabetic conditions might result in reduced a protection against the free radical damage. This is an indication that PBPi may possess some bioactive constituents that are capable of scavenging free radicals, which would then prevent their potential induction of the cellular damage.

In conclusion, the observations from this study confirm that STZ (60 mg/kg bw) has an adverse effect on the serum testosterone levels and on the antioxidant status of the brains and testes of diabetic rats via an induction of lipid peroxidation. Thus, our study proposed that the PBPi showed a modulatory effect by attenuating the above lipid peroxidation in the STZ-induced diabetic rats in a dose-dependent manner, with 400 mg/kg bw identified as having a particular efficacy with no evidence of toxicity. Taken together, these results provide evidence that the PBPi could protect the brain and testicular tissues against oxidative stress induced by STZ via a modulation of the serum testosterone concentration, and also by enhancing the antioxidant defence system in STZ-diabetic rats.

7.5 Acknowledgements

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7.6 Conflict of interest

None declared.

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Table 7.1 Effects of PI on the testes weight of STZ-induced diabetic rats

Testes	Control	PI 200ND	PI 400ND	STZ	STZ I	STZ PI 200	STZ PI 400
Weight (g)	3.29 ± 0.10	3.57 ± 0.25	3.61 ± 0.10	2.60 ± 0.55	3.42 ± 0.08	3.34 ± 0.11	3.40 ± 0.55

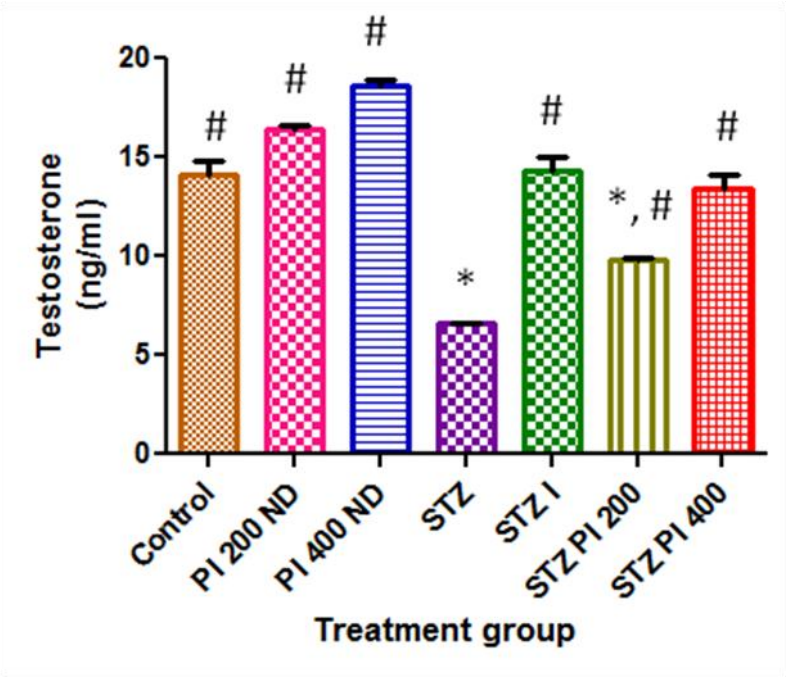

Figure 7.1: Effects of PI on serum testosterone levels of STZ-induced diabetic rats.

Testosterone (ng/ml) data are presented as mean ± S.D. values (n = 10). Mean differences are significant ($P < 0.05$) when compared with the * control group, # STZ only.

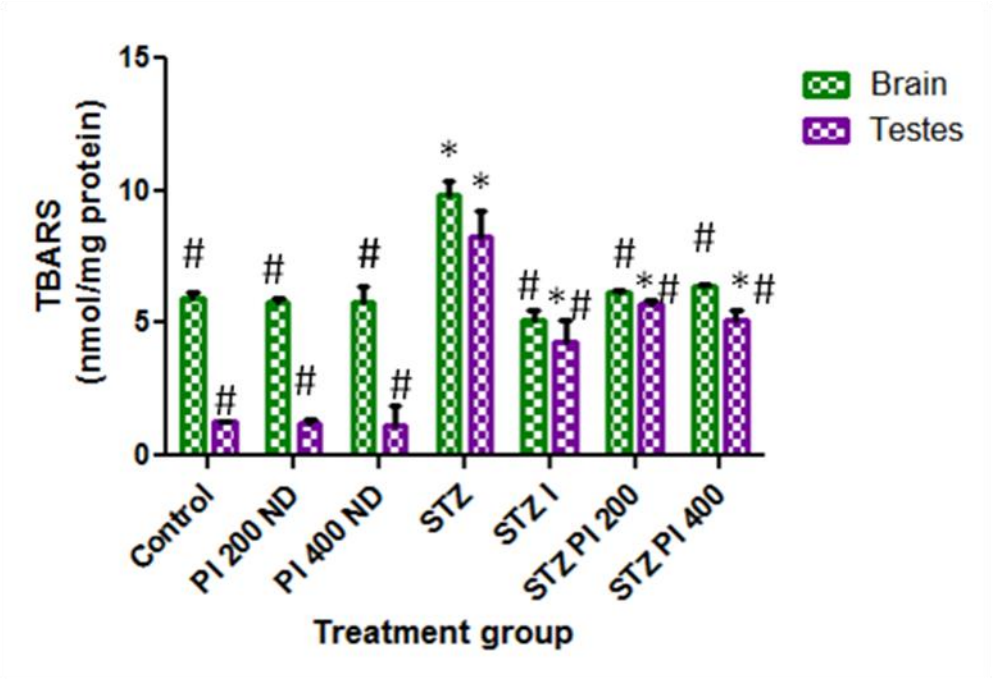

Figure 7.2: Effects of PI on lipid peroxidation levels in the brain and testes of STZ-induced diabetic rats. TBARS (nmol/mg protein).

Data are presented as mean ± S.D. values (n = 10). Mean differences are significant (P < 0.05) when compared with the * control group # STZ only.

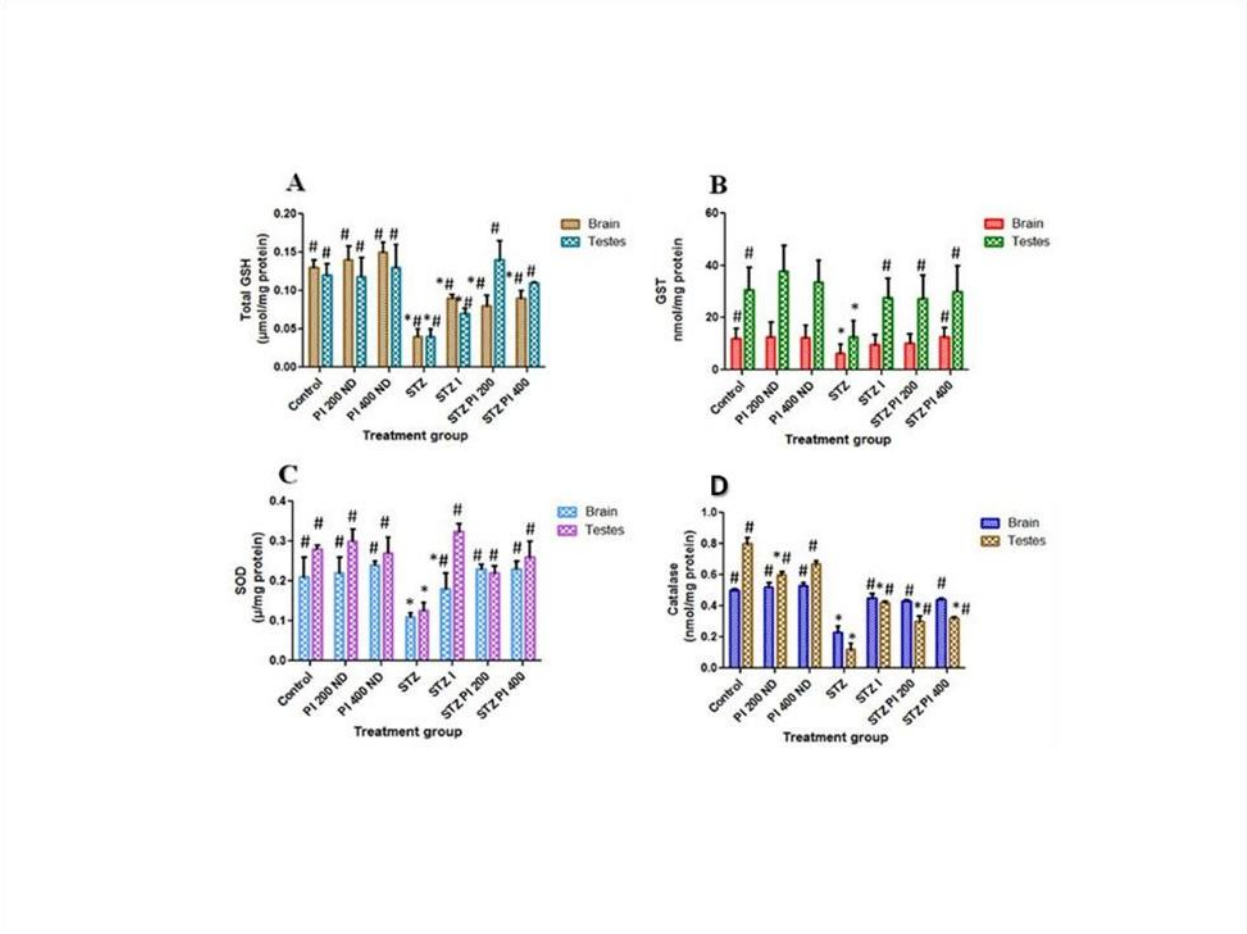

Figure 7.3: Effects of PI on antioxidant parameters in the brain and testes of STZ-induced diabetic rats.

