THE EFFECTS OF *TETRAPLEURA TETRAPTERA* SAPONINS ON NON ENZYMATIC ANTIOXIDANT (GSH) AND PRO-OXIDANT (MDA) LEVELS IN THE TESTES OF STREPTOZOTOCIN-INDUCED DIABETES WISTAR RATS.

BY

AIGBODION INNOCENT EBOSETALE **BMS160169 6**

DEPARTMENT OF MEDICAL BIOCHEMISTRY,
SCHOOL OF BASIC MEDICAL SCIENCE
COLLEGE OF MEDICAL SCIENCES
UNIVERSITY OF BENIN
BENIN CITY

JULY, 2021

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A PROJECT SUBMITTED TO THE DEPARTMENT OF MEDICAL BIOCHEMISTRY, SCHOOL OF BASIC MEDICAL SCIENCES, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE, BSC. (HON) MEDICAL BIOCHEMISTRY OF THE UNIVERSITY OF BENIN, BENIN CITY.

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CERTIFICATION:

We the undersigned certify that Mr. **Innocent Ebosetale Aigbodion** carried out this work, in the Department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin City and we approve same as adequate in scope and quality for the award of Bachelors of Science Degree (B.Sc) in Medical Biochemistry.

Signed:

Prof. A.A Omonkhua **Date**

(Seminar Supervisor)

Prof. A.A Omonkhua **Date**

(Head of Department)

(External Supervisor) **Date**

DEDICATION:

Firstly, I wish to dedicate this work to God Almighty, the giver of wisdom. Also to my family, my parents: Mr. and Mrs. Lucky Aigbodion and my siblings: Mathias, Charles, Maria and Joy for their unconditional love, supports and fervent prayers.

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In putting this work together, I acknowledge the grace and enablement given to me by God Almighty,

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ABSTRACT

Diabetes mellitus is a degenerative disease that has deleterious effects on male reproductive function, possibly through an increase in oxidative stress and protection against this damage can be offered by antioxidant supplementation. This study investigated the effects of oral administration of *Tetrapluera tetraptera* saponins (TTS) on concentrations of lipid peroxide (as malondialdehyde; MDA) and reduced glutathione (GSH) in the testes of rats with diabetes induced by streptozotocin (STZ). Six groups were used (7 animals each) and these animals were allocated to different groups: normal control group, diabetic group. The diabetic group was subdivided into five groups as follows: diabetic control (DC), metformin treated group, 10mg TTS treated group, 20mg TTS treated group and 40mg TTS treated group. The metformin and TTS were also administered for 12 consecutive weeks. The MDA and GSH levels in the harvested testes were determined with comparison made between groups. Although MDA concentration in the testes increased in diabetic control, the TTS administered significantly reduced the pro-oxidant levels ($P<0.05$) to normal levels.. However, in the testes and serum, the reduced glutathione significantly ($p<0.05$) decreased in the diabetic treated groups compared to the diabetic untreated group. The results indicated that increased lipid oxidation may compromise the oxidant-antioxidant balance in the experimental animals.

CHAPTER ONE:

INTRODUCTION

Diabetes Mellitus (DM), represents a universal health issue that is distinguished by hyperglycemia which could stimulate oxidative stress to occur due to the generation of free radicals (El-Barky *et al*., 2016). It gives rise to numerous complications, for instance, retinopathy, neuropathy and peripheral vascular disease (Chehade and Mooradian, 2005). Distinct pathogenic processes are implicated in the progression of diabetes. These vary from autoimmune destruction of the β-cells of the pancreas with resultant in insulin deficiency to abnormalities that result in resistance to insulin action. The basic role of the abnormalities in the metabolism of carbohydrate, fat, and protein in diabetes is due to the deficient action of insulin on target tissues (Rabbani *et al*., 2009). Diabetes is classified into four categories: First category is Type I, also known as Insulin-dependent diabetes mellitus (IDDM), which is identified by absolute insulin deficiency. The main causes of type I diabetes are immune or idiopathic causes, whereas Type II diabetes, the second category is known as Non-insulin-Dependent Diabetes Mellitus (NIDDM) which is recognized by tissue resistance to the action of insulin combined with a relative insufficiency in insulin secretion.

Diabetes mellitus has been recognized as a growing worldwide epidemic by many health's advocacy group including WHO. The WHO has estimated that diabetes will be one of the world leading cause of death and disability with next quarter century. The statistics are alarming; 30 million people were diagnosed with diabetes worldwide in 1985, by 1995 the number had risen to 135 million, and at the current rate there will be some 300 million by the year 2025 as predicted by the WHO (Pandey *et al*., 2011). Furthermore, diabetes mellitus is associated with erectile dysfunction and reduced fertility in men and animal models (Nazıroglu, 2003). Diabetes has also

been reported to be associated with severe changes in the structure and function of the testes 2 wk after the onset of diabetes. The pathophysiological mechanisms of impotence in diabetic patients remain obscure (El-Missiry *et al.*, 1999).

Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of diabetes mellitus. The reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction) (Cay *et al.,* 2001). The production of reducing sugars and their reaction products may lead to tissue damage through a variety of mechanisms (e.g., alteration of the structure and function of tissue proteins and stimulation of cellular responses). Several reports indicate the production of reactive oxygen substances (ROS) from glycated proteins under physiological conditions. ROS are known to stimulate glycation end production by autoxidation of sugars. Oxidative stress has been linked to diabetic complications (Nazıroglu and Çay, 2001). Diabetes has also been reported to be associated with severe changes in the structure and function of the testes. Some correlation has been found between glucose level and testicular damage (Nazıroglu, 2003).

All living organisms also synthesize the series antioxidant enzymes responsible for deactivating the ROS. For example, a selenoenzyme glutathione peroxidase together with superoxide dismutase and catalase protect cells against damage caused by free radicals and hydroperoxides or lipoperoxides (Naziroglu *et al.,* 2000).

In addition, glutathione and reduced glutathione (GSH) are known to play pivotal roles in the cellular oxidant defense system and are indispensable for preventing lipid peroxidation by ROS. Then, glutathione is regenerated from its respective radical by vitamin C (Frei *et al.,* 1990).

The general consensus on treatment of type 2 diabetes is that life style management at the forefront of therapy options. In addition to exercise, weight control and medical nutrition therapy,oral glucose lowering drugs, and injections of insulin are the conventional therapies. Pharmacological treatment is indicated when fasting glucose level exceeds 140mg/dl the postprandial glucose level exceeds 160 mg/dl or HbAlc exceeds 8 % (Pandey *et al*., 2011).

The Limitations of Pharmacological Treatment have made Medicinal plants or Traditional plants a better alternative therapy as they have been reported to have significant anti-diabetic properties with no harmful side effects. They are rich sources of anti-diabetic compounds and have a vast potential in the treatment of various ailments due to the presence of therapeutically important phytochemicals such as flavonoids, alkaloids, phenolic, tannins and saponins that improve the efficiency of pancreatic tissues by increasing the insulin secretion or decreasing the intestinal absorption of glucose (Kooti *et al*., 2016).

Tetrapleura Tetraptera, called “aidan” in the South-western part of Nigeria, and “*ihokiriho*” by the Ngwa people in the South-eastern part of Nigeria, is a deciduous tree belonging to the family *Mimosaceae*. It is a typical example of these medicinal plants. Recent studies have also revealed that the pod possesses antioxidant and amylase inhibitory activities ; the fruits and barks extracts also have antioxidant activities.

Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants have grown throughout the world. To date, a lot of research has been done to study the role of different natural products in male reproductive functions (Mohamed *et al.,* 2011; Mohamed *et al.,* 2012; Mohamed *et al.,* 2013). Some studies have used whole plant extracts, thus taking advantage of the abundance of different phytochemicals which have several intervention targets, while others have used specific compounds isolated from plants. Several natural products significantly reversed DM-induced reproductive impairment in rats (Ghosh *et al.,* 2014; Khaki *et al.,* 2014).

This research was aimed at evaluating the potential and effects of administration of *Tetrapluera Tetraptera* Saponins on concentrations of lipid peroxide (as malondialdehyde; MDA) and reduced glutathione (GSH) activity in the testes of rats with diabetes induced by streptozotocin (STZ).

The specific objectives were:

To determine the antidiabetic effects of *T. tetraptera* saponins in streptozotocin diabetic rats.

To determine the lipid peroxidation levels and antioxidant effect of *T. tetraptera* saponins in streptozotocin-induced diabetic rats.

