USMANU DANFODIYO UNIVERSITY, SOKOTO

(POSTGRADUATE SCHOOL)

EFFECTS OF CO-ADMINISTRATION OF GLIBENCLAMIDE AND AQUEOUS EXTRACT OF *HIBISCUS SABDARIFFA* CALYCES ON OXIDATIVE STRESS MAKER IN STREPTOZOTOCIN-INDUCED DIABETIC RATS.

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DEDICATION

This work is dedicated to Almighty ALLAH (S.W.A), the Most Beneficent and Merciful, my lovely parents, late Alhaji Bello Audu Onuchi and Ayjimoh Sheidu, my wife Jemilatu Aliyu and my children.

CERTIFICATION

This dissertation by BELLO, Audu Bashir (Admission No: 16211226013), has met the requirements for the award of degree of Master of Science in Medical Laboratory Science (Chemical Pathology) of the Usmanu Danfodiyo University, Sokoto, and is approved for its contribution to knowledge.

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LIST OF ACRONYMS

ADA	American Diabetes Association
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BW	Body Weight
CAT	Catalase
DM	Diabetes Mellitus
ELIZA	Enzyme Linked Immunosorbent Assay
FBG	Fasting Blood Glucose
GLIB	Glibenclamide
GPx	Glutathione Peroxidase
H_A	Alternative Hypothesis
Ho	Null Hypothesis
HS	*Hibiscus sabdariffa* ("Sobo")
IDF	International Diabetes Federation
MDA	Malondialdehyde
NAD	Nicotinamide Dinucleotide
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STZ	Streptozotocin
WHO	World Health Organization

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ABSTRACT

This study was aimed at assessing the potential benefits of a combination of *Hibiscus sabdariffa* (HS) and glibenclamide in ameliorating oxidative stress in streptozotocin (STZ)-induced diabetic rats. A total of 25 male albino Wistar rats were used in this study. The rats were divided randomly into five (5) groups: normal (Non-DM), diabetic (DM), diabetic treated with 600µg/kg bw of glibenclamide (DM + GLIB), diabetic treated with 500mg/kg bw of HS (DM + HS), diabetic treated with both 600µg/kg bw of glibenclamide and 500mg/kg bw of HS (DM + GLIB + HS). All the intervention groups were treated for a period of 28 days. The diabetic groups treated with either Glibenclamide, HS or both, showed significant reduction in glucose level compared to DM untreated rats. There is also reduction in plasma antioxidant enzyme (CAT, GPx, SOD) activities across the groups. However, the activity of Malondialdehyde was increased across both DM and all the intervention groups. The micrographic histology of the pancreas demonstrated various degrees of cell injuries across the DM and intervention groups with only the group treated with glibenclamide and HS showing regeneration of islet-cells. Conclusively, the study suggests that HS may have both antidiabetic and antioxidant effects.

CHAPTER ONE

1.0 INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous condition of public health importance with increasing global incidence (Janaka and Luigi, 2016). The number of people with DM was 451 million in 2017 and is estimated to reach 693 million by 2045 (IDF, 2017). In Nigeria, about 1.7 million people are still living with DM (IDF, 2017). Despite the availability of several oral hypoglycaemic agents, DM is still one of the main causes of many micro- and macro-vascular complications such as retinopathy leading to blindness, end stage renal disease and cardiovascular complications (Roglic *et al.*, 2010). DM is characterized by defects in insulin secretion and/or insulin action leading to impairment of glucose, lipid and protein metabolism (Tripathy and Chavez, 2010; Chan and Watts, 2011). In DM, inability of excess glucose to enter insulin dependent tissues, such as skeletal muscles and adipocytes as a result of deficiency of or insensitivity to insulin, leads to accumulation of glucose in the blood and other non-insulin dependent tissues such as pancreas and brain (Czech *et al.*, 2010; Rizza *et al.*, 2010., Piero *et al.*, 2014). In addition to reactive oxygen species (ROS)- generating enzymes, hyperglycaemia increases oxidative stress through a number of mechanisms such as glucose autoxidation, glycation reactions with proteins and lipoproteins or glucose entering the polyol pathway leading to its conversion to sorbitol as well as other mechanisms (Tsuruta *et al.*, 2010; Bravard *et al.*, 2011).

The concentration of free radicals in the body is maintained within physiological levels by the antioxidant enzymes, mainly superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and other antioxidants such as reduced glutathione (Dröge, 2002). However,

hyperglycaemia in DM causes a reduction of the cellular antioxidant defence mechanisms and increases the concentrations of free radicals (Sharma *et al.*, 2010; Tsuruta *et al.*, 2010). Several studies have suggested a role of oxidative stress in the onset and advancement of DM and complications arising from it (Jay *et al.*, 2006; Wei *et al.*, 2009; Giacco and Brownlee, 2010). In both experimental and clinical models of diabetes, antioxidants have been shown to ameliorate markers of oxidative stress (Johansen *et al.*, 2005; Fenercioglu *et al.*, 2010; Guardiola and Mach, 2014). Moreover, some studies have suggested that antioxidants are effective and inexpensive compared to conventional therapy in management of some diseases (Berkson, 1999; Trevithick *et al.*, 2004). Therefore, antioxidants or nutrients with high antioxidant capacity may offer additional health benefits with possibility of limiting the progression of DM and its associated complications (Fenercioglu *et al.*, 2010).

Glibenclamide is one of the most frequently prescribed oral hypoglycaemic agents (Nathan *et al.*, 2009). It stimulates insulin secretion and reduces hepatic glucose production resulting in reduced blood glucose (Rendell, 2004). However, the use of glibenclamide is limited due to prolonged hypoglycaemia, high secondary failure rate and other adverse events (Harrower, 1994; Mukai *et al.*, 2007).

Hibiscus sabdariffa (HS) commonly known as Sobo in Northern Nigeria, Bissap (Senegal), Roselle (English), Oseille de Guinée (French) and Karkadeh (Arabic), is an erect annual herb cultivated for its seeds, petals and leaves (Adegunloye, *et al.*, 1996). It is used in preparation of local non-alcoholic cold or hot drinks. In Nigeria, the production of a non-alcoholic drink called Sobo that is prepared from the red petals of the plant is popular. There are various poly-herbal formulations present in the market which contain HS as a major constituent (Gaya., *et al.*, 2009). HS tea is loaded with antioxidants such as vitamin A, vitamin C and Zinc. Previous reports

showed that the extract of HS contains potent antioxidants (Wang *et al.*, 2000; Ologundudu *et al.*, 2009).

HS aqueous extract was shown to prevent hyperglycaemia, hyperlipidaemia and oxidative stress (Peng *et al.*, 2011). The antioxidant activity of HS has proven to reduce the incidence of liver lesions including inflammation (Da-Costa *et al*, 2014). According to a study conducted by (Aliyu *et al.*, 2014), extract of HS demonstrated blood pressure and heart rate lowering effects. It is postulated that the oxidative stress induced by DM on the pancreas may be reduced if glibenclamide and extract of HS are administered simultaneously.

1.1 STATEMENT OF THE PROBLEM

Chronic hyperglycaemia of DM is associated with long–term damage, dysfunction and failure of different organs such as retinopathy, nephropathy, neuropathy, micro and macro vascular damage (IDF, 2017). The current world prevalence of DM in both adults and children are 8.8% and is predicted to rise to 10.4% in 2040 (Holman *et al.*, 2015). In 2017, about five million deaths were attributable to DM globally (IDF, 2017) and the total global health expenditure was estimated at 850 billion US Dollars (IDF, 2017). Three quarters (75%) of those with diabetes were living in low- and middle-income countries, Nigeria inclusive. Studies conducted by (Boyle *et al.*, 1999; Bruno *et al.*, 2005; Evans *et al.*, 2000 and Holman *et al.*, 2015) have estimated that approximately 87-91% of all people with diabetes have type 2 diabetes, 7-12% have type 1 diabetes and 1-3 % have other types of diabetes.

1.2 JUSTIFICATION

Glibenclamide is an oral hypoglycaemic agent which has limitations due to undesirable adverse effects such as weight gain, secondary failure, and inability to arrest pancreatic degeneration

which has been linked to oxidative stress (Mukai *et al.*, 2007). However, natural products such as HS which is loaded with antioxidants could reduce the pancreatic oxidative stress by potentiating the hypoglycaemic effects of glibenclamide (Ndu *et al.*, 2011).

1.3 AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to investigate the potential benefits of combination of HS and glibenclamide in ameliorating oxidative stress in streptozotocin (STZ)-induced diabetic rats.

1.3.2 Objectives

The specific objectives of this study are as follows:

i To compare the hypoglycaemic effects of HS alone versus HS and glibenclamide in Streptozotocin (STZ)-induced diabetic rats.

ii To compare the effects of HS alone versus HS and glibenclamide on the serum levels of SOD, GPx, MDA and catalase in STZ-induced diabetic rats

iii To compare the effects of HS alone versus HS and glibenclamide on the histology of pancreatic tissues of STZ- induced diabetic rats.