A) Total GSH (µmol/mg protein), B) GST (nmol/mg protein), C) SOD (µ/mg protein), D) CAT (nmol/mg protein). Data are presented as mean ± S.D. values (n = 10). Mean differences are significant ($P < 0.05$) when compared with the * control group # STZ only.

Chapter Eight

8.0 The protective effects of *Parkia biglobosa* protein isolate on streptozotocin-induced hepatic damage and oxidative stress in diabetic male rats

Running title: Effects of *Parkia biglobosa* protein isolate on STZ-induced oxidative stress in rats

Abstract

Bioactive compounds isolated from medicinal plants have become a primary target in the search for new antidiabetic therapies. The present study was designed to investigate the possible protective roles of the *Parkia biglobosa* protein isolate (PI) against the streptozotocin-induced hepatic damage and oxidative stress in diabetic male rats. Before embarking on the animal experiments, an HPLC fingerprint analysis of the PBPi was carried out. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg of body weight). Diabetic rats were orally treated on a daily basis with the PBPi (200 or 400 mg/kg body weight) or insulin (5 U/kg, i.p.) for 28 days. The degree of protection was then evaluated using the various biochemical parameters such as the serum transaminases (ALT and AST), total protein, thiobarbituric acid reactive substances (TBARS), total glutathione (total GSH), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) activities as well as the interleukin-6 (IL-6). Histology of the liver sections was also carried out. The HPLC fingerprint of the PBPi revealed eleven distinct peaks. An oral administration of PBPi at the tested doses significantly attenuated STZ-induced elevated levels of serum IL-6, ALT and AST, as well as the hepatic TBARS levels. The hepatic antioxidants (total GSH, GST, SOD and CAT) as well as the total protein were markedly restored in a dose-dependent manner. Histopathological results strongly supported the protective role of the PBPi. Taken together, these results suggested that PBPi could confer protection by ameliorating the hepatic damages and oxidative stresses caused by the STZ in animal models, possibly via its anti-inflammatory and antioxidant properties.

Keywords: Hepatic antioxidant, histology, oxidative stress, *Parkia biglobosa*, STZ-induced diabetes.

8.1 Introduction

The global day-to-day prevalence of diabetes mellitus, a group of metabolic disorders, has increased at an alarming rate. It has recently been estimated that, by the year 2030, a possibile 366 million people will be diabetic worldwide (Kaveeshwar and Cornwall, 2014; Patel *et al.,* 2012). A significant increase in the morbidity and mortality rates in diabetics has been associated with microvascular (retinopathy, neuropathy, and nephropathy) and macrovascular (heart attack, stroke and peripheral vascular disease) complications (;Deshpande *et al.,* 2008; Tuttolomondo *et al.,* 2015; Irudayaraj *et al*., 2012). At the moment, there is no complete cure for diabetes. Its prevention or reversal without side effects is a key challenge. This complex, multifactorial disease therefore requires a lifelong management (Ogunyinka *et al.,* 2015; Kazeem *et al*., 2013).

In the etiology of diabetes, it is generally accepted that diabetes is characterized by an uncontrolled hyperglycaemia that develops due to the ineffective secretion of insulin, inadequate insulin, or both, as well as due disturbances in carbohydrate, protein and fat metabolism, resulting in free radical induced (especially of the reactive oxygen species, ROS) oxidative stresses and oxidative damaged. This process has been suggested as the mechanism underlying diabetes and its associated complications (Karuna *et al.,* 2011; Ahmed *et al.,* 2013). In addition, the non-enzymatic protein glycation, the impaired antioxidant enzyme status and the production of peroxides which may contribute to severe liver damage and disorder have also been implicated as possible sources of the oxidative stress believed to play an important role in the pathogenesis and progression of diabetes mellitus (Kazeem *et al.,* 2013). Taken together, the excessive hyperglycaemia, oxidative stress and hepatic fat accumulation play a vital role in the development of the hepatocellular injury in diabetics (Afrin *et al*., 2015).

Added to this, a direct relationship has been established between increased cytokines, such as the tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin 10 (IL-10), and excessive hyperglycaemia in both types 1 and 2 diabetes (Frances *et al.,* 2013; Uyanik *et al.,* 2012). IL-6 has emerged as an important systemic alarm signal which is usually elevated in response to almost every kind of

damage or tissue injury (Seghal, 1990). Under hyperglycaemic conditions, the elevated glucose and ROS levels are pro-inflammatory and may increase the levels of IL-6. This evidence confirms the role of cytokines in type 1 and type 2 diabetics (Niture *et al.,* 2012; Elmarakby and Sullivan, 2012). Several pharmacological applications of plant derived foods (nutraceuticals) that contribute towards an improvemet of the immune defence and in reducing the risks associated with diabetes, have been reported in the literature (Ogunyinka *et al.,* 2015). Detailed evaluations of some of these plant derived foods may serve as potential sources for novel therapies in the prevention, reversal and management of diabetes and its associated complications.

P. biglobosa has been identified as one of the plants the possessing hepatoprotective, anti-inflammatory, antioxidative as well as hypolipidaemic and antimicrobial properties (Ogunyinka *et al.,* 2015). *P. biglobosa* is an essential household spice, usually used in Nigeria and many other West African countries, in the preparation of various foods, and as a seasoning agent in soups (Ogunyinka *et al.,* 2015; Ajaiyeoba, 2002; Odetola *et al.,* 2006). This study was designed to evaluate the protective activities of *Parkia biglobosa* protein isolate (PI) in streptozotocin-induced diabetic rats.

8.2 Materials and methods

8.2.1 Chemicals

All the chemicals used this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Merck (South Africa) and Sciencell Research Laboratories (Carlsbad, CA, USA).

8.2.2 Plant material and preparation of the protein isolate

After obtaining an import permit (P0060156) from the Department of Agriculture, Forestry and Fisheries (DAFF; Republic of South Africa), raw and fermented *Parkia biglobosa* seeds used in this study were purchased from a local market in Ijebu-Ode, Ogun state, Nigeria. The seeds were identified and authenticated by the Chief botanist of the Department of Botany, University of Zululand and a voucher

specimen (B07) was deposited at the university herbarium. Prior to separation of the protein isolate, the fermented seeds were oven-dried at 50 °C. Thereafter, the dried seeds were ground into a uniform powder using an electric blender. One kilogram of uniformly powdered seeds was defatted with hexane to obtain the defatted extract.

The defatted extract was air dried and then extracted (1: 10 w/v) with acidic butanol to remove possible anti-nutrients. Protein isolate was extracted from the defatted extract using the method described by Nkosi et al. (2005). Briefly, the dry defatted was filtered to remove debris and the filtrate adjusted to pH 5, followed by centrifugation at 5000 rpm for 15 minutes at 4 °C. The supernatant was discarded while the pellet containing the protein isolate was retained and freeze-dried to yield a brown extract. The lyophilized extract was kept dry until needed.

8.2.3 Induction of diabetes in experimental animals

Seventy healthy, male, Sprague-Dawley rats (250 – 290 g; averaging 12 weeks old) were procured from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were acclimatized for 7 days prior to the commencement of the study and maintained at standard conditions of temperature and relative humidity, with a 12-hour light/dark cycle. The animals were fed with standard rat pellets and water *ad libitum*. All animal experimental procedures were performed following the guidelines for the caring and supervision of experimental animals and were in agreement with the Institutional Animal Ethics Committee and clearance certificate (UZREC 171110-030-RA level 02 Dept 2014/74) issued by the University of Zululand, Research Ethics Committee.

Following an overnight fasting, diabetes was induced in the selected rats by a single intraperitoneal injection of a freshly prepared STZ (Sigma-Aldrich Co.) at a dosage of 60 mg/kg of body weight, dissolved in 0.1M, pH 4.5 ice cold citrate buffer (Hula *et al.,* 2011). Diabetes was confirmed in the rats 72 hours after the STZ administration by measuring the fasting blood glucose (FBG) levels. Rats with FBG level above 300 mg/dl were considered diabetic and selected for the study. Treatment commenced on the 4^{th} day and continued for a period of 28 days.

8.2.4 Experimental design and biochemical analysis

Animals were divided into seven groups of ten animals each and treated as follows: Group 1 (the control) was given citrate buffer only. Group 2 (PI 200ND), non-diabetic rats, was given citrate buffer and the protein isolate (200 mg/kg body weight). Group 3 (PI 400ND), non-diabetic rats, was given citrate buffer and the protein isolate (400 mg/kg body weight). The STZ-induced diabetic rats were divided in four groups (Groups 4–7). Group 4 (STZ) was the diabetic control group. Group 5 (STZ I), the positive control group, was given insulin (5 U/kg, i.p.). Group 6 (STZ PI 200) was diabetic and received protein isolate (200 mg/kg body weight). Group 7 (STZ PI 400) comprised diabetic rats that received the protein isolate (400 mg/kg body weight). Treatments were given orally for 28 days.