To compare these effects with that obtained from a standard hypoglycaemic drug (metformin)

CHAPTER TWO

2.1.0. DIABETES.

2.1.1 DEFINITION OF DIABETES MELLITUS

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Kharroubi *et al.,* 2015). Metabolic abnormalities in carbohydrates, lipids, and proteins result from the importance of insulin as an anabolic hormone. Low levels of insulin to achieve adequate response and/or insulin resistance of target tissues, mainly skeletal muscles, adipose tissue, and to a lesser extent, liver, at the level of insulin receptors, signal transduction system, and/or effector enzymes or genes are responsible for these metabolic abnormalities (Kharroubi *et al.,* 2015). The severity of symptoms is due to the type and duration of diabetes. Some of the diabetes patients are asymptomatic especially those with type 2 diabetes during the early years of the disease, others with marked hyperglycemia and especially in children with absolute insulin deficiency may suffer from polyuria, polydipsia, polyphagia, weight loss, and blurred vision. Uncontrolled diabetes may lead to stupor, coma and if not treated death, due to ketoacidosis or rare from nonketotic hyperosmolar syndrome (American Diabetes Association, 2014; Galtier, 2010).

2.1.2. EPIDEMIOLOGY OF DIABETES

The global prevalence of diabetes in adults (20-79 years old) according to a report published in 2013 by the IDF was 8.3% (382 million people), with 14 million more men than women (198 million men vs 184 million women), the majority between the ages 40 and 59 years and the number is expected to rise beyond 592 million by 2035 with a 10.1% global prevalence

with 175 million cases still undiagnosed, the number of people currently suffering from diabetes exceeds half a billion. The number of youth (0-14 years) diagnosed with type 1 diabetes worldwide in 2013 was 497100 and the number of newly diagnosed cases per year was 78900 (International Diabetes Federation, 2013). These figures do not represent the total number of type 1 diabetes patients because of the high prevalence of type 1 diabetes in adolescence and adults above 14 years of age.

The prevalence of type 2 diabetes, which accounts for 90–95% of all diabetes, was relatively low at 4.9% in Africa (19.8 million individuals with diabetes). Diabetes figures in Africa are projected to increase with the number of individuals with the condition rising from 19.8 million in 2013 to 41.5 million in 2035, representing a 110% absolute rise. The Middle East and North Africa region has the highest prevalence of diabetes (10.9%), however, Western Pacific region has the highest number of adults diagnosed with diabetes (138.2 millions) and has also countries with the highest prevalence (International Diabetes Federation, 2013). Low- and middle income countries encompass 80% of the cases, "where the epidemic is gathering pace at alarming rates" (International Diabetes Federation, 2013).

The sixth edition of IDF diabetes Atlas, shows that Nigeria is the leading country in Africa in terms of the number of people with diabetes, 3.9 million had diabetes with 105,091 diabetes-related deaths in 2013 which is estimated to increase annually by 125,000 between 2010 and 2030 even though the prevalence of 4.99% is far less than that of Reunion (15.38%), Seychelles (12.11%), Gabon (10.71%), Zimbabwe (9.73%), and South Africa (9.27%); in addition, there are still about 1.8 million Nigerians with undiagnosed diabetes in 2013 (Dahiru, et al., 2016).

2.1.3. CLASSIFICATION OF DIABETES MELLITUS

The classification of diabetes as proposed by the American Diabetes Association (ADA) in 1997 as type 1, type 2, other types, and gestational diabetes mellitus (GDM) is still the most accepted classification and adopted by ADA (American Diabetes Association, 2014). Wilkin (2009) proposed the accelerator hypothesis that argues "type 1 and type 2 diabetes are the same disorder of insulin resistance set against different genetic backgrounds"(Canivell and Gomis, 2014). The difference between the two types relies on the tempo, the faster tempo reflecting the more susceptible genotype and earlier presentation in which obesity, and therefore, insulin resistance, is the center of the hypothesis. Other predictors of type 1 diabetes include increased height growth velocity (Lamb *et al*., 2009) and impaired glucose sensitivity of β cells (Franzoni *et al*., 2010). The implications of increased free radicals, oxidative stress, and many metabolic stressors in the development, pathogenesis and complications of diabetes mellitus (Halban *et al*., 2014) are very strong and well documented despite the inconsistency of the clinical trials using antioxidants in the treatment regimens of diabetes (Nebbioso *et al*., 2012). The female hormone 17-β estradiol acting through the estrogen receptor-α (ER-α) is essential for the development and preservation of pancreatic β cell function since it was clearly demonstrated that induced oxidative stress leads to β-cell destruction in ER-α knockout mouse. The ER-α receptor activity protects pancreatic islets against glucolipotoxicity and therefore prevents β-cell dysfunction (Kilic *et al*., 2014).

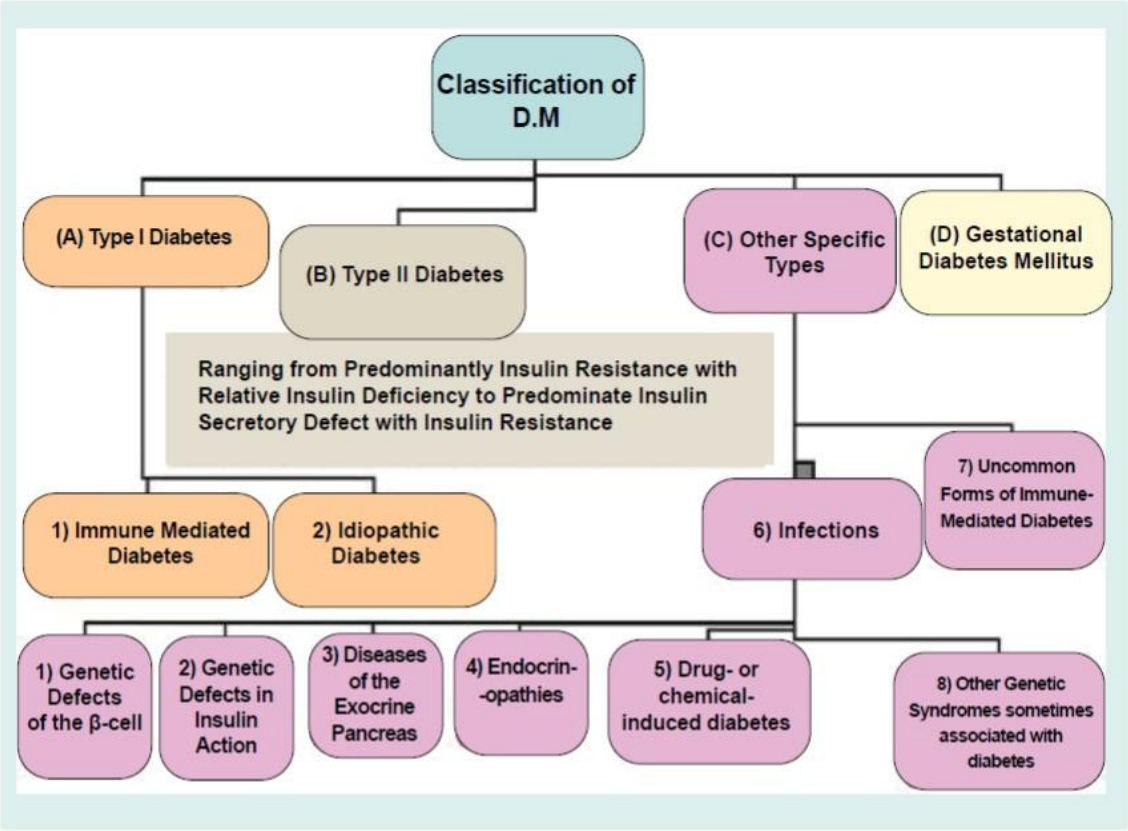


Fig. 2.1: Classification of Diabetus Mellitus.

