HISTOMORPHOLOGICAL AND BIOCHEMICAL STUDIES OF THE PANCREATIC B-CELLS, KIDNEY AND LIVER IN STREPTOZOTOCIN-INDUCED DIABETIC WISTAR RATS TREATED WITH METHANOLIC EXTRACT OF *HIBISCUS SABDARIFFA* (LINN).

BY

ADEYEMI, DAVID OLAWALE

B.Sc. (ILORIN) M.Sc. (IFE)

Submitted to the Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, College of Health Science, Obafemi Awolowo University, Ile Ife

In partial fulfillment of the requirements for the award of Doctor of Philosophy degree in Anatomy

2012

AUTHORIZATION TO COPY

Author:ADEYEMI, David Olawale.

Title: Histomorphological and Biochemical Studies of the Pancreatic β-cells, Kidney and Liver in Streptozotocin-Induced Diabetic Wistar Rats Treated with Methanolic Extract of *Hibiscus sabdariffa* (Linn).

Degree: Ph.D. (Anatomy)

Year 2012

I, **ADEYEMI,** David Olawale hereby authorize the Hezekiah Oluwasanmi Library to copy my thesis in part or in whole in response to request from individuals and or organizations for the purpose of private study or research.

---------- ----------

Signature Date

CERTIFICATION BY SUPERVISOR

I certify that Adeyemi David Olawale carried out this research work during the course of his studies in the Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, Obafemi Awolowo University, Ile Ife.

…….. …….

Dr. O.S Adewole Date

(MBBS., M.Sc., Ph.D.)

Supervisor

DEDICATION

This project is dedicated to my beloved parents- the best parent in the world, Chief and Mrs. T.D. Adeyemi; my sweetheart, Atinuke and my beautiful daughter, Oluwabusayomi. I love you all

ACKNOWLEDGEMENTS

To God is the Glory for He has done great things in my life. The strength He gave me, His protection over me and His Grace upon my life have worked to make this program a success.

My profound appreciation goes to my supervisor, Dr. O.S Adewole, without whose support and painstaking correction, this research would not have been a success. Your encouragement at all times is highly appreciated. I am also grateful to the Head of Department of Anatomy; Prof. L.M. Oginni for his encouragement and unrelenting efforts in providing useful equipments that has helped in the smooth running of the research.

I also appreciate all other members of Staff of the Department of Anatomy and Cell Biology. Mrs. D. O. Osewa, your motherly role in the department is highly appreciated. May your good works be rewarded here on earth and in heaven in Jesus name.

My special thanks also go to my teacher and friend, Dr. Efere Martins Obuotor of Biochemistry Department. You made provision for all facilities I used for my bench work and even made your office available to me day and night to make my work easier. May God in His infinite mercy bless you.

My sincere appreciation also goes to my friends and colleagues who in one way or the other have affected my life for good and contributed to my success. Mr. Gbela, Your contribution to this work in the area of very good slide is appreciated. You are a father.

My special appreciation goes to my sweet heart, Tinuke and my lovely daughter Oluwabusayomi for bearing with me during the course of this research. Finally, my gratitude goes to my parents – Chief and Mrs. T.D. Adeyemi. I shall for ever appreciate you. Thanks for your inestimable love and support in all respect towards my progress in academics and prosperity in life. I cannot imagine what would have

become of me if I was not opportuned to have you as my parents. May you live long to continue to reap the fruits of your labour in Jesus' name. Amen !

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVIATIONS

AAI	antiatherogenic index
AD	Alzheimer's disease
ADP	adenosine triphosphate
ALP	alkaline phosphatase
ALT	alanine amino transferase
ANOVA	analysis of variance
AST	aspartate amino transferase
ATP	adenosine triphosphate
cGMP	cyclic guanylyl monophosphate
D.A.N.	Diabetes Association of Nigeria
DM	diabetes mellitus
DNA	Deoxyribonucleic acid
FPG	fasting plasma glucose
GAD	glutamic acid decarboxylase,
GDM	gestational diabetes mellitus
GFR	glomerular filtration rate
GPx	glutathione peroxidase
GSH	glutathione