At the end of the 28 days of treatment, the rats were fasted overnight and then sacrificed under anesthesia. Blood samples were obtained by cardiac puncture in plain tubes without anticoagulants, left for 1 hour to coagulate, then centrifuged at 3000 rpm for 15 min at 4 °C to obtain the serum. The livers were collected, washed in saline, blotted dry and weighed. Portions of the livers were homogenized in 56 mM Tris-HCl buffer (pH 7.4) containing 1.15 % KCl, and then centrifuged at 10,000 rpm for 15 minutes to obtain the supernatants, which were subsequently stored at −80 °C an until needed for analysis. The other liver portions were preserved in 10 % formalin and used for the histological assessment. Histology studies were carried out at the Vet Diagnostix Laboratories (Pietermaritzburg, SA) by a qualified pathologist having no prior knowledge of the treatment of the samples. This method allowed for an unbiased description of the histological lesions which were present if any in the samples. The liver tissues were stained with haematoxylin and eosin (H & E).

The extent of lipid peroxidation in the liver homogenate was determined spectrophotometrically by measuring the formation of the thiobarbituric acid reactive substances (TBARS) and according to the method described by Varshney and Kale (1990). This was expressed in terms of the malondialdehyde (MDA), which is the end product of the reaction. Serum activity of ALT, AST and the concentrations of the total protein were measured by through the use of commercial kits (Randox Laboratories Ltd., UK) specific to each test. The concentrations of the serum interleukin-6 (IL-6) were estimated using the enzyme-linked immunosorbent assay kit

(Thermo Scientific, USA), while the hepatic levels of the total glutathione (total GSH), as well as the activities of glutathione-S-transferases (GST) superoxide dismutase (SOD) and catalase (CAT) were determined by a use of the corresponding assaying kits (ScienCell Research Laboratories, Carlsbad, USA) an in accordance with the manufacturer's instructions.

8.2.5 HPLC analysis

The HPLC analysis of the *Parkia biglobosa* protein isolate (PBPi) was carried out at the University of the Western Cape. The chromatographic system consisted of: the Beckman HPLC system consisting of a double pump Programmable Solvent Module model 126; a Diode Array detector module model 160; a Samsung computer 386 with management System Gold (V601) software applied by Beckman, a column C18 Bondapak 5 µm and dimensions of 250 x 4.6 mm^2. The chromatographic conditions for the mobile phase were: solvent A of 1 % acetic acid; solvent B of methanol; the mode had a gradient flow rate of 1 min/min; the injection volume was 10 µl; and the UV detection was at 350 nm. The HPLC operating conditions were programmed to give the following: 0 min, solvent B: 20 %; 5 min, solvent B: 40 %; 15 min, solvent B: 60 %; 20 min, solvent B: 80 % at 27 min. The run rate was 30 min (Loonat and Amabeoku, 2014).

8.2.6 Statistical analysis

All obtained data was presented as the mean ± standard deviation. The data were analyzed a by one-way ANOVA followed by the Duncan's multiple ranges (SPSS13.0, Inc., Chicago, IL, USA). The differences were considered significant at $P < 0.05$.

8.3 Results

8.3.1 HPLC analysis

The HPLC fingerprint (Figure 8.1) of the *Parkia biglobosa* protein isolate obtained revealed 11 distinct peaks at the following retention times (in minutes): 2.716, 2.780, 3.040, 3.514, 3.676, 4.225, 4.414, 10.526, 29.616, 29.914 and 30.295; and 5 minor

peaks at the following retention times (in minutes): 6.167, 7.287, 24.135, 28.774 and 31.341.

8.3.2 The effect of PBPi on serum liver function

Presented in Figures 8.2a, 8.2b and 8.2c are the effects of PI on serum liver function indices (AST, ALT and total protein respectively). The serum activities of AST and ALT were significantly elevated with a concomitant decrease in the total protein levels in STZ-induced diabetic rats when compared to the controls. Treatment with the PBPi attenuated these changes in a dose-dependent manner.

8.3.3 The effect of PBPi on serum interleukin-6 (IL-6)

The effect of PI on serum interIeukin-6 is presented in figure 8.2d. In diabetic rats, there was a significant increase in the serum interleukin-6 levels when compared to the controls. When PI was administered to the diabetic rats, there was a significant down-regulation in the production of the interleukin-6. This was exhibited in a dose-dependent manner and comparable to that of insulin (Figure 8.2d).

8.3.4 The effect of PI on the assessment of oxidative stress and antioxidants

The effect of PI on the assessment of oxidative stress and antioxidants in 8.3a-e. STZ caused a substantial increase in the hepatic TBARS content, which was associated with a concomitant significant depiction in hepatic total GSH content as well as the GST, SOD and CAT activities, compared to the control. These changes were ameliorated when the PBPi was administered for 28 days (Figure 8.3).

8.3.5 The effect of PBPi on liver histopathological examination

The histological investigation of the liver tissues showed (Figure 8.4) a normal architecture in the case of the control, PI 200ND and PI 400ND groups. The portal tracts were within the normal limits, with no inflammatory cell infiltrates identified. The hepatocytes also showed a normal histology with no steatosis, inflammation, necrosis or cholestasis. In the case of the STZ group, a histopathological examination of the liver sections demonstrated that the portal tracts were expanded by a severe lymphocytic infiltrate associated with the necrotic hepatocytes focally.

(These are features of lobular and portal tract chronic inflammation and focal hepatocyte necrosis). In the STZ I group, sections showed liver tissue with a mild lymphocytic infiltrate present within the portal tracts. The inflammatory process was also observed within the hepatocytes, with mild focal necro-inflammatory damages. Very mild lymphocytic infiltrate were observed within the portal tracts and the lobules of the STZ PI 200 group, with mild focal necro-inflammatory changes. In addition, there was a very mild macrovesicular steatosis. In the STZ PI 400 group, sections demonstrated liver tissues with the normal architecture. A lymphocytic infiltrate was also identified within the portal tracts, with no evidence of interface hepatitis. Regenerative changes were seen within the hepatocytes even though there were very mild necro-inflammatory foci.

8.4 Discussion

Increasing experimental evidence has demonstrated that an STZ-induced toxicity in animal models is characterized by insulin deficiency and hyperglycaemia (Sartori *et al*., 2009). When this condition is not properly managed, it results in extremely high intracellular ROS production which is capable of interfering with the structural macromolecules that, in turn, facilitates functional abnormalities as well as histological alterations (Hassan *et al*., 2015; Irudayaraj *et al*., 2012; Okechukwu *et al*., 2013).

Serum transaminases (ALT and AST) are important indicators of the hepatocellular injury. These enzymes are usually abundant in the liver, where they play a central role in amino acid metabolism. STZ-toxicity has been characterized by changes in the permeability of the liver membrane and a cellular leakage of ALT and AST from the hepatocytes into the blood stream. In this study, the activities of these enzymes (ALT and AST) in the serum of the STZ-induced diabetic rats were markedly elevated. This suggests that there is a certain degree of damage to the liver. Similar results have also been reported by Adeyemi *et al*., 2014; Luke *et al*., 2013 and Erejuwa *et al.,* 2012. Treatment with the PBPi at both doses (200 and 400 mg/kg b.wt), considerably decreased the elevated levels of ALT and ALT in serum of the STZ-induced diabetic rats. Apparently, PBPi plays a protective role in STZ-induced diabetic rats.

Importantly, lapses in amino acid/protein metabolism as a result of a deficiency in insulin secretion and / or inadequate insulin in STZ-induced diabetes is a more critical factor than hyperglycaemia associated with some diabetic complications (Pradeepa *et al*., 2013). Documented experimental evidence demonstrates that the STZ-diabetic rat models show numerous alterations in amino acid metabolism. This has been linked to the increased muscle proteolysis, reduced protein synthesis, some energy dependent processes in the liver, and a stimulated hepatic gluconeogenesis utilizing gluconeogenic amino acids (Pradeepa *et al*., 2013; Rao and Adinew, 2011).

In this study, a significant reduction in the total serum protein levels was observed in the STZ-induced diabetic rats. The reduction was significantly reversed after a treatment with the PBPi in a dose-dependent manner. This is in agreement with similar studies reported in the literature (Balamurugan *et al.,* 2014; Gopalakrishnan and Dhanapal, 2014). This could be attributed to the nutritional quality (high protein content and a broad spectrum of essential amino acids) of PBPi.

It has been established that the elevated levels of the inflammatory mediators, such as the proinflammatory cytokines (IL-6 and IL-1β) in diabetes and its associated complications, are as a result of hyperglycaemia and these mediators have been considered to be the link between inflammation and insulin resistance (Uyanik *et* al., 2012; Niture *et al.,* 2014). In agreement with similar previous studies (Al-Enazi, 2014; Al-Malki and Rabey, 2015; Kuate *et al.,* 2015), treatment with PI significantly inhibited the elevated levels of the IL-6 in the diabetic rats at the doses tested. The observed effect suggests that PI possess some immunomodulatory and anti-inflammatory properties.

It is believed that in STZ-diabetic conditions, hyperglycaemia produces ROS that exceed the endogenous antioxidant capacity. These ROS may react with polyunsaturated fatty acids of cell membranes, thereby causing lipid peroxidation and membrane damage (Shaikh and Shrivatava, 2014, Ebong *et al.,* 2011). The results of the present study revealed that TBARS levels in the livers of the STZ-induced diabetic rats were markedly elevated when compared with the controls. The concurrent treatment of diabetic rats with the PBPi (200 and 400 mg/kg b.wt) showed a diminished level of the TBARS. Our findings are therefore in agreement with the

earlier experimental reports (Adewole and Ojewole 2009; Murali *et al*., 2013; Singh *et al*., 2013). The observed effect suggests that there was an amelioration of the oxidative stress in diabetic rats, which may be due to the free radical scavenging activity of the PBPi.