2.1.4 TYPE 1 DIABETES MELLITUS

This type of diabetes constitutes 5%-10% of subjects diagnosed with diabetes (Maahs *et al*., 2010) and is due to destruction of β cells of the pancreas hence, autoimmune type 1 diabetes (Devendra *et al*., 2004). Type 1 diabetes accounts for 80%-90% of diabetes in children and adolescents (Dabelea *et al*., 2014). Type 1 diabetes is mainly due to an autoimmune destruction of the pancreatic β cells through T-cell mediated inflammatory response (insulitis) as well as a humoral (B cell) response. The presence of autoantibodies against the pancreatic islet cells is the hallmark of type 1 diabetes, even though the role of these antibodies in the pathogenesis of the disease is not clear. These autoantibodies include islet cell autoantibodies, and

autoantibodies to insulin (IAA), glutamic acid decarboxylase (GAD, GAD65), protein tyrosine phosphatase (IA2 and IA2β) and zinc transporter protein (ZnT8A). These pancreatic autoantibodies are characteristics of type 1 diabetes and could be detected in the serum of these patients months or years before the onset of the disease. Autoimmune type 1 diabetes has strong HLA associations, with linkage to DR and DQ genes. HLA-DR/DQ alleles can be either predisposing or protective (American Diabetes Association, 2014). This autoimmune type 1 diabetes is characterized by the absence of insulin secretion and is more dominant in children and adolescents. In addition to the importance of genetic predisposition in type 1 diabetes, several environmental factors have been implicated in the etiology of the disease (Canivell and Gomis, 2014). Viral factors include congenital rubella, viral infection with enterovirus, rotavirus, herpes virus, cytomegalovirus, endogenous retrovirus and Ljungan virus (Kharroubi *et al.,* 2015). Other factors include low vitamin D levels, prenatal exposure to pollutants, improved hygiene and living conditions decreased childhood infections in countries with high socioeconomic status leading to increased autoimmune diseases (hygiene hypothesis) (Kharroubi *et al.,* 2015), early infant nutrition such as using cow's milk formula instead of breast feeding, in addition to insulin resistance in early childhood due to obesity or increased height *growth* velocity (Knip *et al.,* 2010). The role of environmental factors remains controversial. Recent evidence supported the causative effect of viral infections in diabetes. Type 1 diabetes often develops suddenly and can produce symptoms such as polydipsia, polyuria, enuresis, lack of energy, extreme tiredness, polyphagia, sudden weight loss, slow-healing wounds, recurrent infections and blurred vision (International Diabetes Federation, 2013) with severe dehydration and diabetic ketoacidosis in children and adolescents. The symptoms are more severe in children compared to adults. These autoimmune type 1 diabetes patients are also prone to

other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison' s disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia (American Diabetes Association, 2014). The complete dependence on insulin of type 1 diabetes patients may be interrupted by a honeymoon phase which lasts weeks to months or in some cases 2-3 years (Kharroubi *et al.,* 2015). In some children, the requirement for insulin therapy may drop to a point where insulin therapy could be withdrawn temporarily without detectable hyperglycemia (Lombardo et al.,2002).

2.1.4.1. Idiopathic Type 1 Diabetes

A rare form of type 1 diabetes of unknown origin (idiopathic), is less severe than autoimmune type 1 diabetes and is not due to autoimmunity has been reported (Kharroubi *et al.,* 2015). Most patients with this type are of African or Asian descent and suffer from varying degrees of insulin deficiency and episodic ketoacidosis (Abiru *et al*., 2002). Idiopathic type 1 diabetes is characterized by ketoacidosis soon after the onset of hyperglycemia, high glucose levels (≥ 288 mg/dL) with undetectable levels of serum C-peptide, an indicator of endogenous insulin secretion (Imagawa and Hanafusa, 2011). It has been described mainly in East Asian countries and accounted for approximately 20% of acute-onset type 1 diabetes patients in Japan (5000-7000 cases) with an extremely rapid and almost complete beta-cell destruction resulting in nearly no residual insulin secretion (Shibasaki et al., 2012). Both genetic and environmental factors, especially viral infection, have been implicated in the disease. Anti-viral immune response may trigger the destruction of pancreatic beta cells through the accelerated immune reaction with no detectable autoantibodies against pancreatic beta cells. Association of

fulminant type 1 diabetes with pregnancy has also been reported (Imagawa and Hanafusa, 2006).

2.1.5. TYPE 2 DIABETES MELLITUS

The increased incidence of type 2 diabetes in youth is mainly due to the change in the lifestyle of the children in terms of more sedentary life and less healthy food. Obesity is the major reason behind insulin resistance which is mainly responsible for type 2 diabetes (Kraemer *et al*., 2014). The ADA recommends screening of overweight children and adolescence to detect type 2 diabetes (Reinehr, 2013). The prevalence of obesity in children in on the rise which is probably the main reason for the increased incidence of type 2 diabetes in the young (30.3% overall increase in type 2 diabetes in children and adolescence between 2001 and 2009) (Dabelea *et al*., 2014).

Insulin resistance in type 2 diabetes patients increases the demand for insulin in insulin-target tissues. In addition to insulin resistance, the increased demand for insulin could not be met by the pancreatic β cells due to defects in the function of these cells (Halban *et al*., 2014). On the contrary, insulin secretion decreases with the increased demand for insulin by time due to the gradual destruction of β cells that could transform some of type 2 diabetes patients from being independent to become dependent on insulin. Most type 2 diabetes patients are not dependent on insulin where insulin secretion continues and insulin depletion rarely occurs (Kharroubi *et al.,* 2015). Dependence on insulin is one of the major differences from type 1 diabetes. Other differences include the absence of ketoacidosis in most patients of type 2

diabetes and autoimmune destruction of β cells does not occur. Both type 1 and type 2 diabetes have genetic predisposition, however, it is stronger in type 2 but the genes are more characterized in type 1 (the TCF7L2 gene is strongly associated with type 2 diabetes. Due to the mild symptoms of type 2 diabetes in the beginning, its diagnosis is usually delayed for years especially in countries where regular checkup without symptoms is not part of the culture. This delay in diagnosis could increase the incidence of long term complications in type 2 diabetes patients since hyperglycemia is not treated during this undiagnosed period. In addition to diabetes, insulin resistance has many manifestations that include obesity, nephropathy, essential hypertension, dyslipidemia (hypertriglyceridemia, low HDL, decreased LDL particle diameter, enhanced postprandial lipemia and remnant lipoprotein accumulation), ovarian hyperandrogenism and premature adrenarche, non-alcoholic fatty liver disease and systemic inflammation (Kraemer *et al*., 2014). The presence of type 2 diabetes in children and adolescence who are not obese, the occasional severe dehydration and the presence of ketoacidosis in some pediatric patients with type 2 diabetes had led to the misclassification of type 2 to type 1 diabetes (American Diabetes Association, 2000). Some patients with many features of type 2 diabetes have some type 1 characteristics including the presence of islet cell autoantibodies, or autoantibodies to GAD65, and are classified as a distinct type of diabetes called latent autoimmune diabetes in adults (LADA) (Pozzilli and Mario, 2001). People diagnosed with LADA do not require insulin treatment. In a previous study, (Hawa *et al*., 2002) reported 7.1% of European patients with type 2 diabetes with a mean age of 62 years, tested positive for GAD autoantibodies and the prevalence of LADA was higher in patients diagnosed with diabetes at a younger age. This classification of LADA as a distinct type of diabetes is still controversial (Leslie *et al*., 2008).

2.1.6. GESTATIONAL DIABETES

Gestational diabetes refers to hyperglycemia in pregnancy which usually disappear after the newborn. Mothers with gestational diabetes and babies born to such mothers have increased risk of developing diabetes later in life (Kharroubi *et al.,* 2015). Hyperglycemia in pregnancy is responsible for the increased risk for macrosomia (birth weight ≥ 4.5 kg), large for gestational age births, preeclampsia, preterm birth and cesarean delivery due to large babies(Metzger *et al.,* 2008). Risk factors for gestational diabetes include obesity, personal history of gestational diabetes, family history of diabetes, maternal age, polycystic ovary syndrome, sedentary life, and exposure to toxic factors(Galtier, 2010.). Diagnosis of type 2 diabetes before or during pregnancy is based on this criteria: Fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L) or 2-h plasma glucose ≥ 200 mg/dL (11.1 mmol/L) after a 75 g oral glucose load (Kharroubi *et al.,* 2015). However, gestational diabetes is diagnosed using this method by FPG ≥ 92 mg/dL (5.1 mmol/L), 1-h plasma glucose after a 75 g glucose load ≥ 180 mg/dL (10.0 mmol/L) or 2-h plasma glucose after a 75 g glucose load ≥ 153 mg/dL (8.5 mmol/L (Roglic and Colagiuri, 2014). This criteria is derived from the Hyperglycemia and Adverse Pregnancy Outcome (HAPO).

2.1.7. Monogenic Diabetes

Characterization of the genetic etiology of diabetes enables more appropriate treatment, better prognosis, and counseling (Murphy et al., 2008). Monogenic diabetes is due to a genetic defect in single genes in pancreatic β cells which results in disruption of β cell function or a reduction in the number of β cells. Conventionally, monogenic diabetes is classified according

to the age of onset as neonatal diabetes before the age of six months or Maturity Onset Diabetes of the Young (MODY) before the age of 25 years. However, certain familial defects are manifested in neonatal diabetes, MODY or adult onset diabetes(Schwitzgebel, 2014).