1.3 RESEARCH HYPOTHESIS

1.4.1 Null Hypothesis (Ho):

The concurrent administration of HS and glibenclamide will not reduce diabetes- associated oxidative stress in STZ-induced diabetic rats.

1.4.2 Alternative Hypothesis (H_A):

The concurrent administration of HS and glibenclamide will reduce diabetes-associated oxidative stress in STZ-induced diabetic rats.

CHAPTER TWO

2.0 INTRODUCTION

Diabetes Mellitus (DM) is a heterogeneous, chronic illness that results from an absolute or a relative deficiency of insulin (hormone secreted by the beta-cells of the pancreas) or presence of factors that oppose its (insulin) actions (Punthakee *et al*, 2018). This condition is associated with increased blood glucose (hyperglycaemia), decrease glucose utilization in the tissues, increase in glycogenolysis and stimulation of gluconeogenesis (formation of glucose from non-carbohydrate source (ADA, 2017). Acute complications include diabetic ketoacidosis, nonketotic hyperosmolar coma, while serious long-term complications include cardiovascular disease, foot ulcer, stroke, retinopathy, nephropathy and neuropathy.

There are major geographical and ethnic differences in the prevalence of the different types of DM in tropical countries. Distribution and prevalence of DM are linked to genetic susceptibility and environmental factors. In some areas and populations, changes in lifestyles and westernization are contributing to major prevalence and incidence of diabetes (Baynes, 2015).

2.1 HISTORY OF DIABETES

DM has been known since antiquity, its treatments were known since 5th- 15th century (Patlak, 2002) and the elucidation of its pathogenesis occurred mainly in the 20th century. Non-progressing type 2 diabetes almost went undiagnosed (Piero, 2014). The discovery of the role of the pancreas in diabetes was made by Joseph Von Mering and Oskar Minkowski in 1889. They found that upon complete removal of the pancreas from the dogs, it exhibited all the signs and symptoms of diabetes and died shortly afterwards. In 1910, Sir Edward Albert Sharpey-Schafer of Edinburgh in Scotland suggested that diabetics lacked a single chemical which was normally

produced by the pancreas, the name of this chemical was later proposed to be insulin (Piero, 2014).

2.2 EPIDEMIOLOGY OF DIABETES

Diabetes is a global issue that strikes people at their most productive age, impoverishing families or reducing the life expectancy of older people (ADA, 2017). Diabetes is a common threat that does not respect borders or social class. According to International Diabetes Federation (IDF) about 451million people were affected by the scourge (ADA, 2017). It is estimated that by 2045, this number will increase to 693 million (ADA, 2017), Asians and Africans will be most affected because of changing lifestyle (WHO, 2015). In Nigeria, about 1.7 million people are living with diabetes (IDF, 2017). The prevalence rate of DM in Sokoto in 2017 was 4.3% (Sabir *et al.,* 2017). The burden of diabetes drains national healthcare budgets, reduces productivity, slows economic growth, causes catastrophic expenditure for vulnerable households and overwhelms healthcare systems (Baynes, 2015).

2.3 CLASSIFICATION OF DIABETES

Diabetes can be broadly classified into 2 categories: type 1 and type 2 diabetes (Punthakee *et al.,* 2018). Type 1 diabetes encompasses diabetes that is primarily a result of pancreatic beta cells destruction with consequent insulin deficiency, which makes the patient prone to ketoacidosis. This form of DM includes cases due to an autoimmune process and those for which the aetiology of beta cell destruction is unknown (Shield *et al.*, 2015). Type 2 diabetes may range from predominant insulin resistance with relative insulin deficiency to a predominant secretory defect with insulin resistance (Fatima, 2013). Gestational diabetes mellitus refers to glucose intolerance with onset or first recognition during pregnancy. Other specific types include a wide variety of

relatively uncommon conditions, primarily specific genetically defined forms of diabetes or diabetes associated with other diseases or drug use (ADA, 2017).

2.4 STREPTOZOTOCIN

Streptozotocin(STZ), N-methylnitrosocarbamoyl-α-D-glucosamine, is abroad-spectrumantibiotic derived from the bacterium streptomyces achromagene (Eleazu *et al.*, 2013). It is a DNA alkylating agent often used as an antibacterial as well as anticancer (Raza and John, 2012). However, it is not a preferred drug for the treatment of cancers. This is due to genotoxic effects which lead to drug resistance (Bathina *et al.*, 2016). STZ is known to be a pancreatic beta-cell-specific cytotoxic and is therefore being widely used in large dose (60mg/kg body weight) to induce experimental type 1 diabetes in rat models (Arwa *et al.*, 2017).

STZ is a glucose analogue that is selectively accumulated in pancreatic beta-cells via a GLUT 2 glucose transporter in the plasma membrane (Wu and Yan, 2016). STZ toxicity in beta-cells is dependent on GLUT 2 expression. Hosokawa and his colleagues in 2001 revealed that in transgenic mice, GLUT 2-expressing beta-cells are sensitive to the toxic effects of STZ, whereas GLUT 1-expressing islets are completely resistant (Grieb, 2016). After entering the beta-cells via the GLUT 2 transporter, STZ induces DNA damage due to the DNA alkylating activity of its methyl nitrosourea moiety, this in turn, results in DNA fragmentation (Raza and John, 2012). Subsequently, the fragmented DNA activates poly (ADP-ribose) synthetase to repair DNA. Poly ADP-ribosylation leads to the depletion of cellular NAD^+ and ATP (Zheng, 2015). The decreased synthesis is demonstrated by dephosphorylation which provides more substrates for xanthine oxidase, resulting in the formation of hydrogen peroxide and hydroxyl radicals causing oxidative stress (Bathina, 2016).

2.5 FREE RADICALS

Free radicals can be defined as atoms, molecules or molecular fragments that contain single or more unpaired electrons in their atomic outer most shell. Generally, it is considered that around 10000-20000 free radicals attack every cell every day out of which some are beneficial for health (Ahmed, 2014). These free radicals enable the human body to fight inflammation, kill bacteria, control smooth muscles which regulate the proper functioning of internal organs and blood vessels (Qazi *et al.*,2018). On the other hand, free radicals play vital roles in the pathogenesis of various diseases such as, DM, heart disease, Alzheimer's disease, Parkinson's disease, cancer, arthritis and aging if produced in large or uncontrolled manner (Qazi *et al.*, 2018)

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are free radicals generated physiologically in the human body in different forms as follows.

2.5.1 Reactive oxygen species (ROS)

Superoxide (O_2·), Hydrogen peroxide (H_2O_2·), Hydroxyl radical (HO·), Peroxyl radical (RO_2), Alkoxyl radical (RO·), Hydroperoxyl radical (HO_2), Singlet oxygen (O·), Ozone (O_3·).

2.5.2 Reactive nitrogen species (RNS)

Nitric oxide (NO·), Nitrogen dioxide (NO_2·), Nitrous acid (HNO_2·), Dinitrogen tetroxide (N_2O_4·), Dinitrogen trioxide (N_2O_3·), Peroxynitrite (ONOO·), Peroxynitrous acid (ONOOH·), Peroxynitrites (ROONO·), Nitronium cation (NO_2+), Nitryl chloride (NO_2Cl·).

2.6. FORMATION OF FREE RADICALS

Free radicals are generated from either exogenous or endogenous sources (Ahmed, 2014).

2.6.1 EXOGENOUS SOURCES OF FREE RADICALS

2.6.1.1 Dietary factors

Alcohol, coffee, foods of animal origin, roasted foods, broiled foods, fried foods, grilled foods, browned or burned foods, herbicides and pesticides residue available in food, and hydrogenated vegetable oils etc, accelerate the free radical formation process (Eboh, 2014).

2.6.1.2 Environment factors

Air pollutants (asbestos, tobacco smoke, carbon monoxide, benzene, etc.), chemical solvents (chlorine, formaldehyde, toluene, chloroform, paints, paint thinners etc.), radiations (cosmic radiation, electromagnetic radiation, x-rays, radon gas, solar radiation) are all potent generators of free radicals (Qazi *et al*., 2018).

2. 6.1.3 Toxins

Carbon tetrachloride, Aniline dyes, Toluene etc. are responsible for generation of free radicals.

2.6.1.4 Drugs

Some drugs accelerate free radical formation process such as Adriamycin, Bleomycin, Mitomycin C, Nitrofurantoin, and Chlorpromazine. (Qazi *et al*., 2018).

2.6.2. ENDOGENOUS SOURCES OF FREE RADICALS

These comprise of metabolic and immune system involving mechanisms that are more complex and extensive. Some examples are briefly discussed below.

2.6.2.1 Mitochondria

The mitochondria are the most vital sources of ROS production (Obeagu, 2018). During the physiological process of ATP generation via the respiratory chain, molecular oxygen is reduced to two water molecules (Obeagu, 2018). However, during energy transduction some electrons 'leak' prematurely thereby necessitating the incomplete conversion of about 1-2% of molecular oxygen into superoxide anion radical ((Victor, 2004).). Superoxide radical chemical reactivity is relatively weak due to its inability to pass through lipid membrane and its quick conversion to hydrogen peroxide by the antioxidant superoxide dismutase (Victor, 2004). Nevertheless, H_2O_2 produced may lead to the generation of a more chemically reactive molecule; the hydroxyl radical through the reaction of H_2O_2 with iron in the Fenton reaction (Nwosu *et al.*, 2016).