Besides the elevation witnessed in the TBARS levels in the STZ-induced diabetic rats, our results demonstrated that there was a concomitant decline in the hepatic antioxidant capacity, notably, the total GSH, GST, SOD and CAT. Hyperglycaemia is known to inactivate the activities of the antioxidative enzymes in diabetic model animals, which may involve a non-enzymatic glycosylation (Fatani *et al.,* 2015). Antioxidant enzymes as well as the nonenzymatic antioxidants are the first line of defence against ROS induced oxidative damage in living organisms (Hassan *et al.,* 2015).

Added to this, SOD and CAT play an essential role in attenuating cellular stress by maintaining the physiological levels of oxygen and hydrogen peroxide. SOD scavenges the superoxide radicals by converting them to hydrogen peroxide and molecular oxygen, while the CAT brings about a reduction of the hydrogen peroxides and protects higher tissues from the highly reactive hydroxyl radicals (Shaikh and Shrivastava, 2014; Shilpa *et al.,* 2012). Interestingly, an administration of PBPi to the STZ-induced diabetic rats considerably revert the hepatic antioxidant status in a dose-dependant manner that was near normal when compared to the control groups.

The HPLC fingerprint obtained for the PBPi revealed 11 distinct peaks and 5 minor peaks, which will be helpful in providing new leads in the future identification of the bioactive components that are, individually and/or synergistically, responsible for the protective effect of PBPi in this study. The protective effect of the PBPi was, furthermore, strongly supported by the results of the histopathological examination (Figure 3).

It is apparent from the data obtained in this study that the PBPi possesses some anti-inflammatory and antioxidant properties capable of protecting against the STZ-induced hepatic injuries and oxidative stresses in diabetic animal models. Therefore, PI is a promising candidate in the ongoing search for novel therapies in diabetes management.

8.5 Acknowledgements

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8.6 Conflict of interest

None declared.

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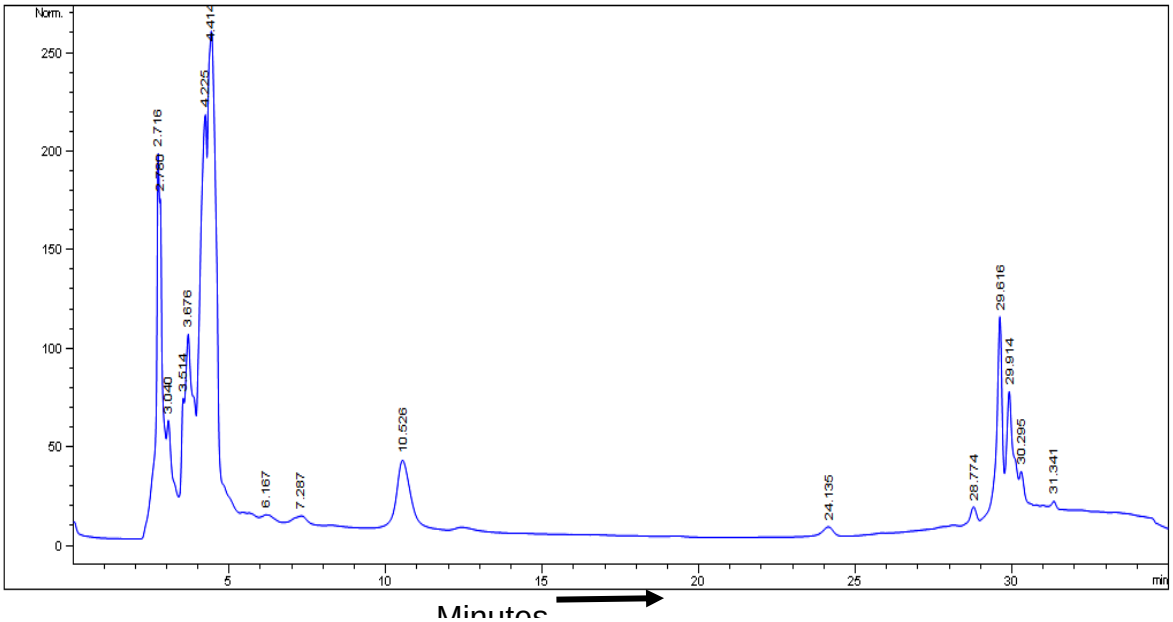

Figure 8.1: HPLC fingerprint of *Parkia biglobosa* protein isolate.

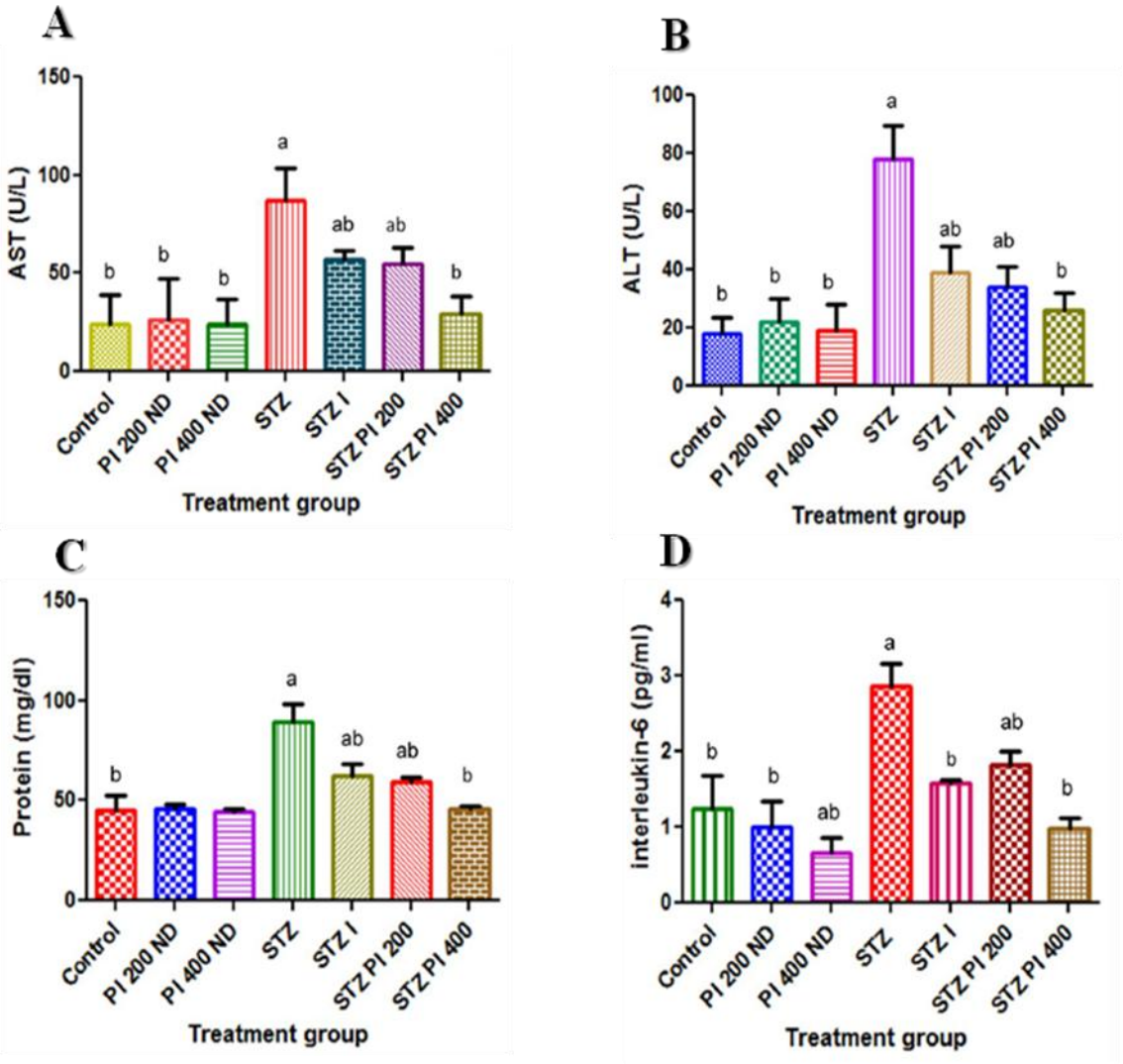

Figure 8.2: Effect of PI on serum liver function and serum interleukin-6 (IL-6).

a) AST (U/L), b) ALT (U/L), c) Protein (mg/dl), d) Interleukin-6 (pg/mL). Data are presented as a mean ± S.D. (n = 10). The mean differences are significant ($P < 0.05$) when compared with [a] control group and [b] STZ only.