H_2O_2	hydrogen peroxide
HDLC	high density lipoprotein cholesterol
HLA	human leukocyte antigen
HNF	hepatocyte nuclear factor
HSCE	*Hibiscus sabdariffa* calyx extract
HSE	*Hibiscus sabdariffa* extract
IAAs	autoantibodies to insulin,
ICAs	islet cell autoantibodies,
IDDM	insulin-dependent diabetes mellitus
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IU	international unit
LD_{50}	median lethal dose
LDLC	low density lipoprotein cholesterol
MDA	malondialdehyde
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like syndrome
MNU	nitrosamide methylnitrosourea
MODY	Maturity-onset diabetes in youth
mRNA	messanger ribonucleic acid

NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized)
NCD	Non-communicable diseases
NDDG	National Diabetes Data Group
NIDDM	non-insulin-dependent diabetes mellitus
NSE	neuron specific enolase
OGTT	oral glucose tolerance test
OH	hydroxyl radical
PAI-1	plasminogen activator inhibitor-1
RER	rough endoplasmic reticulum
ROS	reactive oxygen species
r.p.m.	revolutions per minute
r-RNA	ribosomal ribonucleic acid
SEM	standard error of mean
SOD	superoxide dismutase
SRP	signal recognition particle
STZ	streptozotocin
TBARS	thiobarbituric acid reactive substances

TBA	Thiobarbituric acid
TC	total cholesterol
TCA	Trichloroacetic acid
TGL	triglyceride
TH	tyrosine hydroxylase
t-RNA	transfer ribonucleic acid
VLDLC	very low density lipoprotein cholesterol
W.H.O.	World Health Organization

ABSTRACT

This study investigated the effects of methanolic extract of *Hibiscus sabdariffa L.* on the morphology, morphometry and histochemistry of the pancreatic β-cells, liver and kidney of experimentally–induced diabetic Wistar rats, assessed the effects of this extract on the blood glucose, serum insulin, lipid profiles, liver function markers, Kidney function markers, antioxidants and lipid peroxidation markers of the rats, and compared the efficacy of the extract with that of protamine zinc insulin. This was with a view to determining the anti-diabetic activities of *Hibiscus sabdariffa L.*

Sixty adult Wistar rats were randomly assigned into five groups (A, B, C, D, and E) of twelve rats each. Group A was the control, group B was untreated diabetic group, group C was *H. sabdariffa* -treated diabetic group, group D was insulin-treated diabetic group while group E was the extract control group. Diabetes mellitus was induced in groups B, C and D by a single intra-peritoneal injection of 80 mg/kg streptozotocin (Sigma, USA) dissolved in 0.1 M citrate buffer; groups A and E rats received intra-peritoneal injection of equivalent volume of citrate buffer and all the animals were monitored for another four week period. Daily intra-peritoneal injection of 1738.76 mg/kg b.w. *H. sabdariffa* was administered to group C and E rats for fifteen days, 1 IU/kg/day of insulin was administered to group D rats for fifteen days while rats in groups B were left untreated. The animals were monitored for another four week period. At the end of the experiment, the rats were sacrificed under chloroform anaesthesia and the pancreas, liver and kidneys of each animal were removed and weighed. The pancreas was fixed in Bouins fluid while the kidneys and liver were fixed in 10 % formol saline. The tissues were processed for paraffin embedding and sections of 5µm thickness were produced and stained with H and E for general histological examination of the tissues, and with special stains to histologically demonstrate β-cells of the pancreatic islets, collagen fibres in liver and kidney, reticular fibres in the liver, basement membrane in the kidney and histochemically demonstrate glycogen in the liver and kidney. The data obtained were analyzed with descriptive and inferential statistics.

The result showed a significant decrease ($F = 48.20$; $df = 11$; $p < 0.05$) in the blood glucose concentration of *H. sabdariffa*-treated group (4.26 ± 0.153 mmol/L) when compared to that of the untreated diabetic group (23.80 ± 2.388 mmol/L) and insulin treated group (11.25 ± 1.465 mmol/L). Histopathological examination of the stained pancreatic sections showed degeneration and necrosis of the pancreatic β-cells and vacuolation of the islets in the untreated diabetic group as well as the insulin treated group while in the extract treated diabetic group, the numerical density of pancreatic β-cells increased suggesting regeneration of these cells. Examination of the liver sections revealed hepatic fibrosis and excessive glycogen deposition in the liver. These morphological changes were ameliorated in the extract-treated diabetic group. Examination of the kidney section revealed that extract of *H. sabdariffa* had nephroprotective effects on the STZ diabetic induced nephropathy which is marked by glomerular necrosis, thickening of the glomerular and tubular basement membranes and renal interstitial fibrosis.