Others believe that classification of diabetes as MODY and neonatal diabetes is obsolete and monogenic diabetes is currently used relating specific genetic etiologies with their specific treatment implications (Kharroubi *et al.,* 2015).

2.2.0 DIABETOGENIC CHEMICALS IN DIABETES RESEARCH

Alloxan and streptozotocin are the most prominent diabetogenic chemicals in diabetes research. Both are cytotoxic glucose analogues. Although their cytotoxicity is achieved via different pathways, their mechanisms of beta cell selective action are identical.

2.2.1 Comparing Alloxan With Streptozotocin As Diabetogenic Agents

STZ has notable advantages over alloxan as chemical agents for induction of experimental diabetes, thus, is often preferred to the latter (alloxan). STZ-induced hyperglycemia is relatively more stable and for a longer duration (as much as three months compared to alloxan-induced hyperglycemia that can only be sustained for less than a month) (Ighodaro, 2018). Moreover, the mechanism of STZ diabetogenicity is less associated with cellular toxicity, hence, lesser animal mortality(Ighodaro, 2018). Alloxan on the contrary, induces diabetes by a mechanism characterized by incidences of ketosis, ROS toxicity, and high mortality rate which is particularly a major setback in experimental diabetes studies (Szkudelski, 2001). One reason for this is that STZ is more selective to islet beta cells than alloxan which causes severe damage to other cell types which express GLUT2 (systemic

toxicity). More so, STZ-induce diabetes is associated with well characterized diabetic complications unlike alloxan-induced diabetes (Lenzen, 2008). In addition, compared to alloxan, STZ diabeto-genicity is not severely interfered with by blood glucose level. Overall, STZ diabetogenicity is more effective and with lesser variation with animal species.

2.3.0 OXIDATIVE STRESS IN DIABETES

2.3.1. DEFINITION OF OXIDATIVE STRESS

Oxygen is necessary for complex organisms with high energy demands but it is also a source of toxic compounds due to the formation of incompletely reduced species. During normal metabolism, oxygen is reduced to water (Piconi *et al.,* 2003). The intermediate reduction products, which are known to give rise to cellular damage, are superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). These three species are often referred to collectively as reactive oxygen species (ROS) (Abele, 2002). Cells have developed several enzymatic and nonenzymatic systems to prevent or limit ROS-associated damage. The defense system is complex and involves many compounds with vastly different properties: enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase), macromolecules (albumin, ceruloplasmin) and small molecules (vitamin C, vitamin E, β-carotene, reduced glutathione and uric acid) (Lunec, 1992). The balance between oxidative species production and antioxidant protection species is the redox state of the cell. Oxidative stress generally describes a condition in which cellular antioxidant defenses are inadequate to completely detoxify free radicals that have been generated, due to excessive production of ROS, loss of antioxidant defenses, or, typically, both. Oxygen-derived free radicals are very important mediators of cell injury and death (Piconi *et al.,* 2003). These highly reactive chemical species are involved not only in the aging process but are either directly or indirectly implicated in a wide variety

of clinical disorders, such as atherosclerosis, reperfusion injury, pulmonary toxicity, macular degeneration, cataractogenesis, diabetes and cancer (Knight, 1995).

2.3.2. EVIDENCE OF OXIDATIVE STRESS IN DIABETES

The levels of all biomarkers of oxidative stress are modified in diabetic subjects. Different studies have been designed to determine the levels of stress-related biomarkers both in type 1 and type 2 diabetes. In type 2 diabetes evidence of lipid peroxidation has been observed with high plasma and urine isoprostane levels (Piconi *et al.,* 2003). Also MDA level results were higher than in the normal subjects and correlated with the degree of glycemic control achieved (Altomare *et al*., 1992). Nitrotyrosine formation is increased in plasma of both types of diabetic patients while FRAP level is decreased (Piconi *et al.,* 2003).

2.3.3. THE SOURCES OF OXIDATIVE STRESS IN DIABETES

It is well known that all forms of diabetes are characterized by a chronic hyperglycemia and by the development of specific micro- and macrovascular complications. Microvascular complications involve progressive degenerative process at the level of retina, renal glomerulus and peripheral nerve. Macrovascular diseases involve accelerated arteriosclerosis of arteries that supply the heart, brain and lower extremities (Ceriello and Sechi, 2002). Longterm vascular complications still represent the main cause of morbidity and mortality in diabetic patients . Increasing evidence from both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes complications (Ceriello, 2003). Different hypothesis has been proposed regarding

mechanisms by which hyperglycemia causes micro- and macrovascular complications; these have been recently reviewed by Brownlee who proposed a convincing unifying theory. The increased polyol pathway flux, the increased advanced glycation end product formation (AGE), the activation of protein kinase C (PKC) isoforms and the increased hexosamine pathway flux could all be the effects of a single hyperglycemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain (Piconi *et al.,* 2003). The high $FADH_2$), generating a high potential at the mitochondrial membrane level by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of coenzyme Q (ubiquinone), that protract the reduction of O_2 to superoxide (Du *et al*., 2000). It was demonstrated in in vitro experiments that normalizing levels of mitochondrial superoxide production using a specific inhibitor (TTFA), or an uncoupler (CCCP), or by overexpression of uncoupling protein-1 (UCP-1), and of Mn-SOD, it is possible to prevent effects of hyperglycemia (i.e., PKC activation, AGE formation and sorbitol accumulation) (Nishikawa et al., 2000). Recent evidence emphasizes that, on the one hand, ROS production is increased in diabetes and that this is closely associated with the development of diabetes-specific complications (Baynes, 1991). On the other hand, it has recently been proven that in diabetic patients there is also an abnormal response of the antioxidant genes to hyperglycemia with a reduced expression of the antioxidant enzymes. This condition may lead to a ROS-induced damage especially in postprandial condition, during acute rise of glucose level (Ceriello and Sechi, 2002).

2.4.0. EFFECT OF DIABETES MELLITUS ON MALE REPRODUCTIVE FUNCTION

Several animal models of DM have been used in the study of male reproductive function. They include type 1 diabetic rats [streptozotocin (STZ)–induced diabetic rats, alloxan–induced diabetic rats and BioBreeding (BB) rats], type 2 diabetic rats [nicotinamide+STZ-induced diabetic rats

(simultaneous administration of nicotinamide and STZ), STZ+high fats diet induced diabetic rats and Goto-Kakizaki (GK) rats] (Nna *et al*., 2017). DM affects male reproductive system at pre-testicular, testicular and post-testicular levels, which are summarized in Figure 2.

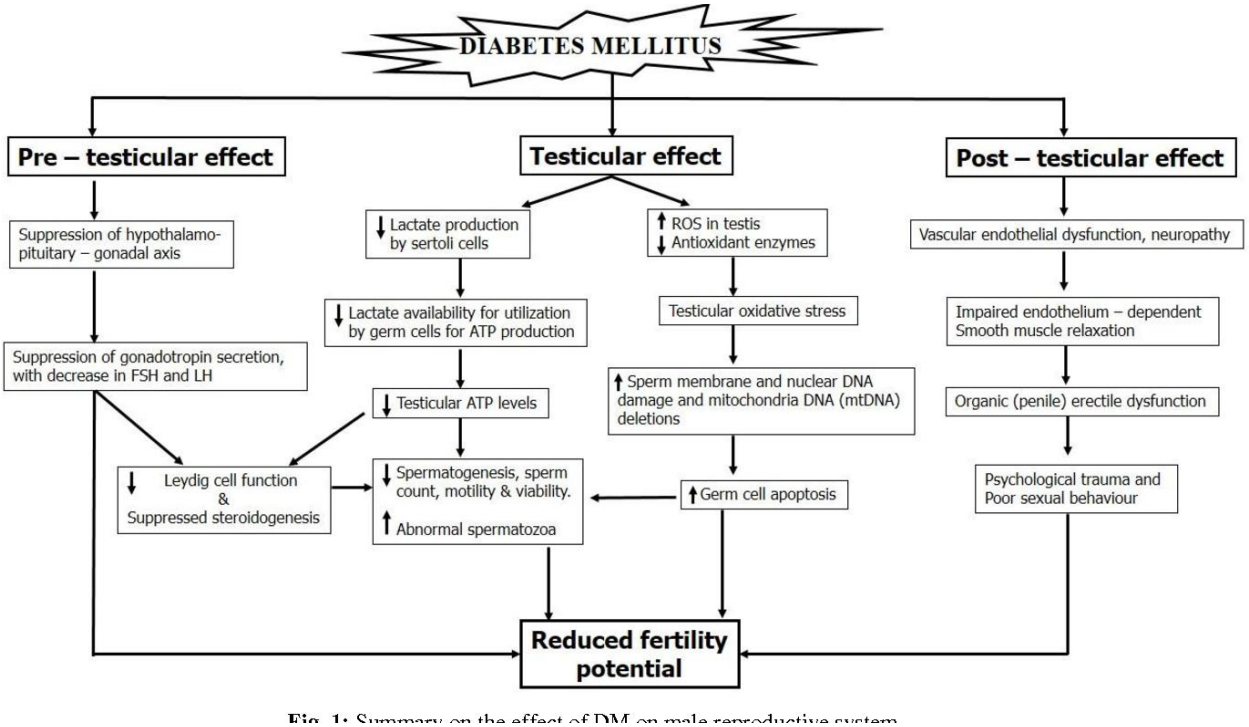


Fig. 1: Summary on the effect of DM on male reproductive system

Fig. 2.2: Summary on the effect of DM on male reproductive system.