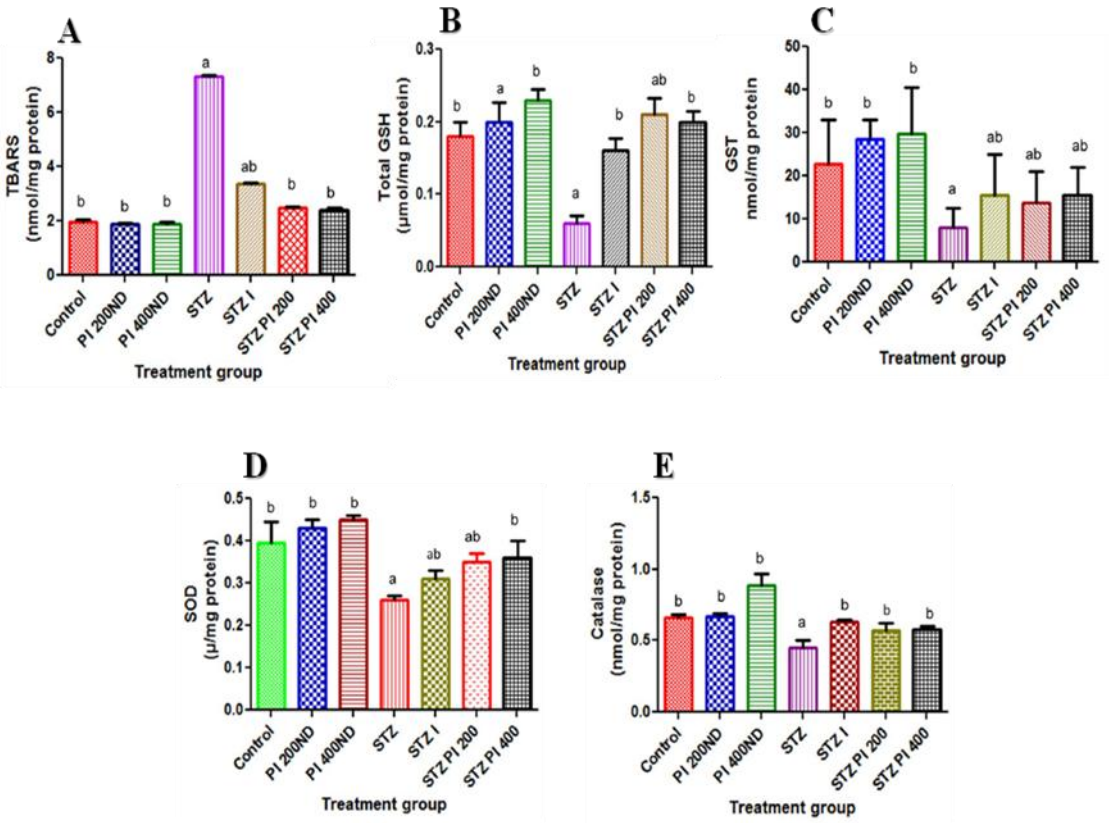

Figure 8.3: Effect of PI on assessment of oxidative stress and antioxidants.

a) TBARS (nmol/mg protein), b) total GSH (µmol/mg protein), c) GST (nmol/mg protein), d) SOD (µ/mg protein), e) CAT (nmol/mg protein). Data are presented as a mean ± S.D. (n = 10). Mean differences are significant ($P < 0.05$) when compared with: [a] control group and [b] STZ only.

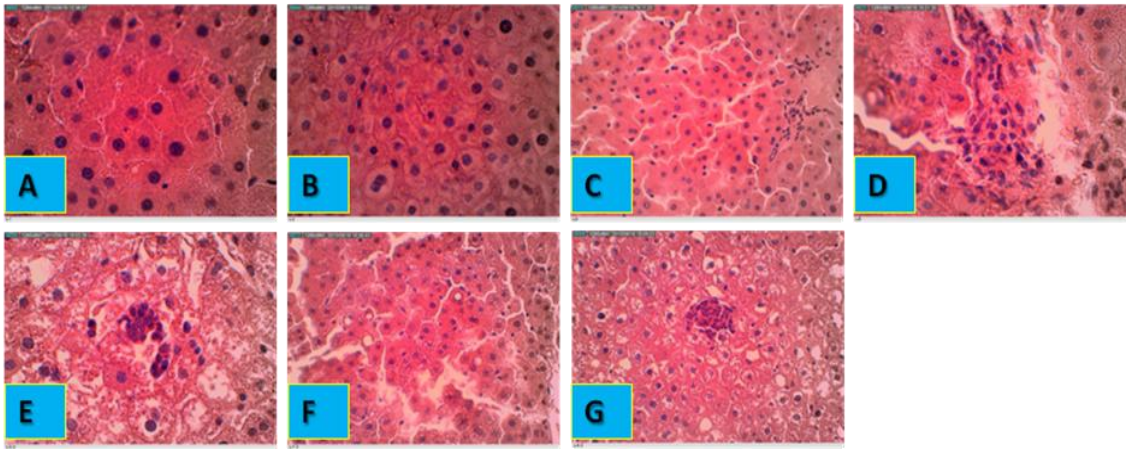

Figure 8.4: Histological examination of rat livers stained with hematoxylin and eosin (H&E).

(A) Control: showing liver tissue with normal architecture; (B) PI 200ND: showing liver tissue with normal architecture; (C) PI 400ND: showing liver tissue with normal architecture; (D) STZ: showing expanded portal tracts by a severe lymphocytic infiltrate associated with necrotic hepatocytes focally; (E) STZ I: showing liver tissue with a mild lymphocytic infiltrate present within the portal tracts (F) STZ PI 200: showing the presence of very mild lymphocytic infiltrate within the portal tracts and the lobules, with mild focal necro-inflammatory changes; and (G) STZ PI 400: showing regenerative changes within the hepatocytes even though there are very mild necro-inflammatory foci.(×400)

Chapter Nine

9.0 Proteomic analysis of the differentially expressed proteins in livers of streptozotocin-induced diabetic rats treated with the *Parkia biglobosa* protein isolate

Running Title: Analysis of liver proteome in STZ-diabetic rats treated with *Parkia biglobosa* protein isolate

Abstract

Protein isolate from *Parkia biglobosa* is believed to possess excellent antidiabetic properties. The purpose of this study was to identify differentially expressed protein in the livers of streptozotocin-induced diabetic rats treated with a *Parkia biglobosa* protein isolate (PBPi). In this regard, total proteins extracted from the rat livers were separated on a one-dimensional polyacrylamide gel electrophoresis (1D SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB) to visualize the protein bands. It was observed that the protein bands in the region of 10 – 15 kDa were altered by the different treatments. These bands were then selected and cut for an in-gel digestion and peptide extraction followed by nLC-MS, MALDI-TOF MS and LIFT MS/MS. Database search with the Mascot algorithm positively identified 4 differentially expressed proteins. These proteins are known to be responsible for diverse biological functions within various organs and tissues. The present result gives an insight and a possible understanding into the molecular mechanisms by which streptozotocin causes various alterations in the liver proteins of diabetic rats and a possible modulatory role of PBPi in the management of diabetes in streptozotocin-induced rats.

Keywords: MALDI-TOF-MS**,** nLC-MS *Parkia biglobosa*, proteomics, rat liver, STZ-diabetes

9.1 Introduction

Multiple-organ failure has emerged as one of the most significant cause of death associated with diabetes mellitus in both the developing and developed countries globally (Singh *et al*., 2013). Diabetes can be classified into two broad categories: type 1 diabetes (insulin dependent diabetes mellitus) and type 2 diabetes (non-insulin dependent diabetes mellitus) (Ogunyinka *et al*., 2015). Studies have shown that in 2010, about 280 million individuals had diabetes, with type 2 comprising of about 90 % of the total cases worldwide; while it has been projected that by 2030, the number of individuals suffering from diabetes would have increased to about 430 million, due to the recent increase in the prevalence of this disease globally (Chang *et al*., 2011; Surya *et al*., 2014).

Diabetes mellitus is an endocrine metabolic disorder identified by an abnormal increase in the blood sugar level (hyperglycemia) as well as an alteration in the lipid, carbohydrate and protein metabolism, which is linked to the low levels of insulin production or an insensitivity of the target organs to insulin (Arumugam *et al*., 2013; Ramachandran *et al*., 2012). Sustained hyperglycemia can lead to disturbances in the cell structure and functions of organs (Karthik *et al*., 2012). It also triggers the activation of the polyol pathway, enhances the sorbitol and fructose accumulation, increases the intracellular formation of the advanced glycation end products, activates protein kinase C and nuclear factor ƙB as well as increasing the hexosamine pathway flux. This cascade of events leads to oxidative stress and as a result of the overproduction of the superoxide radicals by the mitochondrial electron transport chain (Colette and Monnier, 2007; Monnier *et al*., 2006).

Oxidative stress in the biological systems is a state of an imbalance that occurs when the levels of free radical (notably reactive oxygen species) production exceed the cellular antioxidants capacities and usually as a result of the various metabolic abnormalities within the body (Ogunyinka *et al*., 2015; Oyinloye *et al*., 2015). The hyperglycemia-induced oxidative stress apparently accounts for the majority of the diabetic complications (Singh *et al*., 2013). From time immemorial, medicinal plants have been used by traditional healers who believed in their potency for maintaining good health in the treatment of various ailments including diabetes and its associated complications (Karthik *et al*., 2012; Maiti *et al*., 2004). Medicinal plants have constantly been an excellent source of drugs and several of the currently obtainable drugs have surely been derived directly or indirectly from plants (Grover *et al*., 2002). *Parkia biglobosa* is one of them. It is commonly used in Nigeria and many other West African countries as a spice for the flavouring of foods (Ogunyinka *et al*., 2015).

It was recently reported that the *P. biglobosa* (aqueous and methanolic) extracts possess some active phytochemicals which are responsible for this plant nutritional and pharmacological characteristics such as hepatoprotective, hypoglycaemic, hypolipidaemic, antimicrobial and anti-inflammatory activities in experimental animal modes (Adi *et al*., 2013; Odetola *et al*., 2006; Ogunyinka *et al*., 2015). The liver plays a very important part, as it acts as a storehouse for glycogen, which is available for maintaining the normal levels of the blood sugar and the needs of the body. The liver acts to equilibrate the uptake and storage of glucose via glycogenesis and regulates the release of glucose by activating the process of glycogenolysis and gluconeogenesis (Visweswara Rao *et al*., 2013). To understand the underlying molecular and cellular mechanisms of diabetes and systematic cellular alterations in

health and diseases, it is important to evaluate the levels of alterations in the composition of proteins in cells and tissues (Karthik *et al*., 2012; Karthik and Ravikumar, 2011).