The study demonstrated that *H. sabdariffa* possesses anti-hyperglycaemic, anti-hyperlipidaemic, antioxidant, hepatoprotective and nephropretective activities as well as the ability to induce regeneration of insulin producing pancreatic β-cells of STZ-induced diabetic rats, justifying its ethnomedicinal use.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Diabetes mellitus (DM), a metabolic disorder affecting carbohydrate, fats and protein metabolism, is one of the most common metabolic disorders with a worldwide prevalence estimated to be between 1% and 5% of the world population (Kameswararao *et al.,* 2003; Petal and Rybczynski, 2003). It is considered to be at an epidemic level by the World Health Organization (Petal and Rybczynski, 2003). In 2000, according to the World Health Organization, at least 171 million people worldwide suffer from diabetes, or 2.8% of the population (Wild *et al.,* 2004). Its incidence is increasing rapidly, and it is estimated that by 2030, this number will almost double (Wild *et al.,* 2004). The vast majority of cases of diabetes fall into two broad etiopathogenetic categories. In one category (type 1 diabetes), the cause is an absolute deficiency of insulin secretion (Gavin *et al.,* 1997). Individuals at increased risk of developing this type of diabetes can often be identified by serological evidence of an autoimmune pathologic process occurring in the pancreatic islets and by genetic markers (Atkinson and Maclaren, 1994). In the other, much more prevalent category (type 2 diabetes), the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. In the latter category, a degree of hyperglycaemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be present for a long period before diabetes is detected.

The prevalence of diabetes mellitus is rising worldwide in both developed and developing countries (Dunstan *et al.,* 2002). Its worldwide prevalence is about 2%, and the prevalence in Nigeria is 2.2%, which means that about 2.6 million Nigerians are diabetic (The Expert Committee on NCD, 1997). It is known that 50% of the affected individuals (about 1.3

million Nigerians) do not even know that they have the disease (The Expert Committee on NCD, 1997). Complications of diabetes mellitus have been found to set in long before clinical manifestation of the disease (Young and Mustard, 2001; Harris *et al.,* 1998). Diabetes is likely to remain a significant threat to public health in the years to come. In the absence of effective and affordable intervention for either type of diabetes, the frequency of the disease will escalate worldwide, with a major impact on the populations of the developing countries (Marix, 2002).

Diabetes is associated with vascular and renal dysfunction characterized by hypertension, dyslipidaemia and arteriosclerosis (Freener and King, 1997). Increased free radical generation and oxidative stress are hypothesized to play an important role in pathogenesis of diabetes and its late complications (Anuradha and Svaardsudd, 2001). Possible sources of oxidative stress and damage to proteins in diabetes include free radicals generated by auto-oxidation reactions of sugars and sugars adducts to proteins and by auto-oxidation of unsaturated lipids in plasma and membrane proteins. The oxidative stress may be amplified by a continuing cycle of metabolic stress, tissue damage, and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger systems, which further exacerbate the oxidative stress (Baynes, 1991). Indeed, there is widespread acceptance of possible role of reactive oxygen species (ROS) generated as a result of hyperglycaemia in causing many of the complications of diabetes such as nephropathy, retinopathy, neuropathy (Giugliano *et al.,* 1996), and cardiomyopathy (Rodrigues *et al.,* 1992). Glycation reaction in diabetes occurs in various tissues including *β*-cells (Myint *et al.,* 1995; Tajiri *et al.,* 1997). The activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which is low in islet cells when compared to other tissues, becomes further worsened under diabetic conditions (Kawamura *et al.,* 1994). Further, the presence of higher glucose or glycated protein concentration enhances lipid peroxidation (Hicks *et al.,* 1989), and furthermore lipid peroxides may increase the extent of advanced glycation products (Tiedge *et al.,* 1997).