2.4.1. PRE-TESTICULAR EFFECTS OF DIABETES MELLITUS (HYPOTHALAMIC-PITUITARY-GONADAL AXIS)

Several reports have linked Diabetes Mellitus with disruption of the hypothalamic–pituitary–gonadal (HPG) axis, thus altering the concentrations of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in males. Baccetti *et al.* (2002) have shown that DM significantly suppresses the HPG axis and lowers FSH and LH responses to exogenous gonadotropin releasing hormone (GnRH) in T1DM patients. Suppressed testosterone secretion as a result of diminished Leydig cell function (Pitteloud *et al.,* 2005) has been reported in T2DM. Decreased serum concentrations of reproductive hormones (testosterone, FSH and LH) have also been reported in DM

animal models induced by STZ, alloxan and nicotinamide+STZ, and in BB rats (Cameron *et al.,* 1990). These studies show a unified pattern of events irrespective of the diabetic model used.

The effect of DM on the HPG axis may not be type specific since the data from both T1 and T2DM models have shown similar findings.

2.4.2. TESTICULAR EFFECTS

2.4.2.1. Energy Metabolism In The Testis

The Sertoli cells (otherwise called nurse cells) are greatly involved in testicular energy metabolism, and form one of the major components of the blood – testis barrier. Testicular energy metabolism shows some form of specificity in which lactate is the main substrate for energy (ATP) production in germ cells (Oliveira *et al.,* 2012). *In vitro* studies with cultured Sertoli cells have shown that removing either insulin (Oliveira *et al.,* 2012) or glucose (Riera *et al.,* 2009) from the culture medium results in adaptation of the Sertoli cells to glucose transport as seen in modulated gene expression of glucose transporters (GLUT1 and GLUT3). However, Oliveira *et al.,* (2012) have reported that the insulin-deprived Sertoli cells in their *in vitro* experiment (which closely mimics T1DM) have reduced glucose utilization even when the gene expression of the glucose transporters is increased. Additionally, the authors have reported down-regulation of the genes associated with lactate metabolism and transport. Recent study by Rato *et al.* (2015) has demonstrated that exposure of cultured Sertoli cells (obtained from T2 diabetic rats) to testosterone increases glucose consumption and up-regulates GLUT3, but down regulates GLUT1 gene expression compared with Sertoli cells culture obtained from pre-diabetic rats. There is also a decrease in protein expression of monocarboxylate transporter-4 (MCT4) and lactate dehydrogenase (LDH), as well as a decrease in LDH activity in Sertoli cell culture obtained from T2 diabetic rats, compared with Sertoli cell culture obtained from pre-diabetic rats (Rato *et al.,* 2015).

In vivo studies using STZ-induced T1DM have reported that DM reduces testicular LDH activity (Kyathanahalli and Manjunath, 2014), thus suggesting a likely similar trend of events in T1 and T2DM. This ultimately implies that the germ cells may be deprived of lactate in diabetic conditions.

2.4.3. TESTICULAR OXIDATIVE STRESS

The presence of antioxidants in the testis ensures that the two most important events in the testis namely steroidogenesis and spermatogenesis are not negatively affected by oxidative stress. These antioxidants in the testis are of major significance since oxidative stress is currently regarded as the most important cause of impaired testicular function underlying the pathological consequences of a wide range of conditions including DM. Studies with both T1 and T2 diabetic animal models have shown significant decreases in antioxidant enzymes and a significant increase in lipid peroxidation in the testis. Reports from studies using STZ-induced T1DM rats have shown significant decreases in testicular superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, and a significant increase in malondialdehyde (MDA) level (Mohasseb *et al.,* 2011; Kanter *et al.,* 2013).

In another study using alloxan-induced T1DM rats, Ghlissi *et al.* (2012) have reported significant decreases in the activities of antioxidant enzymes (SOD, CAT and GPx) and a significant increase in thiobarbituric acid-reactive substance level in the testis. A similar trend of results is reported by Hamden *et al.* (2008) using alloxan-induced T1DM rats. On the other hand, Gobbo *et al.* (2015) have reported that DM did not significantly alter the testicular antioxidant defence system as shown by the insignificant alterations in the activities of antioxidant enzymes (SOD, CAT, GPx) and MDA level after 1 week and 8 weeks in STZ-induced T1DM rats.

2.4.4. STEROIDOGENESIS AND DIABETES MELLITUS

Steroid hormones including testosterone are synthesized from cholesterol through a series of tightly regulated steps. After formation of testosterone, the Leydig cells are involved with its secretion to support spermatogenesis. Studies have reported a significantly increased testicular cholesterol concentration in STZ-induced T1DM model (Saumya and Basha, 2016). Significant decreases in 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD activities are found in the testis of STZ-induced T1DM rat model (Reddy *et al.,* 2016). Furthermore, there are significant decreases in expressions of steroidogenic acute regulatory protein (StAR) and cytochrome P450 enzyme (CYP11A1, the first enzymatic step in steroidogenesis) in the testis of STZ-induced T1DM rats.

The increased testicular cholesterol concentration (Saumya and Basha, 2016) may be as a result of down-regulation of the cholesterol transporter, StAR, thus leading to cholesterol accumulation. These results suggest that the reduced serum testosterone concentration in DM amidst high testicular cholesterol concentration may be due to down-regulations of steroidogenic transport protein (StAR) and marker enzymes (CYP11A1, 3β-HSD and 17β-HSD) expressions.

2.4.5. EFFECT OF DIABETES MELLITUS ON SPERMATOGENESIS

Experimental and clinical evidences have shown that DM negatively affects spermatogenesis and sperm – related parameters. Several studies using STZ-induced T1DM animal model have shown decreased daily sperm production, sperm count and motility and increased percentage of spermatozoa with abnormal morphology (Kanter *et al.,* 2013). Studies using alloxan-induced T1DM animal models (Hafez 2010; Ghlissi *et al.,* 2012) have also yielded similar results.

Reports from clinical studies have failed to completely corroborate those from experimental animals, and most of them have yielded ambiguous results. Vignon *et al.* (1990) have previous reported a

significant increase in sperm concentration in TIDM patients, while Garcia-Diez (1991) has reported a significant decrease in sperm count, motility and percentage of spermatozoa with normal morphology. However, Agbaje *et al.* (2007) have found an insignificant increase in sperm concentration and total sperm output with an insignificant decrease in sperm motility, and normal sperm morphology. The authors suggested Leydig cell hyperplasia as the cause of the enhanced spermatogenesis, though with poor motility. These inconsistent findings might be as a result of the lack of information with regards to the duration of exposure to DM and the various control measures adopted by the subjects in each population studied, since most of these studies were conducted using patients reporting to fertility clinics, rather than untreated diabetic volunteers.

2.4.6. TESTICULAR HISTOLOGY AND GERM CELL APOPTOSIS IN DIABETES MELLITUS

Oxidative stress, which is one of the hallmarks of DM, has been implicated in DM – induced distorted testicular morphology in diabetic animal models. Studies using T1 and T2 diabetic rats have yielded similar results, indicating that testicular histology is negatively affected irrespective of the type of DM. Studies using STZ (Kanter *et al.,* 2013; Al-Roujeaie *et al.,* 2016), alloxan (Shalaby and Hamowieh, 2010; Hafez, 2010) and BB (Cameron *et al.,* 1990) T1DM models have all reported varying degrees of morphological abnormalities, comparable to the study using T2DM (STZ + High fats diet) model (Long *et al.,* 2015). Mean Johnsen's score, a measure of the degree of spermatogenesis, is significantly lower in STZ- induced T1DM model (Kanter *et al.,* 2012; 2013). Testicular atrophy has also been reported in DM with significant decreased volume and diameter of the seminiferous tubule, and decreased numbers of Sertoli and Leydig cells (Kanter *et al.,* 2013; Al-Roujeaie *et al.,* 2016). Kanter *et al.* (2013) have also reported a significantly decreased population

of germ cells including spermatogonia, spermatocytes and spermatids at various stages which may explain the decreased testicular weight in DM.