Differential protein expression analysis is the most recent and powerful experimental approach that allows a systematic and comparative analysis of proteomic changes (Karthik *et al*., 2012; Karthik and Ravikumar, 2011) This approach uses a combination of two-dimensional gel electrophoresis (2-DE), image analysis, matrix-assisted laser desorption / ionization-time of flight (MALDI-TOF), mass spectrometry (MS), and bioinformatics analyses to comprehensively resolve, identify and characterize proteins in the cells, tissues and animal models (Nagappan *et al*., 2013). Karthik and co-workers (2011), documented that the term ‘‘differentially expressed protein’’ is used to indicate that a protein which plays a precise biological role was significantly decreased or increased in concentration when compared with normal control. Differential expression can be an outcome of disease-related alterations in transcription, protein synthesis, transport, degradation and / or covalent modification (Karthik and Ravikumar, 2011).

This proteomic approach has been employed to fish out proteins that are responsible for or are related to some abnormal functions in various cells and tissues (Karthik and Ravikumar, 2011). In our own proteomic study, we have analyze the proteomic changes in the livers of STZ-induced diabetic rats in relation to the possible effects of the *Parkia biglobosa* protein isolate treatments at varying dosages.

9.2 Materials and Methods

9.2.1 Chemicals

All the chemicals and reagents used this study were procured from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (South Africa), they were analytical grade or equivalent.

9.2.2 Plant material and extract preparation

Fermented *Parkia biglobosa* seeds were obtained from a local market in Ijebu-Ode, Ogun state, Nigeria. An import permit (P0060156) from the Department of Agriculture, Forestry and Fisheries (DAFF; Republic of South Africa) was obtained. The seed sample was authenticated in the Department of Botany, University of Zululand and a voucher specimen (B07) was deposited in University herbarium.

The fermented seeds of *Parkia biglobosa* were oven-dried (50 °C) and pulverized using an electric blender to obtain a coarse powder. One kilogram of a uniform powdered seeds was defatted with hexane to obtain the defatted extract. The defatted extract was air dried and then extracted (1: 10 w/v) with acidic butanol to remove any possible anti-nutrients. A protein isolate obtained from the defatted extract using the method described by Nkosi et al. (2005). Briefly, the dry defatted extract was re-suspended in distilled water at pH 10. The resultant suspension was then filtered to remove debris and the filtrate adjusted to pH 5, followed by centrifugation at 5000 rpm for 15 minutes at 4 °C. The supernatant was discarded while the pellet containing the protein isolate was retained and freeze-dried to yield a brown extract. The lyophilized extract was kept dry until needed.

9.2.3 Experimental animals and experimental design

The Sprague-Dowley rats (weighing about 250 – 290) used in this study was obtained from the animal house of the Department of Biochemistry and Microbiology, University of Zululand. The animals were kept for acclimatization for 7 days prior to the commencement of the study; they were maintained at standard conditions of temperature and relative humidity, with a 12-hour light/dark cycle. The animals were provided with standard rat pellets and water *ad libitum*. The animal experimental protocols were in accordance with the recommendations of the institutional animal ethical committee (UZREC 171110-030-RA level 02 Dept 2014/74).

9.2.4 Induction of diabetes and experimental design

Following an overnight fasting, diabetes was induced in the selected rats by a single intraperitoneal injection of freshly prepared STZ (Sigma-Aldrich Co.) at a dose of 60 mg/kg body weight; dissolved in 0.1M, pH 4.5 ice cold citrate buffer (Hule *et al*., 2011). Diabetes was confirmed in rats 72 hours after STZ administration by measuring the fasting blood glucose (FBG) levels. Rats with FBG level above 300 mg/dl were considered diabetic and selected for the study. Treatment commenced on the fourth day and continued for a period of twenty-eight days. Forty healthy, male rats (averaging 12 weeks old), were divided into four groups of ten animals each and treated as follows: Group 1 (control) was given citrate buffer only. Group 2 (STZ), diabetic control. Group 3 (STZ PI 200) was diabetic and received protein isolate (200 mg/kg body weight). Group 4 (STZ PI 400) diabetic rats that received protein isolate (400 mg/kg body weight). Treatments were given orally for 28 days.

9.2.5 Processing of tissue samples and protein extraction

At the end of the 28 days of treatment, the rats were fasted overnight and then sacrificed under anesthesia. The livers were removed, washed in saline, blotted dry

and weighed. Total proteins from the liver of the experimental animals were extracted from approximately 800 mg of the freshly collected liver samples from each group. The liver samples were homogenized on ice with an IKA T25 Ultra Turrax high speed homogenizer in ice cold RIPA (protein extraction) buffer comprising of (50 mM Tris-HCl, 1 % Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.02 % NaN^3, 100 mM PMSF) pH 7.4; this was then prepared by adding some freshly prepared protease inhibitors and DTT (10 mM). The homogenized samples were kept on ice for 30 minutes and vortexed at intervals. Subsequently, the homogenates were centrifuged at 13,200 rpm for 10 min at 4 °C to pellet the debris. The supernatant was immediately collected and the total protein concentration estimated by measuring absorbance at A 280 nm with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Thereafter, the supernatants containing the protein samples were stored at – 80 °C, and until needed for further use.

9.2.6 1-D SDS-PAGE, In-gel digestion and peptide extraction

Twenty micrograms of the liver proteins of the diabetic rats and healthy controls were separated on 1-DS-PAGE gels. Gel bands were destained with 200 µl of 50 % acetonitrile/25 mM ammonium bicarbonate (NH_4HCO_3) until clear. Samples were dehydrated and desiccated with 100 µl acetonitrile (ACN) before a reduction with 2 mM triscarboxyethyl phosphine (TCEP) in 25 mM NH_4HCO_3 for 15 minutes at room temperature with agitation. Excess TCEP removed and cystein residues were carbamidomethylated with 20 mM iodoacetamide (Sigma) in 25 mM NH_4HCO_3 for 30 minutes at room temperature and in the dark. After carbamidomethylation the gel pieces were washed with 25 mM NH_4HCO_3 followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in trypsin (Promega) solution (10 ng/uL) and incubating at 37 °C overnight. Peptides were extracted from the gel

pieces once with a 30 % acetonitrile; 0.1% trifluoroaceticacid (TFA) solution (Sigma) for 45 minutes at room temperature and with occasional vortexing. The samples were dried down to remove any residual NH_4HCO_3 and were re-dissolved in to 0.1 % TFA and were purified and concentrated using a C_{18} ZipTip® and in accordance with the manufacturer's instructions. The purified samples were eluted in 50 % acetonitrile/H_2O containing a 0.1 % trifluoroacetic acid (TFA) and dried in a speed vac followed by a resuspension in 10 ul 0.1 % TFA.

9.2.7 The nLC procedure

Peptides from the purified samples (bands in the region of 10 – 15 kDa: lane 2, lane 4, lane 6 and lane 8) were separated using an nLC-MS. All experiments were performed on a Thermo Scientific EASY-nLC II connected to a Proteineer fc II protein spotter controlled through HyStar software. For liquid chromatography, separation was performed on an EASY column (2 cm, 75 µm ID, 5 µm, C 18) pre-column followed by an analytical column (10 cm, 75 µm ID, 3 µm, C 18) with a flow rate of 100 µl/hr under a 48-minute gradient run.

9.2.8 Mass spectrometry

MALDI-TOF MS and LIFT MS/MS was performed using an UltrafleXtreme MALDI ToF/ToF system (Bruker Daltonics, Bremen, Germany) with an instrument control Flex control 3.4. Peptides were ionized with a 337 nm laser and spectra acquired in a reflector positive mode at 28 kV using 100 laser shots per spectrum with a scan range of m/z = 700 -4000. Spectra were internally calibrated using a peptide calibration standard II (Bruker Daltonics, Bremen, Germany). Peptide spectra of accumulated 4,000 shots were automatically processed using WARP LC 3.2 software (Bruker Daltonics, Bremen, Germany).

9.2.9 Data analysis

Database interrogation was performed with the Mascot algorithm using the SwissProt database on a ProteinScape 3.0 workstation. The search parameters were as follows: Taxonomy- *Rattus*, Enzyme-trypsin, Missed cleavages-1, Fixed modification-carbamidomethyl (C), Variable modification - Oxidation (M) and Deamidation (NQ), Precursor tolerance-50 ppm, Fragment tolerance -0.7 Da. Candidate protein matches with a molecular weight search (MOWSE) score of greater than 22 were considered as the identified proteins.

9.3 Results

Total protein extracts from the livers of the experimental animals were separated on 1D SDS-PAGE. The molecular weight distribution of the total proteins from the livers of the experimental animals (healthy control, untreated diabetic rats and treated diabetic rats; 200 or 400 mg/kg bw PBPi) are shown in Figure 9.1 The proteins were well separated, the protein bands in the region of 10 – 15 kDa were altered by the different treatments; these bands were then selected and cut for the subsequent in-gel digestion and peptide extraction followed by nLC-MS, MALDI-TOF MS and LIFT MS/MS in order to fully identify the proteins.

Protein identification was carried out by database interrogation. The peptide masses of each protein sample obtained by the MALDI-TOF MS and LIFT MS/MS were submitted to MASCOT and searched against the Swiss-Prot database. This resulted in the positive identification of four (4) differentially expressed proteins, Table 9.1 and 9.2 (candidate protein matches with a MOWSE score greater than 22 were considered as identified proteins).