2.6.2.2 Cellular oxidase

Although mitochondrial respiratory chain is the main source of superoxide, this free radical specie can also be produced by one electron reduction of oxygen by numerous different oxidases under certain conditions (Obeagu, 2018). These oxidases include NAD(P)H oxidase (NOX family) and Xanthine oxidase (XO) (Droge, 2002). NOX enzymes can be found in the lymphocytes, fibroblasts, endothelial cells, myocytes and chondrocytes where moderate amount of ROS are produced and act as a regulator of cellular response on exposure to infections and microbial invasion (Ray and Shah, 2005). An activation of NOX family enzymes is followed by "respiratory burst" which leads to increased oxygen consumption, glucose utilization and increased production of reduced nicotinamide phosphate dinucleotide (NADPH) by the pentose phosphate pathway (Ray and Shah, 2005).

Another distinguished source of superoxide anion is Xanthine Oxidase (XO), a non-heme enzyme that is usually found in the cytosol especially during hypoxic conditions (Nwosu *et al.*, 2016). Under physiological condition, XO which is a Xanthine oxidoreductase (XOR) exists in the dehydrogenase form but during hypoxia it is converted to an oxidase form that have the capability of producing superoxide (. O^{-2}) and hydrogen peroxide (H_2O_2) by using oxygen as an electron acceptor (Ray and Shah, 2005).

2.6.2.3 Metal catalysed reactions

The hydrogen peroxide produced during hypoxia by the XO is likely to face different cellular fates, such as detoxification to H_2O and O_2 by the glutathione peroxidase (GPx) (in the mitochondria, together with glutathione reductase) and catalase in peroxisomes or it can act as a precursor for more reactive species such as hydroxyl radical (HO) (Lushchak, 2014). HO• produced from this reaction is the strongest oxidizing agent known and reacts with organic molecules through diffusion limited rates (Phanjendra and Jestadi, 2014).

Haber and Weiss demonstrated that accumulation of superoxide and H_2O_2 leads to the production of the highly deleterious hydroxyl radical (HO•) and initiates the oxidation of organic substrates by HaberWeiss reaction (Droge, 2002).

$O_2 + H_2O_2 = HO\cdot + OH^- + O_2$

(Haber - Weiss Reaction).

However, for this reaction to produce HO, it requires a metallic constant (CU^{2+}or CU^{3+}) to proceed and is a combination of transition metal mediated chemical reactions called Fenton reaction (Lone *et al.*, 20013)

$Fe^{3+} + . O_2 \longrightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + HO\cdot$

(Fenton reaction).

2.6.2.4 Myeloperoxidase

Myeloperoxidase is predominantly found in neutrophils with lower levels in monocytes and eosinophils. As shown in the reaction below, hydrogen peroxide (H_2O_2) can also be converted to another free radical HOCl by reacting with chloride (Cl-) ions through enzyme myeloperoxidase (MPO) – catalysed reaction (Droge, 2002).

$H_2O_2 + Cl \longrightarrow HClO + HO\cdot$

Radicals produced from oxygen represent the most important class of free radicals generated in living organism (Valko *et al.*, 2007). During respiratory process, O_2 is progressively reduced by a controlled supply of four electrons to yield H_2O. During this reduction process in normal biological system, the electrons are moved either from the electron transfer chain (4- electron reduction) or at random from the organic/inorganic species in their immediate position (1-electron reduction). But, the incomplete reduction of O_2 is sure and often leads to production of chemical entities that are still potent oxidants. Depending on if it is a one-, two- or three-electron reduction, O_2 may generate successively superoxide radical anion ($O^{\cdot -}_2$), hydrogen peroxide (H_2O_2) or hydroxyl radical (OH •). The present use of the term reactive oxygen species comprises of both oxygen radicals and non-radicals which are easily converted into free radicals (O_3, H_2O_2, $1O_2$) (Halliwell, 1994).

2.6.3. OXIDATIVE STRESS

Oxidative stress refers to a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage (Lone *et al*., 2014). Such damage is often called oxidative damage, which has been defined as the bio molecular damage caused by attack of reactive species upon the constituents of living organisms (Halliwell, 1997). Increased oxidative damage can result not only from more oxidative stress, but also from failure to repair or replace damaged biomolecules (Eboh, 2014). Oxidative stress can result from decreases in antioxidant levels, e.g. mutations decreasing the levels of MnSOD; depletions of dietary antioxidants and other essential dietary constituents (e.g. copper, iron, zinc, and magnesium) can also cause it. For example, children with the protein deficiency disease, Kwashiorkor, suffer oxidative stress, involving low GSH levels (lack of sulfur-containing amino acids in the diet) and iron overload (inability to make enough transferrin (Phanjendra and Jestadi, 2014). Oxidative stress can also be due to increased ROS production for instance, by exposure to elevated O_2; the presence of toxins that produce ROS (e.g. paraquat), or excessive activation of natural systems producing ROS, e.g. inappropriate activation of phagocytes (Halliwell and Gutteridge, 2006). What do cells do when under oxidative stress? It depends on the cell and the level of stress applied. Several cell types respond to mild oxidative stress by proliferating, which can be good in wound healing but bad if it leads to tissue fibrosis (Halliwell and Gutteridge, 2006). Cells may adapt to the stress by up-regulation of defence and/or repair systems. This may completely protect against damage, to some extent but not completely, or sometimes overprotect (Fereshtech *et al*., 2017). These cells are then resistant to higher levels of oxidative stress imposed subsequently. Adaptation need not always involve increases in antioxidants; there can be decreases in ROS-producing systems, increases in other protective mechanisms (such as chaperones), or changes in oxidative damage

targets (e.g. E. coli under oxidative stress, can replace a fumarase enzyme sensitive to inactivation by O_2 with one that resists O_2). Moderate oxidative stress usually halts the cell cycle or can drive cells into senescence; the cell survives but can no longer divide (Lone *et al.*,2013). Severe oxidative damage, especially to DNA, may trigger death by apoptosis, necrosis, or mechanisms with features of both. Indeed, ROS act as triggers of apoptosis, and as participants in apoptosis induced by other mechanisms, in both plants and animals (Eboh, 2014).

2.6.3.1 Effects of oxidative stress on DNA, lipids and proteins

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko *et al.*, 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and the deoxyribose backbone (Halliwell and Gutteridge, 2006). The most extensively studied DNA lesion is the formation of 8- OH-G (8-hydroxy-2-deoxyguanosine). Reaction of 2_deoxyguanosine with hydroxyl radicals, radical adducts followed by reduction to 7-hydro-8-hydroxy2_deoxyguanosine, and by oxidation to 8-hydroxy-2- deoxyguanosine (8-OHdG) or its tautomer 8-oxo-7-hydro2_deoxyguanosine (8-oxodG).

Permanent modification of genetic material resulting from “oxidative damage” incidents represents the first step involved in mutagenesis, carcinogenesis and aging as depicted in the figure below.

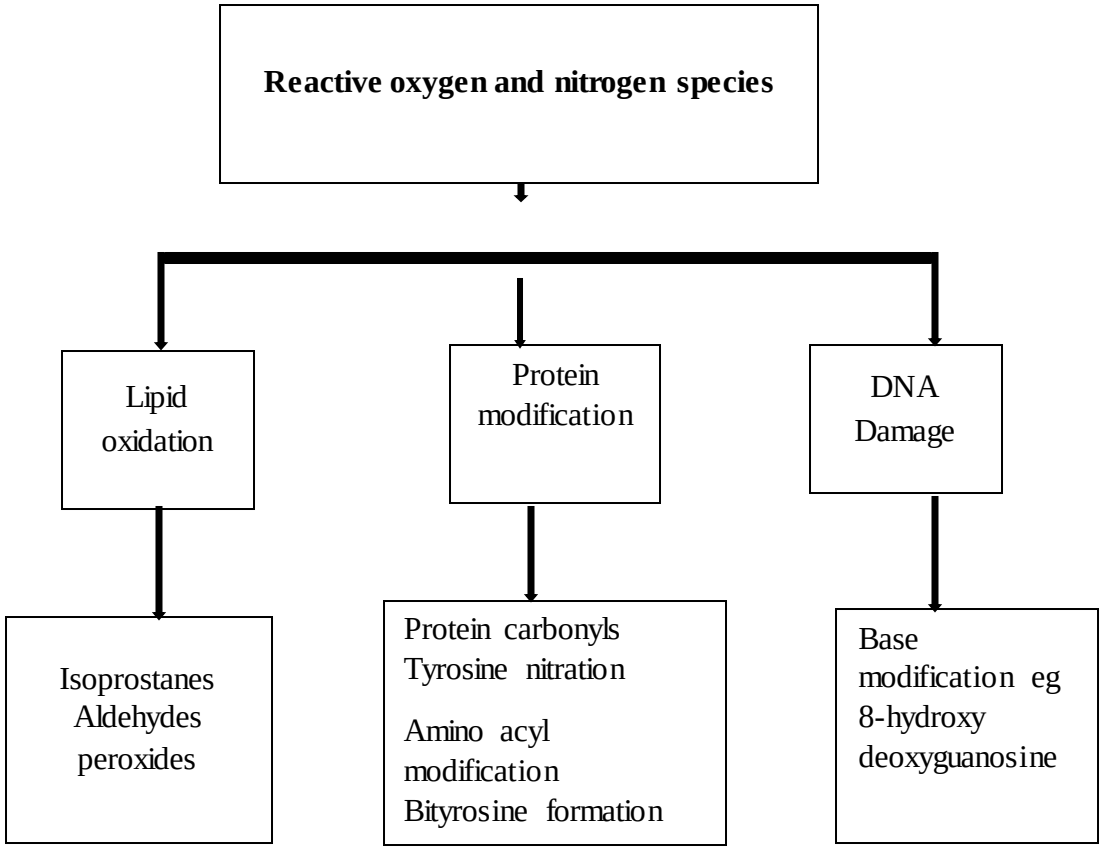

Figure 2.1: Various biomarkers of oxidative damage

It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems *et al.*, 1995). Once formed, peroxyl radicals (ROO•) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Wang *et al.*, 2000). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy- 2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. Mechanisms involved in the oxidation of

proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed (Stadtman, 2004). The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Dalle-Donne *et al.*, 2005). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphide between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (S-glutathiolation). The concentration of carbonyl groups generated by many different mechanisms is a good measure of ROS-mediated protein oxidation. Several highly sensitive methods have been developed for the assay of protein carbonyl groups (Dalle-Donne *et al.*, 2005). Advanced glycation end products (AGEs) ares a class of complex products. They are the results of a reaction between carbohydrates and free amino groups of proteins. The intermediate products are known, such as Amadori, Schiff Base and Maillard products, named after the researchers who first described those (Obeagu, 2018).

2.7. GLINBENCLAMIDE

Glibenclamide, also called Glyburide, is an oral hypoglycaemic agent which belongs to a class of sulfonylurea drugs used in the management of DM (Seena, *et al.*, 2017). This drug is chemically represented as 5- chloro-N-[2-[4-cyclohexyl carbamoyl sulfamoyl) phenyl] ethyl]-2-methoxy benzamide, which acts on pancreatic and extra pancreatic tissue, thereby regulating insulin release (Seena, *et al.*,2017).

2.7.1 MECHANISM OF ACTION

The mode of action of glibenclamide in hyperglycaemic condition is to lower blood glucose via stimulating insulin production from the existing beta cells of the pancreas (Rajasekaran, *et*

al.,2005). Besides this, it also shows extra pancreatic effects by reducing hepatic glucose release. This drug binds to the sulfonylurea receptor 1(Sokolovaska, *et al*.,2012), a regulatory subunit of ATP-sensitive potassium channel in the pancreatic beta cells (Sharma and Kar, 2014). This inhibition causes cell membrane depolarization and open the voltage- dependent calcium channels. This thereby increases intracellular calcium concentration in the beta cells and subsequently stimulates insulin release (Nazaroglu, *et al*.,2009). The onset of action is 30-60 minutes with the duration of action as 18 -24 hours. Sulfonylurea drugs include glipizide (Glucotrol) and glyburide (DiaBeta, Micronase).

2.7.2 SIDE EFFECTS OF GLIBENCLAMIDE

About 1- 3% of people on Glibenclamide have shown hypoglycaemia that led to coma and death. Other side effects include vomiting, weight gain, constipation, headache, rashes, transient leucopenia, photosensitivity and purpura (Senna, *et al*, 2017).

2.8. HIBISCUS SABDARIFFA LINN (ROSELLE)

HS, commonly known as Sobo in Northern Nigeria, Bissap (Senegal), Roselle (English), Oseille de Guinée (French) and Karkadeh (Arabic) is a tropical wild plant belonging to the Malvaceae family (Okereke, 2015). It has a long history of edible and medicinal uses spanning from Egypt, Sudan, Trinidad and Tobago, Mexico, China, Thailand and Malaysia (Patel, 2013). Though the calyx is the most frequently used portion of the plant, the leaves and seeds are often made into salads, curries and potherbs (Okereke, 2015). They are rich in vitamins, natural carbohydrates, proteins, tannins, gums and other antioxidants including minerals. The chemistry of the calyx revealed that per 100 g, it contained 49 calories, 84.5% water, 1.99 protein, 0.1 g fat, 12.3 g total carbohydrate, 2.3 g fibre, 1.2 g ash, 1.72 mg calcium, 57 mg phosphorus, 2.9 mg iron, 300 g vitamin A equivalent and 14mg ascorbic acid (Adegunloye, 1996).

2.8.1 MORPHOLOGY

HS is an erect annual or perennial plant with red stems, serrate leaves which is cultivated for its seeds, petals and leaves (Obouayeba *et at*., 2014). It grows up to 8 ft (2.4m) with nearly smooth, cylindrical typically red stem. The leaves are alternative, 3 to 5 inches (7.5 – 12.5cm) long, green with reddish veins and long or short petioles. Flowers, borne singly in the leaf axils, are up to 5 inches (12.5cm) yellow or buff with a roe or maroon eye, and then turn pink as they wither at the end of the day (Da- costa, 2014).

Figure 2.2: Photo of a typical *Hibiscus sabdariffa* plant

2.8.2 PHYTOCHEMICAL ANALYSIS

Phytochemicals are a group of non-nutrient bioactive compounds naturally found in plant parts such as flowers, leaves, fruits, roots, barks, spices and medicinal plants (Obouayeba, 2014).

HS distilled water extract contains saponin, glycoside and phenol. Ethanol extract contains saponin, glycoside, phenol and flavonoid while methanol extract contained saponin, glycoside, phenol, flavonoid and alkaloid (Okereke, 2015).

2.8.3 NUTRITIONAL AND PHARMACEUTICAL USE OF *HIBISCUS*

The quest to turn into phyto-drugs is gaining momentum as acute and chronic ailments threaten human health. There is draw back that is associated with conventional therapy because of their adverse effects and cost compared to natural products (Piero *et al*., 2012). HS holds abundant potential due to its antioxidant, hypocholesterolaemia, antidiabetic, diuretic, immunomodulatory, anticancer, hepato-protective, antimicrobial, reno-protective and urolithiatic properties in the body (Patel, 2013).

2.9. ANTIOXIDANTS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1992). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Several studies both in vitro (Hirunpanich *et al*., 2005) and in vivo (Youssaf, & Mackawy, 2011) have shown that extracts of HS have a potent antioxidant effect. The antioxidant activity of the extract is due to its strong

scavenging effect on reactive oxygen and free radicals and inhibition of lipid peroxidation (Da-Costa *et al.*, 2014).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 ANIMALS

A total of 25 males adult Wistar albino rats aged 10-12 weeks (150- 200g) were purchased from the animal house, Usmanu Danfodiyo University, Sokoto. The animals were caged in group of five rats per standard cage with 12-hour light/dark cycles at an ambient temperature. The animals were maintained on balanced and standard rats' pellets from vital feeds and had access to water *ad libitum*. The rats feed was obtained from vital, a subsidiary of UAC Food Company in Jos, Plateau state, Nigeria. It has the following nutritional composition: crude protein 13%, fat 8%, crude fibre 15%, calcium 0.9%, phosphorous 0.35% and metabolisable energy of 2,600kcal/kg. The rats were allowed to acclimatize to the animal room conditions for at least five days prior to the induction. All animal experimental protocols were conducted in compliance with animal care standards outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2 PLANT MATERIAL

Dried calyces of *H. sabdariffa* were purchased from the central market, Sokoto and taken to Botany Department of Usmanu Danfodiyo University for authentication. A voucher number (**UDU/ANS/0219**) was collected and the plant specimen deposited at the herbarium of the same department of the institution.

3.3 PREPARATION OF PLANT EXTRACT

The calyces were dried and pulverised with mortar and pestle. About 210gram of powder was extracted in 1400ml of distilled water at 95^{o}c for two hours. The extracted powder was filtered (Whatman no1 filter paper) and concentrated at a temperature of 70^{o}C using hot air ovum (Hospibrand, USA). The residual powder extracts that were recovered yielded 116g and preserved at 4^{o}c till use.

3.4 REAGENTS

All reagents for the study were of good analytical grade.

3.5 EXPERIMENTAL DESIGN

The animals were randomly allotted to groups and treated as follows:

Group 1: Non- diabetic control rats, administered distilled water (0.5ml) orally (n=5).

Group 2: Diabetic control rats administered distilled water (0.5ml) orally (n=5).

Group 3: Diabetic rats, administered glibenclamide 600µg/kg body weight (n=5).

Group 4: Diabetic rats, administered HS extract 500mg/kg body weight (Ibrahim, *et al.*,2017). (n=5).

Group 5: Diabetic rats, treated with HS (500mg/body weight) and glibenclamide 600µg/body weight (Erejuwa, *et al.*,2011) (n=5).

The distilled water, glibenclamide alone and glibenclamide with HS were administered once daily via oral gavage for four weeks. Fasting blood glucose was measured weekly from each rat.