The increased population of apoptotic germ cells observed in diabetic rats is suggestive of the implication of oxidative stress that usually accompanies DM. Mohasseb *et al.* (2011) have reported an increased activity of the executioner caspase (caspase-3) in the testis of STZ-induced diabetic rats. Up-regulation of the pro-apoptotic protein (Bax) and down-regulation of the anti-apoptotic protein (Bcl-2) have been reported in T1 (Ghosh *et al.,* 2014) and T2 (Long *et al.,* 2015) diabetic rats, with an increase in expression of caspases 8 and 3 (Jiang *et al.,* 2013) in T1 diabetic rats. Zhao *et al.* (2011) have reported up-regulation of the pro-apoptotic genes, p38 and p53 in the testis of STZ-induced T1DM rats. Studies in detecting apoptotic germ cells in the testis of T1 diabetic rats using TUNEL staining technique have reported an increased expression of TUNEL-positive cells (Kanter *et al.,* 2012; 2013).

In the light of the above results, it is clear that all the methods and/or markers used to assess DM-induced germ cell apoptosis show uniformity in the trend of results obtained, thus confirming the increased apoptotic germ cell population in diabetic state.

2.4.7. POST-TESTICULAR EFFECTS OF DIABETES MELLITUS (SEXUAL BEHAVIOUR AND FERTILITY OUTCOME)

Studies have linked DM with poor sexual urge as a consequence of poor penile erectile function. Studies have shown that DM reduces penile cGMP concentration, prolongs mount, intromission and ejaculatory latencies, and decreases mount and intromission frequencies (Al-Roujeaie *et al.,* 2016). The decreases in sexual behavioural parameters are linked to the reduced Leydig cell secretion of testosterone (Al-Roujeaie *et al.,* 2016). In another study, diabetic male rats have demonstrated subfertility when mated with healthy female rats (Suresh and Prakash, 2012; Reddy *et al.,* 2016).

Reddy *et al.* (2016) have reported an increase in the percentage of pre- and post-implantation losses, and a decrease in the number of live foetuses/dam. DM does not negatively affect pregnancy rate, but prolongs conception time, which is attributed to poor sperm characteristics secondary to DM (Reddy *et al.,* 2016). Taken together, these reports implicate abnormal sexual behaviour in DM- mediated subfertility.

2.5. TETRAPLURAL TETRAPTERA

Tetrapleura tetraptera, called "aidan" in the South-western part of Nigeria, and "ihokiriho" by the Ngwa people in the South-eastern part of Nigeria, is a deciduous tree belonging to the family Mimosaceae.It is generally distributed in the lowland forest of tropical Africa. The fruits, made up of a fleshy pulp with small, brownish-black seeds, are green when tender and dark-brown when fully ripe and possess high nutritional value (Irondi and Chukwuma, 2013). When dry, they have a pleasant aroma, and therefore are used as spice in Central and West Africa (B.M. Mousette *et al*., 2015). This spicy property makes them valuable for preparing soup for nursing mothers from the first day of birth to prevent post-partum contraction (Ojewole and Adewunmi, 2004). Previous studies have demonstrated that different parts of the plant are used in ethnomedicine for the treatment of several ailments including diabetes mellitus, hypertension, intestinal parasites, malaria, asthma, epilepsy, schistosomiasis, wound healing and arthritis (Atawodi *et al*., 2014) Recent studies have also revealed that the pod possesses antioxidant and amylase inhibitory activities ; the fruits and barks extracts also have antioxidant activities (Irondi *et al.,* 2016).

2.6. SAPONINS AND THEIR POTENTIAL ROLE IN DIABETES MELLITUS

Saponins are amphipathic glycosides secondary metabolites which synthesized by many different plant species, have high molecular weight, consisting of a sugar moiety united to a triterpenoid or

steroid sapogenins. Saponin has received numerous attention due to their various biological activities that including hepatoprotective, antitumor, antimicrobial, and anti-inflammatory activities. Marine organisms such as starfish, sponges and sea cucumbers are now considered a rich source of saponin (Moghimipour *et al*., 2015). Saponins have been known to possess the anti-diabetic property and are promising compounds with potential to be developed into new drugs for anti-diabetes (El-Barky *et al.,* 2017).

2.6.1. CHEMICAL STRUCTURE OF SAPONIN

Saponins chemically consist of two parts aglycone and glycone, the aglycone part is also known by sapogenin which classified to either triterpenoid (C-30), neutral or alkaloid steroids (C-27). The aglycone part covalently linked to one or more glycone (sugar) (Augustin *et al*., 2011), which may be glucose, galactose, glucuronic acid, xylose or rhamnose, the oligosaccharide is attached at the C3 position but in some saponins, additional sugars are attached at C26 or C28 positions (Francis *et al*., 2002).

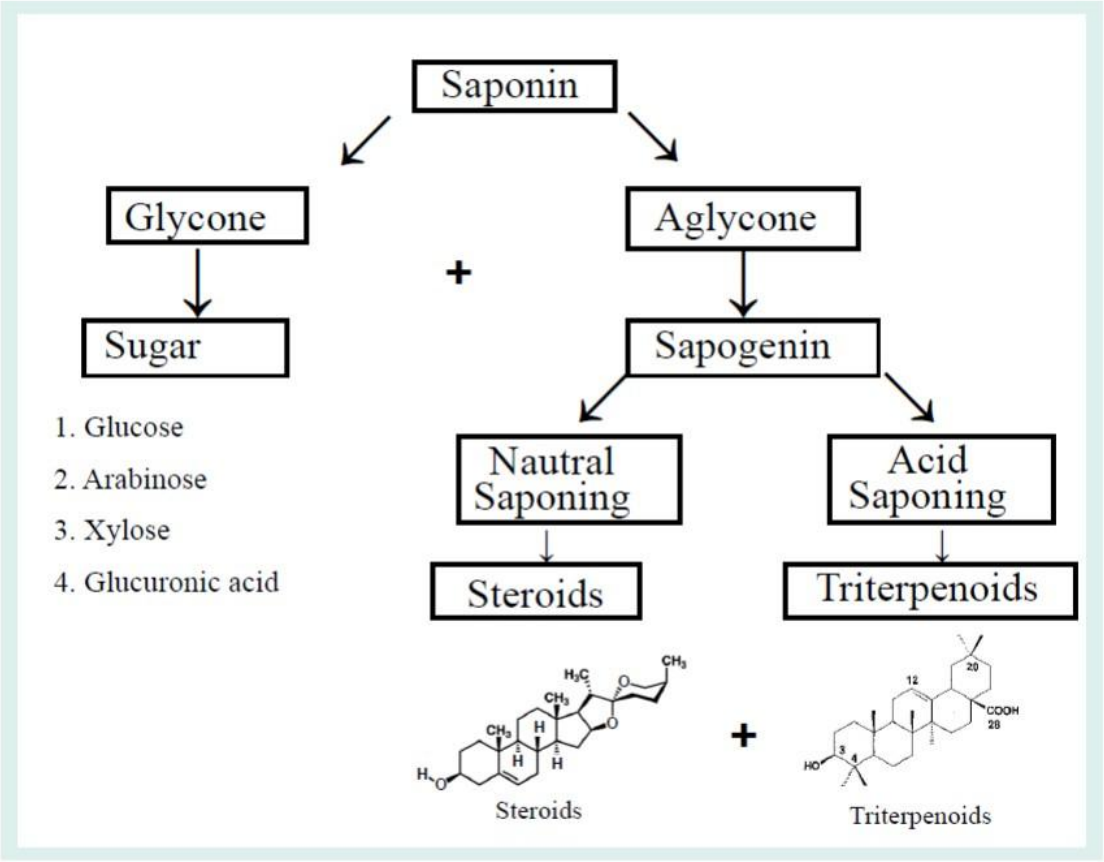


Fig. 2.3: Saponin structure

2.6.2 MECHANISM OF ACTION OF SAPONIN IN DIABETES

Diabetes mellitus (DM) is a serious chronic endocrine disturbance that is a main source of malady worldwide, it is commonly accompanied by distinct complications for instance retinopathy, neuropathy, nephropathy and cardiovascular disease (Elekofehinti *et al*., 2013). Diabetes is associated with severe long-term micro and macrovascular and significantly raises the number of morbidity and mortality. Oxidative stress plays a fundamental role in the development of DM which increases complications with increased free radical formation. Oxidative stress also produces reactive oxygen species, which resultant toxic effect on cell development, growth and

survival (Karasu, 2010). Oxidative stress is created with the formation of advanced glycation end

product which has a strong association with the diabetic complications. Free radicals which resulted from oxidative stress stress-mediated and promoted programmed β-cell death. Oxidative stress also, reacts with polyunsaturated Fatty Acids of lipid membrane and cause lipid peroxidation (Rother, 2007). Saponins have been reported to possess a wide range of biological activities. For instance, saponins were known to be bioactive against diabete (Abdel–Hassan, 2000). Saponin which has been extracted from *Holothuriathomasi,* sea cucumber, has a potent hypoglycemic effect in