9.4 Discussion

Diabetes mellitus is a group of metabolic irregularities characterised by hyperglycaemia that is emerging at an alarming rate globally with serious health complications (Surya *et al*., 2014; Zhang *et al*., 2015). Many of the current therapies for diabetes (insulin and other oral anti-diabetic agents) have some limitations and side effects, such as hypoglycemia, diarrhea, liver and kidney failure, just to mention a few (Zhang *et al*., 2015). At the moment, there are a considerable number of studies on the potential benefit of plants and plant extracts in the prevention, treatment and management of diabetes and its related complications (Dinić *et al*., 2013; Ogunyinka *et al*., 2015; Pandey *et al*., 2011). It has recently been believed that any alterations at molecular (protein structure, function, production and interactions) level play an important role, in part if not completely, in the pathogenesis of many metabolic diseases including diabetes; and contributes significantly to the underlying mechanisms of these diseases (López-Villar *et al*., 2015).

In view of the fact that diabetes is a polygenic disease with expected alterations in many proteins; proteomics is therefore a powerful approach to study the pathophysiological mechanisms of alteration in these proteins as well as the mechanism of action of PBPi remedies in STZ-induced diabetic rats (Awdeh *et al*., 2006; Gerich, 1998; Lao *et al*., 2014;). The present study demonstrated that STZ-induced diabetes resulted in a decreased expression of most of the proteins identified in this study. Treatment with PI (200 or 400 mg/kg bw) substantially up-regulated their expression in a dose-dependent manner. This complemented the previously identified protective role of *Parkia biglobosa* protein isolate in human health and diseases.

The thioredoxin OS=Rattus norvegicus GN=Txn PE=1 SV=2 (THIO_RAT) was one of the differentially expressed proteins identified in the present study. The biological system possess elaborate defence mechanisms which prevent against cellular damage, this involves metabolic enzymes such as the superoxide dismutase, catalase and glutathione peroxidise for free radical detoxification as well as non-enzymatic molecules such as thioredoxin, thiols and vitamins E, C and trace metals, such as selenium, which function as direct scavengers of ROS (Mishra and Vijayakumar, 2014). Thioredoxin alterations have been previously reported in many diseases including diabetes and its related complications (Maulik and Das, 2008).

Hyperglycaemia promotes the thioredoxin/Txnip interaction leading to a functional inhibition of thioredoxin antioxidative role. This particular interaction alters the cellular redox balance and enhances intracellular oxidative stress (Schulze *et al*. 2004). We thought that the down regulation of thioredoxin in the treated groups is as a result of the overwhelming effects of ROS generation on the endogenous thioredoxin system. Therefore, we suggest that one of the mechanisms by which STZ may elicit its deleterious metabolic effect is through the down regulation of the endogenous thioredoxin activity.

The profilin-1 OS=Rattus norvegicus GN=Pfn1 PE=1 SV=2 (PROF1_RAT) was also found to be differentially expressed in this study. Profilin-1 is a small ubiquitous actin-binding protein, (12 to 15 kD) widely distributed in various types of cells with very highly conserved sequences (Pae and Romeo, 2014; Li *et al*., 2013). It binds to G-actin and motivates the exchange of ADP to ATP on the G-actin, thus controlling the pool of ATP–actin within the cell (Pae and Romeo, 2014). Profilin-1 plays a significant role in the processes of endothelial dysfunction and atherosclerosis; and

endothelial dysfunction has been identified and implicated as an underling factor preceding the structural alterations in diabetic vascular diseases, eventhough its molecular bases is still poorly understood (Romeo and Kazlauskas, 2008).

Added to this, the up-regulation of profilin-1 has also been documented in the aortic endothelium of diabetic individuals and in STZ-induced diabetic animal models (Romeo *et al*., 2004). In this study, an induction of diabetes using STZ, also increased the expression profice of the profilin-1 in the diabetic untreated groups while a treatment with PBPi at the tested dosages decreased the expression profice of the profiling-1. This is an indication that PBPi can prevent or delay various complications associated with diabetics.

Furthermore, mitochondrial pyruvate carrier 1 OS=Rattus norvegicus GN=Mpc1 PE=2 SV=1 (MPC1_RAT) was was differentially expressed by the different treatments in this study. Mitochondrial pyruvate carriers (MPC) are extremely conserved proteins that are essential for pyruvate uptake at the mitochondrial inner membrane (Timón-Gómez *et al*., 2013). The MPC complex comprises two paralogous subunits, MPC1 and MPC2; approximately 12 kDa and 14 kDa with 2 and 3 predicted transmembrane domains respectively (Gray *et al*., 2016). Even though their existence has been known for over 40 years, it is only recently that these proteins (MPG) are being identified at the molecular level (Divakaruni *et al*., 2013; Schell and Rutter, 2013). Their contribution in the control of cell fate in cancer and gluconeogenesis in type 2 diabetes models has been demonstrated in recent documented reports. Therefore, biochemical evaluations of MPC activities are foundational for a better understanding of the roles of pyruvate transport in health and diseases (Gray *et al*., 2016).

Though little is known about how pyruvate is transported into mitochondria in β-cells; it is well established in literature that mitochondrial metabolism of pyruvate is crucial for insulin secretion (Patterson *et al*., 2014). Loss of either MPC1 or MPC2 protein may result in a destabilization and / or degradation of the other and consequently a loss of the MPC complex. It has recently been demonstrated that the MPC may be a point of altered metabolic regulation in disease states such as cancer, obesity, and type 2 diabetes (Gray *et al*., 2016). Pyruvate is the end product of glycolysis and a fundamental metabolite linking lipid synthesis, amino acid biosynthesis, and gluconeogenesis (Bricker *et al*., 2012). Although gluconeogenesis plays a vital role in blood glucose homeostasis during fasting, it has also been implicated in sustaining hyperglycemia in insulin resistance and type 2 diabetes; therefore, maintaining a critical balance between energy demand and supply is thus very essential for health (Bender and Martinou, 2016).

Owing to the central metabolic position occupied by the MPC, alterations in MPC activities, either during post-translational modifications or protein abundance, may intensely regulate overall cellular metabolism. In this study, it is worth noting that MPC 1 was not expressed at all in the control, STZ and STZ PI 200 groups. Interestingly, treatment with PI 400 mg/kg bw did contribute towards the enhanced abundance of this protein in STZ PI 400 group. Taking into account the MOWSE score, number of peptides and the sequence coverages of this protein in our diabetic animal model in this experiment, we believe that the increased expression of this protein is not detrimental as it will contribute positively towards the metabolic regulation of pyruvate in the diseased state. We suggest that this is one of the mechanisms by which PI exhibits its protective effects in the prevention and management of diabetics.

Additionally, fatty acid-binding protein, liver OS=Rattus norvegicus GN=Fabp1 PE=1 SV=1 (FABPL_RAT) was also found to be differentially expressed by the different treatments in this study. Fatty acid binding proteins (FABPs) are abundantly expressed as 14–15 kDa proteins. They are intracellular lipid chaperones whose biological role and mechanisms of action are still poorly understood; but are believed to be a group of molecules that coordinate lipid responses in cells and are also strongly connected to the metabolic and inflammatory pathways (Furuhashi and Hotamisligil, 2008; Maeda *et al*., 2005). FABPs are also known to facilitate the utilization of lipids in metabolic pathways in adipocytes and other cells. It has been suggested that they play essential role, probably a link between lipid metabolism, hormone action and cellular functions in adipocytes and other cell types, where they contribute towards the systemic energy homeostasis (Maeda *et al*., 2005).

In the present study, the results obtained demonstrated that the expression of fatty acid-binding protein (FABPL_RAT) in the liver was enhanced in the PBPi treated animals in a dose-dependent manner while the expression of this protein was suppressed in the untreated STZ animals. This is similar to the findings of Melki and Abumrad (Melki and Abumrad, 1993). The result suggests that PBPi does contribute towards the enhanced abundance of FABPs in this study. Taken together, this study provides some significant insights into some of the mechanisms underlying the efficacy of PBPi treatment in the management of diabetes in STZ-diabetic animal models.

9.5 Acknowledgements

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9.6 Conflict of Interest: None declared.

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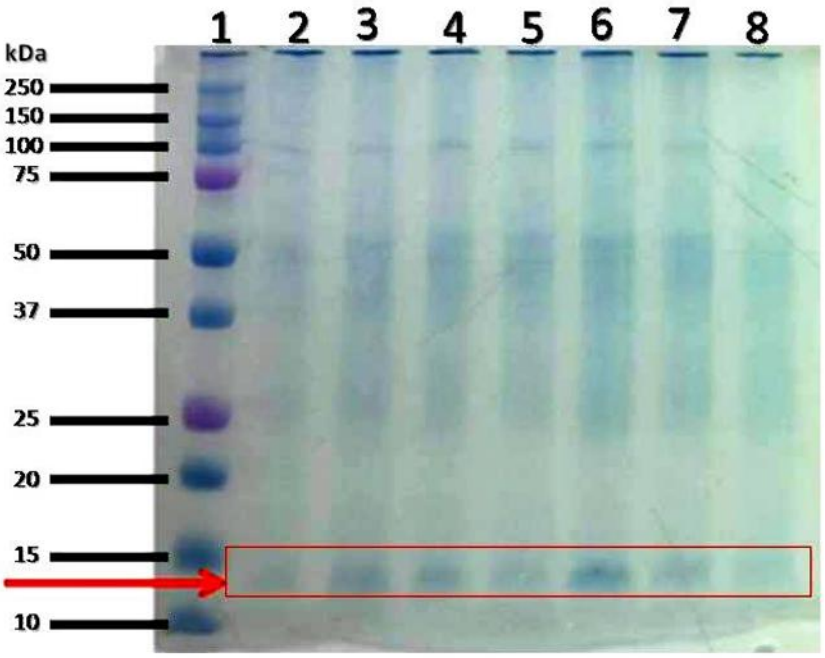

Figure 9.1: 1D SDS-PAGE from the liver of the experimental animals. Proteins (20 µg) were separated by 1D SDS-PAGE and visualized by CBB staining. The protein profiles of four representative samples are shown in this figure. Lane 1: Molecular marker; lane 2: STZ PI 200, lane 4: STZ, lane 6: Control and lane 8: STZ PI 400.