3.6 INDUCTION OF DIABETES

DM was induced by intraperitoneal administration of STZ (60 mg/kg body weight, which was dissolved in 0.1M citrate buffer, pH 4.5) to rats following 12 hours fasting. Another group of rats was injected with citrate buffer alone without STZ. This group served as control. Three days after STZ injection, diabetes was established and measurements of blood glucose were made on blood obtained via a prick to the tail vein using lancets and a calibrated Accu- check (Roche, Germany). Rats with blood glucose concentrations of ≥12 mmol/L were considered diabetic (Mardiah *et al*., 2013).

3.7 SAMPLE PREPARATION AND HISTOLOGY OF THE PANCREAS

At the end of the treatment period, rats were fasted for 12hrs and anaesthetised using light ether soaked with cotton wool enclosed in plastic container. About 5ml of blood was collected through cardiac puncture into fluoride oxalate bottles for biochemical analysis. Following cardiac puncture, the pancreas was rapidly excised and fixed in 10% formalin. Histological analysis of the pancreas was done by using haematoxylin and eosin. The organ was brought out of fixative and examined macroscopically on cutting bench. The pancreas grossed and placed in a pre-labelled cassette. The tissues were dehydrated, cleared and impregnated using automatic tissue processor (Leica TPO102 model, China), after which they were embedded using embedding machine (Leica EG1 160 model, China). Section of the embedded tissue blocks were cut at 3µm using rotary microtome (Leica RM212 RT, China) and then floated out on labelled glass slides. The cut sections were allowed to dry on hot plates for 15 minutes and stained in haematoxylin and eosin stains. Stained sections were examined microscopically using x 10 and x 40 objective

lenses. Photomicrographs of the pancreatic tissue sections of all the intervention rats were taken using an eye-piece-mounted camera and presented alongside with the control sections.

3.7.1 Reagents

Blood glucose was estimated by using Accu-check glucometer strips from Roche Germany.

Protein was estimated by using Biuret kit from Prestige Diagnostics, UK.

All the antioxidant enzymes assay kits were from Sigma Aldrich, USA.

3.7.2 Equipment

The equipment and glass ware that were used for the studies include the following:

I. Universal 320 Hettich, Zentrifuge (Italy) was used for spinning samples

II. Spectrophotometer Mc Jefferson 722G (Germany).

III. Rayto microplate reader (RT 6000c)

IV. Test tubes

V. Micropipette

VI. Cuvette and pipette tips

VII. Water bath

VIII. Microtome (Leica RM 2125RT, China)

IX. Microscope and camera (Olumpus by Carl-Zeiss, Model Axioskop 40, Germany)

X. Glass slide

XI. Accu- check Glucometer (Roche, Germany)

3.7.3 Biochemical analysis

3.7.4 Estimation of serum glucose concentration

Glucose estimation was done in accordance with (Roche, 1958).

3.7.4.1 Principle

The glucose in the blood reacts with an enzyme electrode containing glucose oxidase. The enzyme is re-oxidized with an excess of mediator reagent, such as a ferricyanide ion, a ferrocene derivative or osmium bipyridyl complex. The mediator in turn is re-oxidized by reaction at the electrode, which generates an electric current. The total charge passing through the electrode is proportional to the amount of glucose in the blood that has reacted with the enzyme.

3.7.4.2 Procedure

The test strip was removed from the container and inserted into the glucometer. It was ensured that the numeric display of 888 (mg/dl) or (mmol/ L) were seen. The tail end of rats was pricked with lancet and a drop of blood was applied to the centre of the orange field on the strip. This took about five seconds to display the results either in mg/dl or mmol/L.

3.7.5 ESTIMATION OF PROTEIN

Protein estimation was done according to the method of Biuret (Biuret 1949).

3.7.5.1 Principle

Divalent copper reacts in alkaline solution with protein peptide bond to form the characteristic purple coloured Biuret complex. The intensity of the complex was measured spectrophotometrically at 546nm.

3.7.5.2 Procedure

A total of 1000µl of the protein reagent was dispensed into cleaned blank, standard and sample test tubes. The standard and sample (each 20µl) were dispensed respectively into standard and sample test tubes. The mixture was shaken gently and incubated at 37˚C for 10 minutes, the absorbance of the sample and standard against the reagent blank were read at 546nm.

3.7.5.3 Calculation

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of Standard (40g/L)}$$

3.7.6 Estimation of Lipid peroxidation

Lipid peroxidation assay was done using methods described by Niehaus Samuelson,1968.

3.7.6.1 Principle

The assay was based on the reaction of malondialdehyde with thiobarbituric acid, forming an MDA-TBA_2 complex that will be absorbed strongly at 535nm.

3.7.6.2 Procedure

A volume of 0.1ml of serum was added and treated with $2cm^3$ of (1:1:1 ratio) TBA-TCA-HCl reagent (Thiobarbituric acid 0.37%, 0.25N HCl and 15% TCA). The tube was placed in a water-

bath for 15 minutes, cooled and centrifuged at room temperature for 10 minutes at 1000rpm.The absorbance of clear supernatant was measured against reference blank at 535nm.The concentration of TBARS was calculated using the molar extinction coefficient of malondialdehyde ($1.5x10^5$mol/l/cm). The MDA levels were expressed as nmol per mg protein.

3.7.7 ESTIMATION OF GLUTATHIONE PEROXIDASE ACTIVITY

Glutathione peroxidase estimation was done using Cayman's catalase assay kit (Paglia and valentine,1967).

3.7.7.1 Principle

This assay measures glutathione peroxidase activity indirectly by a coupled reaction with glutathione reductase. Oxidised glutathione produced upon reduction of hydro peroxidase by glutathione peroxidase, is recycled to its reduced state by glutathione reductase and NADPH.

$$R.O.O.H + 2GSH \xrightarrow{\text{Glutathione peroxidase}} R.O.H + GSSG + H_20$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{Glutathione reductase}} 2GSH + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm.

3.7.7.2 Procedure

Three wells were designated as sample, non-enzymatic and control wells. To sample well, 100µl of acid buffer, 50µl of co-substrate mixture, and 20µl of serum were added. To non-enzymatic well, 120µl of assay buffer and 50µl of co-substrate mixture were added and to positive control well, 100ul of assay buffer, 50µl 0f co-substrate, and 20µl of diluted GPx were added. The

reaction was initiated by adding 20µl cumene hydroperoxide to each well and the plate was carefully shaken for few seconds to mix. The absorbance was read at 340nm using Rayto (RT2100c) plate reader (manufacturer details please) once every three minutes.

$$\Delta \text{ Abs/min} = \frac{\text{Abs (time 2)} - \text{Abs (time 1)}}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

$$\text{GPx activity} = \frac{Abs/\min}{0.00373\mu/M} \text{ X } \frac{Abs/\min}{0.00373\mu/M} = \text{nmol/min/ml}$$

3.7.8 ESTIMATION OF SUPEROXIDE DISMUTASE

Superoxide dismutase estimation was done using Cayman's superoxide dismutase assay kit (Marklund, 1980).

3.7.8.1 Principle

The assay utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.

3.7.8.2 Procedure

Two wells were designated as standard and sample. To each well 200µl of diluted radical detector, 10µl of prepared standard to the standard well and 10µl of serum to the sample well was added. 20µl of diluted xanthine oxidase was added to both standard and sample wells to initiate the reaction. The plate was shaken for a few seconds to mix and covered with cover plate. The plate was incubated on a shaker at room temperature for 20 minute and absorbance was read at 450nm using Rayto (RT 200c) plate reader and the serum superoxide dismutase activity was extrapolated from the standard curve plotted.

3.7.9 ESTIMATION OF CATALASE ACTIVITY

Catalase estimation was done using Cayman's catalase assay kit (Johansson and Borg,1988).

3.7.9.1 Principle

The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2.The formaldehyde produced was measured spectrophotometrically with 4-amino-3- hydrazine-5-mercapto-1,2,4-triazole(purpald) as the chromogen. Purpald specifically forms a bicyclic hetrocycle with aldehydes, which upon oxidation changes from colourless to purple colour.

3.7.9.2 Procedure

Three wells were designated as sample, standard and control. To each well, 100µl of assay buffer and 30µl of methanol were added. To standard well, 20µl of prepared standard was added and to sample well 20µl of serum was added. A quantity (20µl) of H_2O_2 was added to each well to initiate the reaction. The plate was covered with plate cover and incubated on a shaker for 20 minutes at room temperature. To each well, 30µl of potassium hydroxide was added to terminate the reaction and 30µl of purpald was then added. The plate was covered once again and incubated for 10 minutes at room temperature on a shaker. To each well, 10µl of potassium peroxide was added, covered and incubated for 5 minutes on a shaker once again. The absorbance was read at 540nm using Rayto (RT2100c) plate reader.

3.8 STATISTICAL ANALYSIS

Data were analysed using IBM Statistical Package for Social Science (SPSS) version 20.0. The results were reported as mean ± SEM. One-way ANOVA was used to compare differences among groups. Differences were considered significant at $p < 0.05$.