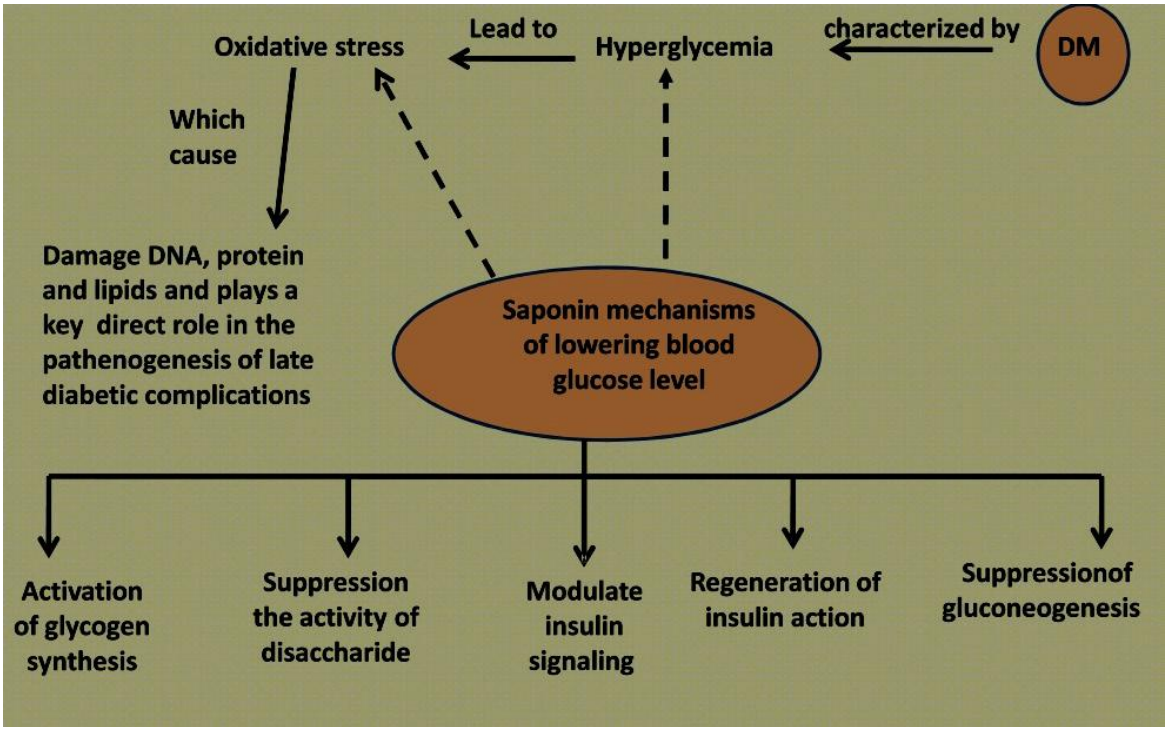


Fig. 2.4: Mechanism action of Saponin in Diabetes.

STZ-diabetic rats as it stimulates secretion, the action of insulin, regeneration of beta cells islets and activate the enzymes which are responsible for glucose utilization. Charantin which is a steroidal saponin is composed of a mixture of beta-sitosterol-beta-D-glucoside and 5.25 stigmadien-3-beta-ol glycoside, utmost quantity of charantin is found in the fruit of bitter melon which characteristic by hypoglycemic effect (Spasov *et al*., 2008). *Momordicacharantia* extract enhances liver glycogen storage, supports healthy insulin secretion by the islets of Langerhans, promotes peripheral glucose

utilization, and healthy serum protein levels. Saponin of *Momordica charantia* increase glucose utilization by the liver, decrease gluconeogenesis via inhibition the two key enzyme glucose-6-phasphat and fructose-1,6 bisphosphatase and improve glucose oxidation by activating glucose-6-phasphate dehydrogenase via the shunt pathway (Kumar, 2010). Charantin also upgrades insulin release from beta cells pancreatic islets and promote a new growth of insulin-secreting beta cells. The antidiabetic mechanism of this compound is a diminish blood glucose and an increase plasma insulin levels. The ability of saponin to decrease blood glucose level makes saponin an excellent efficient antioxidant in the remediation of diabetes mellitus. The hypoglycemic action of saponin is via rejuvenation of insulin, amendment insulin signaling, release insulin from the beta cell islets, inhibition the activity of disaccharide, activation of glycogen synthesis, Inhibition of gluconeogenesis, inhibition the activity of α-glucosidase, inhibition of mRNA expression of glycogen phosphorylase and glucose 6-phosphatase and increase the expression of Glut4 (McAnuff *et al*., 2005).

2.7. Justification

Past examinations exhibited the *in vivo* antidiabetic, antihyperlipidaemic and antioxidant impacts of aqueous extracts of T. *tetraptera* root bark. The presence of saponins is effectively perceptible in T. *tetraptera* root bark. A few reports have shown that saponins from various sources have antidiabetic, antihyperlipidaemic and antioxidant properties; this study was along these lines intended to find out whether the results observed from past studies are owing to saponins. This will help narrow down the phytochemicals that apply the noticed expected remedial effects of T. *tetraptera* root bark, which can last prompt the explanation of the specific antidiabetic substance in this plant.

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS

3.1.1 Plant Collection

Tetrapleura Tetraptera (Schum. and Thonn) Taub was obtained from Akungba-Akoko, Ondo State, South-Western Nigeria and identified in the Department of Plant Science and Biotechnology, University of Benin, Nigeria. Herbarium specimen will be deposited at the Herbarium of the University of Benin, Nigeria.

The plant materials were washed thoroughly under running water, shade dried and then pulverized.

3.1.2 Chemicals/Reagents preparation and Equipment

All chemicals were of an analytical grade and are supplied from Sigma Chemical Co. USA. Distilled water was used in all biochemical assays.

i. Streptozotocin

ii. Ellman's Reagent

iii. Trichloroacetic acid (TCA)

iv. Thiobarbituric acid (TBA)

v. sulfosalicyclic acid

vi. Reduced glutathione (GSH)

vii. Acetic acid, and other analytical grade reagents

Equipment:

i. Spectrophotometer

ii. Centrifuge

iii. Freeze dryer

iv. Rotary evaporator

v. Glucometer

vi. Electronic digital weighing balance

vii. Sensitive weighing balance

viii. Beakers and other laboratory glasswares

3.1.3. Extraction of saponins

The scheme below was employed for the extraction of *T. tetraptera* total saponins (TTS) fractions