Table 9.1: Summary of all differentially expressed proteins identified form rat liver by nLC-MS

S/N	Accession No	Protein Name	Observed MW(kDa)/pI
1	**THIO_RAT**	Thioredoxin OS=Rattus norvegicus GN=Txn PE=1 SV=2	11.70/4.64
2	**PROF1_RAT**	Profilin-1 OS=Rattus norvegicus GN=Pfn1 PE=1 SV=2	14.90/9.43
3	**MPC1_RAT**	Mitochondrial pyruvate carrier 1 OS=Rattus norvegicus GN=Mpc1 PE=2 SV=1	12.40/10.40
4	**FABPL_RAT**	Fatty acid-binding protein, liver OS=Rattus norvegicus GN=Fabp1 PE=1 SV=1	14.30/8.96

Table 9.2: Differentially expressed proteins identified form rat liver in each experimental group by nLC-MS

		Control		**STZ**		**STZ PI 200**		**STZ PI 400**	
S/N No	**Accession**	**MOWSE Score**	**No. of Peptides / Seq. Coverage**	**MOWSE Score**	**No. of Peptides / Seq. Coverage**	**MOWSE Score**	**No. of Peptides / Seq. Coverage**	**MOWSE Score**	**No. of Peptides / Seq. Coverage**
1	**THIO_RAT**	65.90	1/8.60 (%)	___	___	___	___	___	___
2	**PROF1_RAT**	24.62	1/7.90 (%)	266.82	6/36.70 (%)	___	___	___	___
3	**MPC1_RAT**	___	___	___	___	___	___	23.47	1/7.30 (%)
4	**FABPL_RAT**	275.54	7/33.10 (%)	23.51	1/4.80 (%)	68.58	1/14.20 (%)	126.67	3/31.50 (%)

Chapter Ten

10.1 General Discussion and conclusion

Diabetic mellitus is a metabolic disease associated with chronic hyperglycemia, the major risk factor in diabetic complications (International Diabetes Federation, 2011). Despite the improved knowledge in synthesis of anti-diabetic drugs, mortality related to diabetic diseases is still increasing unabated (Inzucchi *et al.,* 2012). Most currently used anti-diabetic drugs have been reported with side effects and drug resistance. Plant-derived foods therefore have gained prominence in the management of metabolic diseases based on their physio-chemical properties.

In the exploration of anti-diabetic potential of *Parkia biglobosa* seed protein isolate of was investigated in this study. The results revealed that protein isolate of this plant seed has a high protein content. This is similar to reports from other previous studies (Al-Numair and Ahmed, 2008; Olawuni *et al.,* 2012). Proteins play an important role in the natural synthesis and maintenance of body tissues, enzymes and hormones as well as other substances required for healthy functioning (Hayat *et al.,* 2014). Streptozotocin (STZ), a widely accepted diabetogenic agent has the ability to selectively destroy pancreatic insulin-secreting β-cells resulting in tissues having a poor glucose utilization (Lenzen, 2008). Even though there was no attempt to determine the apparent mechanisms of the observed hypoglyceamic effect of PI, the high protein content and excellent amino acid spectrum of this isolate could have prevented the loss in body weight by a reduction in protein degradation and replenishing of protein loss in diabetic

rats. It is worth noting that the protein isolate also contained very high ash and low fat and carbohydrate contents which are all desirable anti-diabetic properties.

The anti-diabetic potential of the PI was further confirmed by the antioxidant activity that it exhibited. Chronic hyperglycaemia in diabetic mellitus deplete the activity of antioxidant enzymes, thus increasing the generation of reactive oxygen species (ROS), which cause cellular damage (Haskins, 2003). Antioxidant enzymes have been demonstrated to improve endothelial dysfunction in diabetic rats (Mohora, 2007). PI significantly ameliorated the low activity of antioxidant enzymes (SOD, CAT, GST and total GSH) and high level of TBARs in the heart tissues of diabetic induced rats. Antioxidant activities of plant protein isolates have also been previously reported (Nkosi et al, 2005, 2006a, b)

Enzymatic antioxidants play a very important role in protecting the cell against the overwhelming deleterious effects of ROS in hyperglycaemia-induced oxidative stress sustaining (Ullah *et al*., 2015). The hepato-protective effects of these protein isolates have been reported in literature (Nkosi *et al.,* 2005) and have been attributed to their antioxidant properties. Brain cell injury or impairment as well as reproductive dysfunction are the common complications of diabetes (Matough *et al*., 2012; Ramzy *et al*., 2014). The protective effect of the PI was further portrayed in its protective effect on diabetic rats' brain and testes. It is apparent that the PI is not toxic in that its consumption decreased hepatocellular injury in diabetic rats as indicated by the results of the PI on liver function enzymes (AST, ALT).

The HPLC fingerprint obtained for PI revealed 11 distinct peaks and 5 minor peaks, which will be helpful in providing new leads for the possible future identification of the bioactive components that are, individually and/or synergistically, responsible for the protective effect of PI in this study.

Database search with the Mascot algorithm using the SwissProt database on a ProteinScape 3.0 workstation, positively identified 4 differentially expressed proteins from PI protein bands. These proteins are known to be responsible for diverse biological functions within various organs and tissues.

10.2 Conclusion

Chronic hyperglycaemia is implicated in diabetic mellitus, and this promotes generation of oxidative stress and inflammatory molecules that contribute to vascular and organ damages in diabetic complication. Protein sources have previously been used in the management of diabetic mellitus. This study revealed that the PI is a good source of protein with essential amino acids, essential fatty acids and vital mineral contents that play important role in the body system. PI normalized the hyperglycemia, dyslipidemia and markers of oxidative stress in diabetic rats' hearts. Likewise, PI also dose dependently attenuated the adverse effects of which on serum testosterone levels and antioxidant status in testis and brain tissues of diabetic rats. The high levels of ALT, AST and inflammatory marker in the serum of diabetic rats were reversed by administration of PI. Therefore, these properties of PI could be considered in the treatment and management of diabetic complications.

10.3 Recommendation for further studies

This research work has demonstrated the anti-diabetic potentials of PI, therefore it is important to further investigate the mechanisms of action by which the protein isolate from *Parkia biglobosa* brings about the ameliorative effects shown.

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APPENDIX A

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Plant Health and Quality

Permit No. P0060156

PERMIT FOR THE IMPORTATION OF CONTROLLED GOODS

In terms of the provisions of section 3(1) of the Agricultural Pests Act, 1983 (Act 36 of 1983) and subject to the conditions stated here under, authorisation is hereby granted to-

OGUNYINKA IDIAT BOLAJOKO Tel No: 072 657 7539

P.O. BOX 10456

GRANTHAM PARK

EMPANGENI

3880

to import into the Republic the following controlled goods **REGULATED ARTICLES FOR LABORATORY ANALYSIS**

PARKIA BIGLOBOSA 10 KG

Name and address of foreign supplier **NIGERIA**

Conditions **1. AS ATTACHED**

Port of Entry : **O R TAMBO INTERNATIONAL AIRPORT**

Import authorized from **2013/07/17** TO **2014/07/17**

IMPORTANT : This permit does not exempt the holder from the provisions of any other Act, ordinance or agreement

DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES
DIRECTORATE PLANT HEALTH
2013 -07- 15
DIRECTORATE PLANT HEALTH

.....
Date

.....
Executive Officer

Reference Number 9/13/100

INQUIRIES : TEL.: (012) 319 6363 (Shashika Maharaj) FAX: (012) 319 6370

APPENDIX B

Ethical clearance certificate

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-30- RA Level 02)

RESEARCH & INNOV
Website: http://www.unizulu
Private Bag X1001
KwaDlangezwa 3886
Tel: 035 902 6887
Fax: 035 902 6222
Email: MangeleS@unizulu.ac.za

ETHICAL CLEARANCE CERTIFICATE

Certificate Number	**UZREC 171110-030-RA Level 02 Dept. 2014/74**						
Project Title	**Biochemical and proteomic effects of *Stretozotocin* induced diabetes in male Sprague-Dowley rats treated with *Parka biglobosa* seed protein isolate**						
Principal Researcher/ Investigator	BI Ogunyinka						
Supervisor and Co-supervisor	Dr MA Kappo			Prof AR Opoku			
Department	Biochemistry and Microbiology						
Nature of Project	Honours/4th Year		Master's		Doctoral	x	Departmental

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions:

(1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
(2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.
(3) Cervical dislocation will be done and it is acceptable and blood will be removed, Dr Fourie must supervise the induction of the diabetes

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC

Page 1 of 1

- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
	X				
Low Risk		Medium Risk		High Risk	
				X	

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

Documents	Considered	To be submitted	Not required
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation	X		
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants	X		
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.

Professor Rob Midgley
Deputy Vice-Chancellor, Research and Innovation
Chairperson: University Research Ethics Committee
30 October 2014

CHAIRPERSON
UNIVERSITY OF ZULULAND RESEARCH ETHICS COMMITTEE (UZREC)
REG NO: UZREC 171110-30
13 -11- 2014
RESEARCH & INNOVATION OFFICE