Post hoc test was carried out using Tukey-Kramer method for multiple comparisons.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of HS aqueous calyx extract and glibenclamide on the initial and final body weights of streptozotocin-induced diabetic rats

The initial and final body weights of streptozotocin-induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both (HS and Glibenclamide) are presented in Figure 4.1.

Except for the DM vs DM + HS groups that were statistically different ($p = 0.023$, ANOVA), the initial body weights of the rats were similar ($p > 0.05$, ANOVA) across the other treatment groups.

The final body weights of the rats in the Non-DM group were significantly higher than those of the untreated DM ($p = 0.000$), DM + GLIB + HS ($p = 0.000$) and DM + HS ($p = 0.011$, ANOVA) treatment groups. Diabetic rats treated with glibenclamide (DM + GLIB) were significantly heavier ($p = 0.010$, ANOVA) than their counterparts that were treated with both glibenclamide and HS.

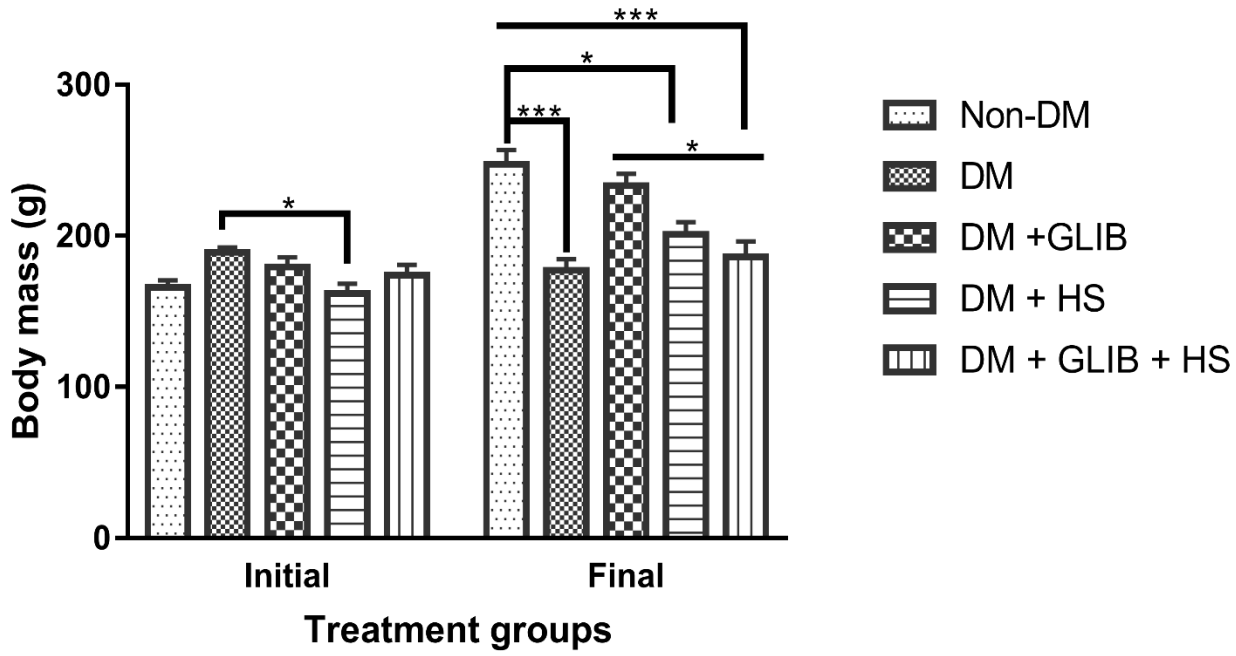

Figure 4.1: Initial and final body weights of streptozotocin-induced diabetic rats

* = means are significantly different at $p < 0.05$.

*** = means are significantly different at $p < 0.0001$.

Initial= initial body mass, Final= final body mass, Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

4.2 Effect of HS aqueous calyx extract and glibenclamide on the baseline and final fasting blood glucose concentration of streptozotocin-induced diabetic rats

Table 4.1 shows the mean post induction baseline (FBG1) and final (FBG 2) fasting blood glucose concentration of streptozotocin-induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both.

While the initial post-induction fasting blood glucose concentration of non-diabetic rats was significantly lower ($p < 0.001$, ANOVA) than that of the rats in the other treatment groups, that of untreated diabetic rats was significantly higher ($p = 0.000$, ANOVA) when compared to all the other treatment groups.

Administration of glibenclamide alone and HS alone and in combination significantly lowered ($p < 0.001$, ANOVA) the final fasting blood glucose concentration of the rats in the respective treatment groups.

Table 4.1: Initial and final fasting blood glucose concentration of streptozotocin-induced diabetic rats

Treatment groups	FBG1 (mmol/L)	FBG2 (mmol/L)
Non-DM	4.32 ± 0.32^{a}	4.68 ± 0.24^{a}
DM	27.14 ± 2.99^{b}	24.00 ± 2.08^{c}
DM + GLIB	19.70 ± 2.48^{b}	8.70 ± 1.14^{b}
DM + HS	22.10 ± 3.74^{b}	9.38 ± 0.95^{b}
DM + GLIB + HS	25.60 ± 2.29^{b}	6.58 ± 0.72^{ab}

a,b,c = means with different superscripts are significantly different at $p \leq 0.005$ across columns. FBG1= baseline fasting blood glucose concentration, FBG2= final fasting blood glucose concentration. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

4.3: Effects of HS aqueous calyx extract and glibenclamide on plasma activities of catalase, glutathione peroxidase and superoxide dismutase

The plasma activities of catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in streptozotocin induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both are presented as Table 4.3.

The plasma activity of CAT of Non-DM rats was significantly higher compared to the DM ($p = 0.000$, ANOVA) and DM + GLIB ($p=0.006$, ANOVA) rats respectively. However, the activity of CAT in DM + HS ($p=0.076$, ANOVA) and DM + GLIB + HS ($p=0.0597$, ANOVA) were significantly higher compared to DM untreated rats.

Similarly, the plasma activity of GPx was significantly higher ($p= 0.000$, ANOVA) in Non-DM rats compared to all the other treatment groups. Diabetic rats that received HS (DM + HS) and those that received glibenclamide and HS (DM + GLIB + HS had significantly higher ($p = 0.011$, ANOVA) plasma activity of GPx compared to the untreated diabetic rats. However, diabetic rats that were treated with either HS alone, glibenclamide alone or a combination of HS and glibenclamide had similar ($p> 0.05$, ANOVA) level of activity of GPx.

The plasma activity of SOD of Non-DM was significantly higher ($p = 0.000$, ANOVA) when compared to all the other treatment groups. However, though the activity of SOD in the untreated diabetic group was similar ($p > 0.05$, ANOVA) to that of DM + GLIB and DM + GLIB + HS, it was significantly lower than those in the DM +HS ($p= 0.0494$, ANOVA) group.

Table 4.2: Plasma activities of catalase, glutathione peroxidase and superoxide dismutase

Treatment groups	CAT (nmol/mg)	GPx (nmol/mg)	SOD (nmol/mg)
Non-DM	0.55 ± 0.02^{a}	1.50 ± 0.12^{a}	8.26 ± 0.23^{a}
DM	0.19 ± 0.03^{b}	0.60 ± 0.03^{b}	3.19 ± 0.14^{be}
DM + GLIB	0.32 ± 0.06^{bc}	0.78 ± 0.06^{bd}	3.97 ± 0.21^{bc}
DM + HS	0.39 ± 0.04^{ac}	0.98 ± 0.03^{cd}	4.40 ± 0.86^{cd}
DM + GLIB + HS	0.46 ± 0.02^{ac}	1.15 ± 0.07^{cd}	4.03 ± 1.96^{bc}

a, b,c,d,e= means with different superscripts across columns are statistically different at $p \leq 0.05$.

CAT= catalase, GPx = glutathione peroxidase, SOD= superoxide dismutase. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

4.4: Effects of HS aqueous calyx extract and glibenclamide on plasma levels of malondialdehyde

The plasma levels of malondialdehyde in streptozotocin induced diabetic rats that were treated with either an aqueous calyx extract of HS, glibenclamide or both are presented as in Figure 4.4. The plasma level of malondialdehyde in Non-DM (control) rats was significantly lower ($p= 0.000$, ANOVA) when compared to the other treatment groups. However, diabetic untreated rats had significantly higher ($p= 0.000$, ANOVA) plasma malondialdehyde level compared to all the other treatment groups. Diabetic rats that were administered with both HS and GLIB had similar ($p =0.000$, ANOVA) plasma level of malondialdehyde with the non-diabetic control rats. However, Combined administration of HS and GLIB to DM rats has resulted in significantly lower ($p\leq.008$) plasma level of malondialdehyde compared to DM untreated rats.