Currently available therapy for diabetes mellitus includes insulin and various oral anti-diabetic agents such as sulfonylureas, metformin, α-glucosidase inhibitors, etc. These drugs are used as monotherapy or in combination to achieve better glycaemic control. Each of the above oral agents suffers from a number of serious adverse effects (Williams and Pickup, 1991; Zhang and Moller, 2000). In addition, these antidiabetic drugs manage only hyperglycaemia, a feature of diabetes mellitus leaving the pathogenesis of this disorder. Consequently, there continues to be a high demand for new oral anti-diabetic drugs. The WHO Expert Committee on diabetes recommended further evaluation of the folkloric methods of managing this disease because of the high mortality and morbidity arising from its attendant complications and problems associated with the use of conventional antidiabetic agents (WHO Expert Committee on Diabetes Mellitus, 1980). Several indigenous medicinal plants are employed in the traditional management of diabetes mellitus but there is a need to conduct pharmacognostic and pharmacological studies to ascertain their therapeutic values (Ahmad *et al.,* 2004).

For a long time, it was believed that the endocrine pancreas belonged to a category of tissues that were finally differentiated and irreplaceable in the adult. This was mainly supported by the low replication rate of the cells of endocrine glands in adulthood (Swenne, 1992). In the light of many recent data, this point of view has been drastically changed, and nobody disputes today that endocrine pancreas is a plastic organ and that β-cell mass is dynamic especially because of its significant capacity for adaptation to changes in insulin demand (Bonner-Weir, 2000). This property has been demonstrated in physiological as well pathophysiological conditions such as pregnancy (Scaglia *et al.,* 1995) and obesity (Klöppel *et al.,* 1985). Increase in β-cell mass may occur through increased β-cell replication, increased β-cell size, decreased β-cell death, and differentiation of β-cell progenitors (neogenesis) (Finegood *et al.,* 1995). Neogenesis is an important component of β-cell mass expansion during development, and has been shown to contribute to increase in β-cell mass in juvenile and adult rodent models (Finegood *et al.,* 1995; Rosenberg, 1995; Bouwens and Klöppel; 1996). After 90% partial

pancreatectomy in rats, age five – six weeks, focal areas consisting of small duct-like structures appear to give rise to new endocrine and exocrine pancreatic tissue (Bonner-Weir *et al.,* 1993).

Among the numerous substrates, hormones, and growth factors involved in endocrine pancreas plasticity and β-cell renewal, the roles of glucose and insulin emerge and have been extensively studied (Bernard-Kargar and Ktorza, 2001). In several species including humans (Tyrberg ***et al.,*** 1996), glucose appears to play a key regulatory role in pancreatic plasticity because it is a potent stimulus of pancreatic β-cell growth both *in-vivo* (Bonner-Weir *et al.,* 1989; Bernard *et al.,* 1998) and *in-vitro* (Chick, 1973; Schuppin *et al.,* 1993).

For a long time, diabetes has been treated with several medicinal plants (such as *Mormodica charantia, **Vernonia amygdalina, Securidaca longipedunculata, Hibiscus sabdariffa, Annona muricata, Bidens pilosa e.t.c.*) or their extracts based on folklore medicine (Akhtar and Ali, 1984). Oral hypoglycaemic agents can produce serious side effects and in addition, they are not suitable for use during pregnancy (Larner, 1985). Therefore, the search for more effective and safer hypoglycaemic agents has continued to be an important area of active research. Furthermore, after the recommendations made by WHO on diabetes mellitus (WHO Expert Committee on Diabetes Mellitus, 1980), investigations on hypoglycaemic agents from medicinal plants have become more important.

Streptozotocin (STZ) is a broad-spectrum antibiotic with oncogenic and diabetogenic properties (Evans *et al*, 1965). The diabetogenic action is mediated by selective destruction of pancreatic beta cells and has been widely utilized as a method for inducing diabetes mellitus in experimental animals and for treatment of malignant beta cell tumours and other neoplasms in human (Rakieten *et al*, 1968). Although the mechanism of the β-cell cytotoxic