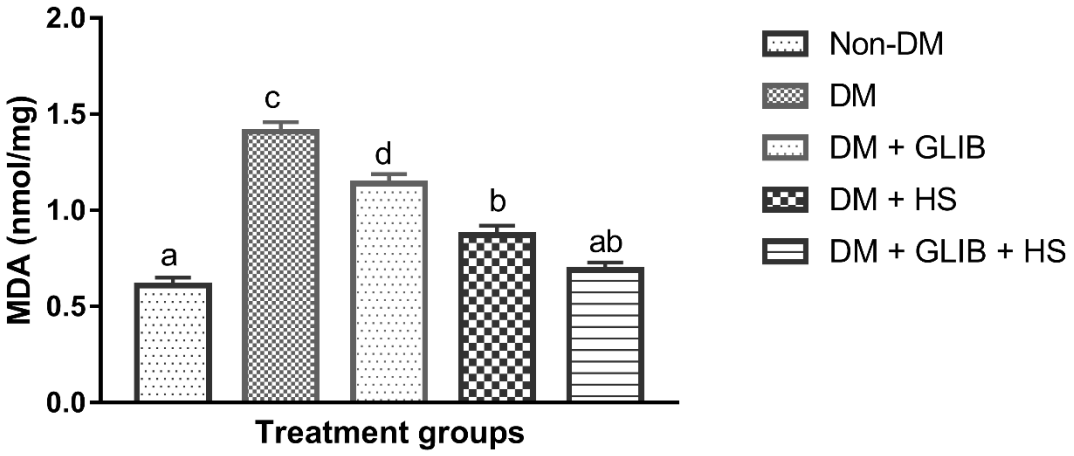

Figure 4.2: **Plasma levels of malondialdehyde (MDA)**

a, b, c, d = means various level of malondialdehyde activity across the rats group induced with streptozotocin treated with either aqueous extract of HS, glibenclamide or both, MDA = malondialdehyde. Non-DM= administered with distilled water only, DM= administered streptozotocin 60mg/kg body weight, DM + GLIB= administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, DM + HS= administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, DM + GLIB + HS =administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Data expressed as mean ±SEM, n= 5 per group.

4.5: Effects of HS aqueous calyx extract and glibenclamide on the pancreatic histology of streptozotocin induced diabetic rats

Figure 4.5 shows representative micrographs of pancreatic histology showing various degree of endocrine gland (beta cell) destruction on different group of diabetic rats induced by streptozotocin. The **slide A** is a Non-DM group, which display normal pancreatic histology with intact endocrine and exocrine glands. **Slide B** represents the DM group which was administered (60mg/kg body weight) of streptozotocin. The histology shows destruction of islet cells with areas of fibrosis of the endocrine gland**. Slides C and D** are representative slides of diabetic rats that were administered with GLIB and HS respectively. They also show some degree of destruction of islet cells. **Slide E** is a representative slide of diabetic rats that received a combination of GLIB and HS. It shows destroyed and regenerating islet cells.

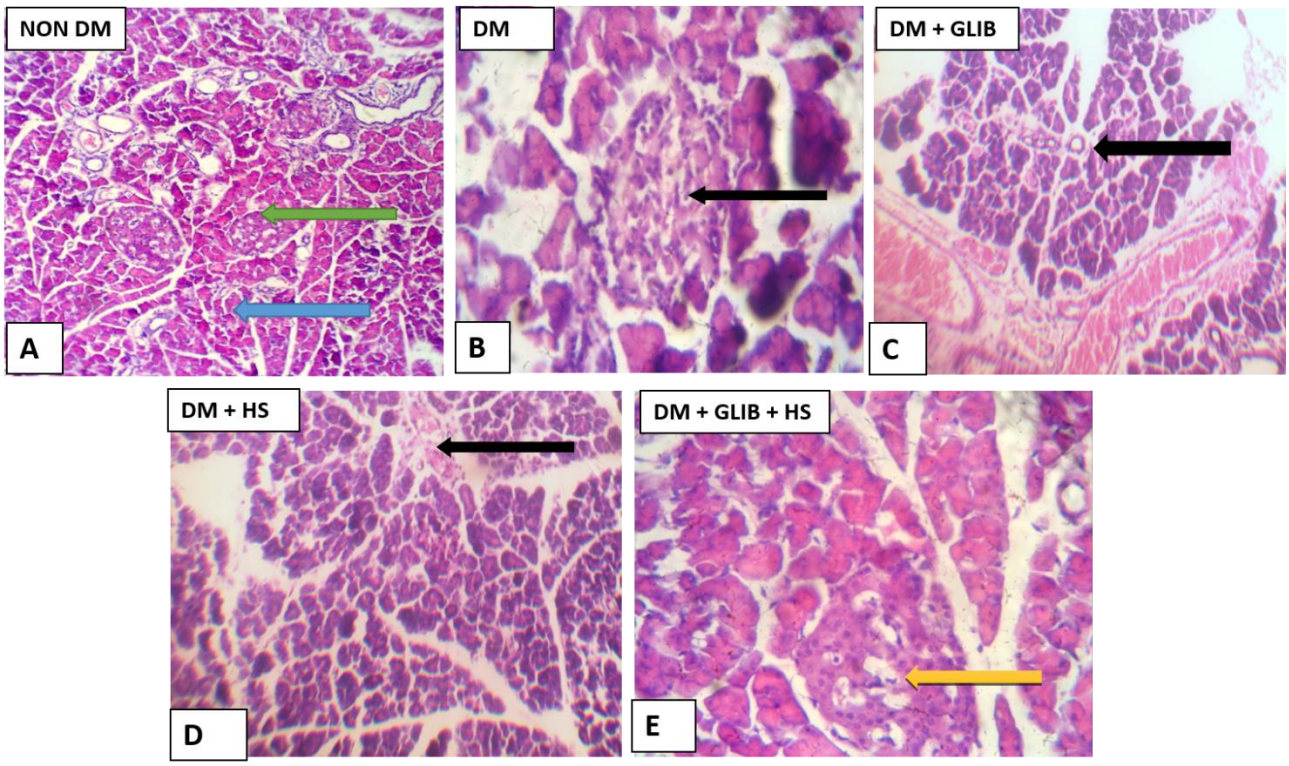

Figure 4.3: Micrographs of pancreas histology (H and E staining, x 400) of streptozotocin-induced diabetic rats.

The green arrow points to normal endocrine gland, the blue arrow points to normal exocrine gland, the black arrow points to destroyed endocrine gland and yellow arrow shows regenerating islet cells. A= Non-DM; administered with distilled water only, B= DM; administered streptozotocin 60mg/kg body weight, C=DM + GLIB; administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight, D= DM + HS; administered streptozotocin 60mg/kg body weight + HS 500mg/kg body weight, E= DM + GLIB + HS; administered streptozotocin 60mg/kg body weight + glibenclamide 600µg/kg body weight + HS 500mg/kg body weight. Tissue section = 3-5µm.

CHAPTER 5

5.0 DISCUSSION

The study was designed to investigate the effects of HS and/or glibenclamide on STZ-induced diabetes and its complications in a rat model. Upon induction of diabetes using STZ, the fasting blood glucose (FBG1) levels of the rats were all significantly elevated compared to the Non DM (control) rats. Streptozotocin is a specific cytotoxic drug that destroys the pancreatic β-cells, there by denying secretion and regulatory action of insulin (Patrick.*et al.*, 2014). The diabetic effect results in production of reactive oxygen species (ROS). Excessive ROS attacks protein, lipids, cellular membrane; and organs like pancreas, kidney and liver (Bathina, *et al.*, 2016).

Hypoglycaemic drugs are either too expensive or have undesirable side effects including haematological, neurological (e.g. coma) and disturbances of liver and kidney functions (Grieb, 2016). Controlling diabetes without any side effects is still a growing challenge in the health sector. This leads the quest to search for effective, safer and affordable antidiabetic natural products like plant extracts (Entsar *et al.*, 2015).

In the current study, the untreated diabetic rats had significantly lower final body weight compared to their non-diabetic counterparts (control). The administration of HS and/or GLIB on the other hand improved significantly the body weights of the diabetic rats. The significant reduction in body weight of the diabetic rats was probably as a result of impaired glucose metabolism due to absent or insufficient insulin hormone. In diabetes, lack of insulin will activate sensitive lipase which acts on adipose tissue, causes its break down and thus leads to rapid weight loss as observed in DM untreated rats (Wu and Yan, 2016).

Previous studies made similar observation of weight loss in diabetic animal models Videla, 2009; Entar, *et al*., 2015; Nazratun, *et al*.,2017). It is suspected that because of the anti-diabetic effect of both HS and glibenclamide, they were able to improve weight gain in the rats by improving glucose utilisation and limiting the breakdown of adipose tissue (Adisakwattana *et al*., 2012). It is important to mention that the administration of GLIB alone to diabetic rats was more effective in improving the body weight of the rats than that of HS alone or HS and glibenclamide combined. Normal weight is an indicator of good diabetic control and glibenclamide is known to cause weight gain (Kasolo *et al*., 2019). Thus, with regards to body weight, glibenclamide may be more beneficial than HS in diabetic rats.

Following the induction of diabetes, the fasting blood glucose concentrations of the rats were significantly elevated compared to Non-DM (control) rats. However, when the rats were treated with either an aqueous extract of HS, glibenclamide or a combination of both HS and glibenclamide, the blood glucose concentration reduced to levels almost similar to that of the control rats. A combination of HS and glibenclamide was more effective in lowering the blood glucose concentration compared to when they were administered independently. HS has been shown to possess anti-diabetic properties in previous studies. For example, when HS extract was administered to diabetic rats at 200mg/kg body weight, it led to a drastic reduction in hyperglycaemia in the rats (Peng *et al*., 2011). HS has also been shown to inhibit the enzyme pancreatic α- amylase leading to a slowing down of digestion of carbohydrates to more absorbable forms of monosaccharides (Adisakwattana *et al*., 2012). Huang *et al*. (2009) have also previously demonstrated that an HS extract suppressed the high glucose stimulated cell proliferation and migration in a dose dependent manner. Additionally, HS contains phytochemicals such as, saponins, glycosides and flavonoids which are believed to have

hypoglycaemic effects, thus reducing hyperglycaemia (Muhammed *et al.*, 2012). There are other studies that buttressed the hypoglycaemic effects of HS in diabetic rats by (Patel, 2014; Ghislain, *et al.*, 2016).

In this interventional study, diabetes significantly reduced the activities of the antioxidant enzymes assayed (catalase, superoxide dismutase and glutathione peroxidase). However, treatment with HS and/ or glibenclamide improved the activities of the enzymes in diabetic rats. The findings in this study corroborate those of other studies that also found diabetes to reduce the serum activities of antioxidant enzymes (Szkudelski, 2001; Sepici- Dincel *et al.*, 2007; Singh *et al.*, 2017).

Under physiological conditions, free radicals generated are scavenged by a repertoire of antioxidants enzymes like SOD, CAT, GPx thus preventing the development of oxidative stress (Entsar, *et al.*, 2015). But in diabetes and its complications, there is increased generation of superoxide, hydrogen peroxide anion and lipid peroxide radicals (Gaya, *et al.*, 2009; Guardiola and Mach, 2014). Thus, causing reduction of antioxidant enzymes due to their increased utilisation to combat the oxidative stress.

CAT is an antioxidant that breaks down hydrogen peroxide into H_2O and O_2. The reduced activity of CAT observed in the diabetic rats might be a consequence of elevated O^{2-} since increased O^{2-} is known to inactivate catalase enzyme (Kono and Fridovich, 1983; Erejuwa *et al.*, 2011).

Glutathione peroxidase is an antioxidant enzyme involved in the detoxification of hydrogen and lipid peroxides (Brigelius-Flohe *et al.*, 2003) and acts as a peroxynitrite reductase (Sies *et al.*, 1997). The activity of GPx in the plasma that was initially reduced in the diabetic rats was

improved with treatment with HS and/ or glibenclamide. However, the activity was more improved in the rats that were administered with both HS and glibenclamide. Generally, antioxidants protect tissues against oxidative damage (Halliwell, 1997). Several studies have reported on the antioxidant potentials of HS (Farombi and Fakoya, 2005; Olaleye and Rocha, 2007; Mossalam *et al.*, 2011). HS is thought to exert this antioxidant activity by scavenging free radicals and reactive oxygen species, inhibition of xanthine oxidase activity (Tseng *et al.*, 1998) and prevention of cell damage via lipid peroxidation (Farombi and Fakoya, 2005).

SOD metabolizes superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) (Fridovich, 1995). Since the non-diabetic rats had normal SOD activity, so reduced SOD activity in the serum of diabetic rats might indicate high levels of O_2^- being generated as result of chronic hyperglycaemia. Reduced SOD in the serum of STZ induced diabetic rats has also been reported by (Qazi *et al.*,2018). This study contradicts a previous study which reported an increased in SOD activity in pancreas of diabetic rats (Balasubramanin *et al.*,2004).

In our study, the levels of lipid peroxidation (MDA) in untreated DM rats were significantly higher than the other treatment groups. However, though treatment with both HS alone and glibenclamide alone reduced the level of peroxidation, the combination of HS and glibenclamide together was more beneficial in this regard. This is similar to what had been previously reported by (Coskun *et al.*, 2005; Turner *et al.*, 1999). This finding indicates that GLIB alone will not offer protection against lipid peroxidative damage but requires multi-therapy approach for effective results. HS extracts have previously been reported to inhibit the formation of malondialdehyde (Usoh *et al.*, 2005) and formation of thiobarbituric reactive substances (Hirunpanich *et al.*, 2005). Therefore, HS is indeed useful in alleviating the lipid peroxidation associated with diabetes.

In the current study, untreated diabetes resulted in fibrosis around the endocrine gland on the very few glands which signify cell injury as a result of cytotoxic effects of administered STZ. STZ is a glucose analogue that is selectively accumulated in pancreatic beta-cells via a GLUT 2 glucose transporter in the plasma membrane (Wu and Yan, 2016).

Administration of glibenclamide alone, HS alone and a combination of glibenclamide and HS all showed pancreatic cellular injuries of varying degrees due to different intervention on the rats. However, the pancreatic tissues of the rats treated with HS alone and those treated with a combined HS and glibenclamide showed signs of regeneration of islet cells probably as a result of antioxidant property of aqueous extract of HS. Hyperglycaemia is associated with pancreatic β-cell damage due to its toxic effects (Erejuwa *et al.*, 2010). Interestingly, a recent study has demonstrated that treatment with HS in a type 1 diabetes rodent model improved the volume of the pancreatic islets and the numerical density of the β-cells depleted by STZ (Adeyemi and Adewole, 2019). Glibenclamide has also been shown to protect pancreatic cells damage through antioxidant mechanisms especially when it is administered in combination with natural polyphenols (Erejuwa *et al.*, 2010). It is therefore safe to assert that HS and glibenclamide when combined produce a more potent protection in the pancreatic cells against the oxidative damage effects of diabetes.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

At the end of this study the following conclusions were made:

1. That the aqueous extract of *H. sabdariffa* exhibited a hypoglycaemic effect on streptozotocin induced diabetic rats but showed no difference between streptozotocin induced DM treated with HS and that of DM treated with HS and GLIB.

2. The antioxidant potential was capable of alleviating oxidative stress induced by hyperglycaemia in experimental rats.

3. This study further suggests that complication of hyperglycaemia and oxidative stress are detrimental to organs like the pancreas, kidney and liver.

6.2 RECOMMENDATION

Considering the finding of this study, it can be recommended that:

1. There is need for periodic evaluation of serum blood glucose, because early diagnosis is a key in stemming down hyperglycaemia, its complication and possible death.
2. Further research is needed on how to complement the aqueous extract of HS and other oral hypoglycaemic drugs for the management of diabetes mellitus because monotherapy has not been able to completely solve diabetes complication.
3. There should be advocacy to pharmaceutical and beverage industry to fortify their products with HS because of its health benefits.

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APPENDIX I

The equipment and glass ware that were used for the studies include the following:

I. Universal 320 Hettich, Zentrifuge (Italy) was used for spinning samples

II. Spectrophotometer Mc Jefferson 722G (Germany).

III. Rayto microplate reader (RT 6000c)

IV. Test tubes

V. Micropipette

VI. Cuvette and pipette tips

VII. Water bath

VIII. Microtome (Leica RM 2125RT, China)

IX. Microscope and camera (Olumpus by Carl-Zeiss, Model Axioskop 40, Germany)

X. Glass slide

XI. Accu- check Glucometer (Roche, Germany)

XII. Lithium Heparin vacutainer

APPENDIX II

Reagents and kits used for the studies are:

I Catalase Assay kit (cayman chemical, USA)

II Superoxide Dismutase Assay Kits (Cayman chemical, USA)

III Glutathione Peroxidase Assay Kit (Cayman chemical, USA)

IV Malondialdehyde Reagent (Cayman chemical, USA)

V Streptozotocin power (Alderide, USA)

VI Total protein Reagent (Labmann, UK)

VII Accu- check glucometer strip (Roche, Germany)

APPENDIX III

PLANT IDENTIFICATION

Plant English Name: Roselle.

Plant Botanical Name: Hibiscus Sabdariffa.

Identification Number: **UDUH/ANS/0219.**

Department of Botany, Usmanu Danfodiyo University, Sokoto.

Identified by: Mr AbdulAzeez Salihu, Department of Botany, Usmanu Danfodiyo University, Sokoto.

APPENDIX IV

HISTOLOGY REPORT

HISTOLOGY	
GROUP A (Non-DM)	**MICROSCOPY**
Pancreas	Normal pancreatic acini and islets of Langerhans cells
GROUP B (DM)	
Pancreas	Normal pancreatic acini with areas of islets of Langerhans cells destructions
GROUP C (DM + GLIB)	
Pancreas	Normal pancreatic acini with areas of minimal islets of Langerhans cells destructions
GROUP D (DM + HS)	
Pancreas	Normal pancreatic acini with areas of minimal islets of Langerhans cells destructions
GROUP E (DM + GLIB +HS)	
Pancreas	Normal pancreatic acini and regenerating islets of Langerhans cells

Reported by: Dr Muhammed Umar, Department of Morbid Anatomy and Forensic Medicine, Faculty of Basic Clinical Sciences, College of Health Sciences, Usmanu Danfodiyo University, Sokoto.