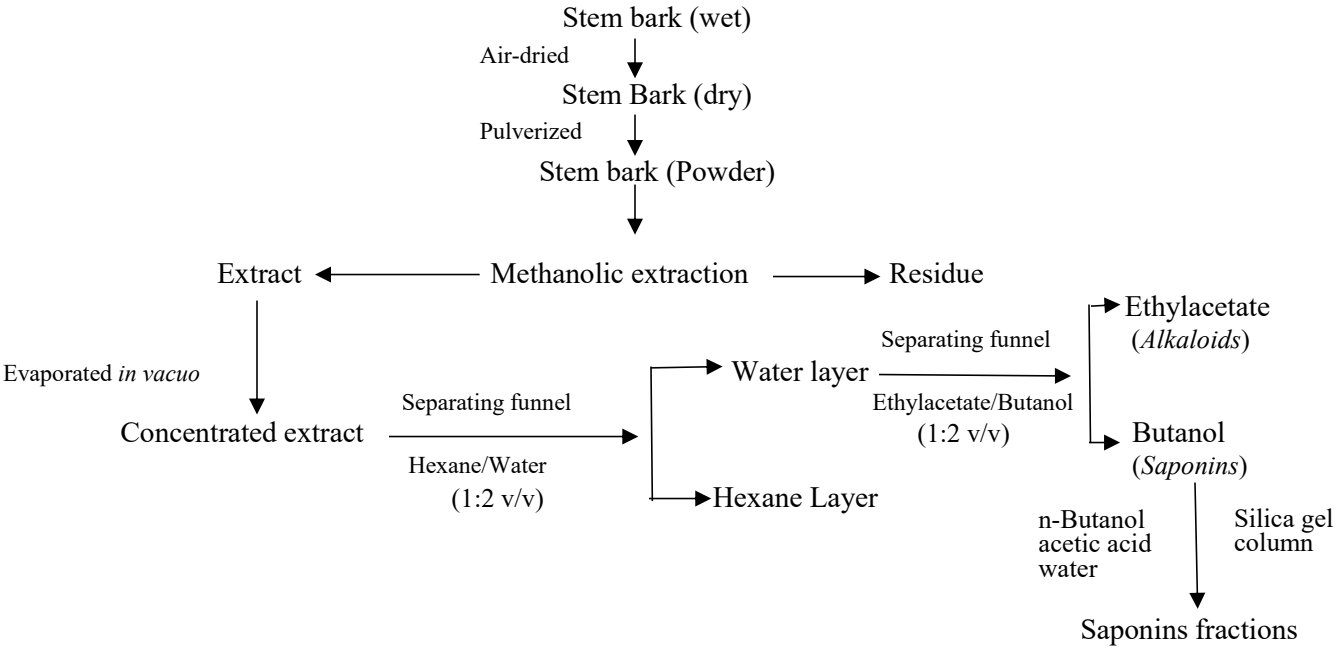


Figure 3.1: Method used to fractionate saponins from *T. tetraptera* stem bark, adapted from Hostettmann *et al.* (1991)

3.1.4 Preparation of plant extract

The *T. tetraptera* total saponins (TTS) fractions was concentrated under pressure and then freeze dried; the TTS was reconstituted appropriately in distilled water.

3.2.1 *In vivo* Antidiabetic Studies

In vivo studies, using streptozotocin diabetic rat models, were done to ascertain the antidiabetic, antihyperlipidaemic and antioxidant effects of *T. tetraptera* saponins (TTS). This phase of the study consist of the following:

i **The induction of experimental diabetes in rat models**

Normal and streptozotocin diabetic rats were divided into the following groups of eight (8) rats each:

a. Normal control (untreated normal rats)
b. Diabetic control (untreated diabetic rats)
c. Positive control (diabetic rats treated with metformin)
d. Diabetic rats treated with 10 mg/kg body weight of TTS
e. Diabetic rats treated with 20 mg/kg body weight of TTS
f. Diabetic rats treated with 40 mg/kg body weight of TTS

ii **Adiministration of Extracts**

The extracts were administered orally for twelve (12) weeks (by *gavage*), after which the rats were sacrificed. Biochemical and histological investigations were conducted

iii **The biochemical investigations**

Non-enzymatic antioxidant analysis - -reduced Glutathione (GSH), and malondialdehyde (MDA) – by standard biochemical methods.

3.3.1. Animals and Experimental Protocol

A total of forty-two (42) adult male rats of the Wistar strain, with average weight of 120 g were purchased from the Department of Animal Science, University of Ibadan, Nigeria. They were kept in a well aerated room (Department of Anatomy Animal House, University of Benin), with 12h light and 12h dark cycles. They were fed twice a day (standard pelleted feed) and given water *ad libitum*. The animals were acclimatize for two weeks before the commencement of the study. Treatment of the animals conformed to the guidelines for the Care and Use of Laboratory Animals (NAS, 2011). An ethical clearance on animal handling and care with the code, EC/FP/020/19 was obtained for this study.

3.3.2. Induction of diabetes

Streptozotocin was dissolved in acidified (pH 4.5) normal saline and administered to the rats, by intra-peritoneal injection, at a dose of 65mg/kg body weight after a 12-hour fast. Diabetes was confirmed after seven (7) days of STZ administration by measuring fasting blood sugar (FBS). After stable diabetes was established (FBS > 180 mg/dl), TTS and metformin administration to rats commenced and lasted for 12 weeks.

3.3.3. Administration of plant extract

Different doses of TTS and metformin, at 100 mg/kg body weight were administered orally (by *gavage*) daily for 12 weeks.

3.3.4. Periodic determination of the weight of animals

The weights of the animals were determined using an electronic weighing balance every 4 weeks to measure the change in body weight over the period of administration.

3.3.5. Blood collection

Blood for monitoring fasting blood sugar was drawn from the tail vein of each rat once in 2 weeks and assessed with a glucometer. At the end of 12 weeks, the rats were sacrificed by decapitation and blood was collected through the trunk; the thoracic/abdominal were opened to expose the heart and other organs. Blood for glucose assays (2 mls) was collected in fluoride bottles while that for other biochemical analyses (2 mls) was collected in plain bottles. The blood samples were allowed to clot on ice and centrifuged at 1,000 X g for 5 minutes; the serum was separated for analysis.

3.3.6. Preparation of serum

The animals were weighed and sacrificed twenty-four hours after treatment with the different doses of the plant isolates by decapitation. Blood samples were collected into EDTA anticoagulant bottles for hormonal assay and into nonheparinized tubes. The blood samples in the nonheparinized tubes were allowed to stand for few minutes in order to clot. Serum was separated by centrifuging the clotted blood at 3,000rpm for 15minutes with a table centrifuge. The clear serum (supernatant) was stored at $4^{o}C$.

3.3.7 Tissue homogenization

The liver, heart, kidneys, pancreas and testes were removed and blotted with cotton wool. Portions for tissue homogenization were washed in ice cold normal saline, and homogenized in ice cold normal saline 4:1 w/v. The homogenate was centrifuged at 1,000 X g for 5 minutes; the separated supernatant was stored in a freezer until analysis.

3.4 BIOCHEMICAL ANALYSIS

3.4.1 Serum Antioxidant Assay

3.4.1.1 Estimation of Reduced Glutathione (GSH) level

The method of Beutler *et al.*, (1963) was followed in estimating the level of reduced glutathione (GSH).

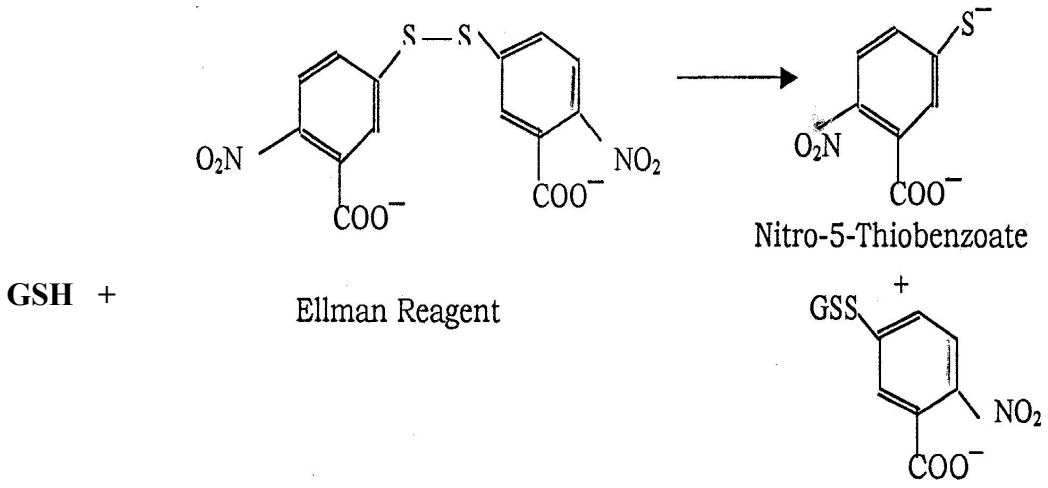


Fig. 3.2: Reaction of GSH with Ellman's Reagent

PROCEDURE: About 0.1ml of test sample was diluted with 0.9ml of distilled water to give 1 in 10 dilutions. 3ml of of 4% sulfosalicyclic acid solution (precipitating agent) was added to the diluted test sample to deproteinize it. The mixture was centrifuged at 3,000rpm for 10minutes. Thereafter, 0.5ml of the supernatant was added to 4ml of 0.1M phosphate buffer and finally 4.5ml of Ellman's reagent was added. A blank was prepared with reaction mixture of 4ml of 0.1M phosphate buffer, 0.5ml of the diluted precipitating solution (addition of 3ml of precipitating solution and 2ml distilled water) and 4.5ml of Ellman's reagent. The concentration of reduced glutathione, GSH level was determined using absorbance at 412nm.

All readings were taken within 5 minutes, as colour developed is not stable after that duration, following addition of Ellman's reagent. Serial dilutions of the GSH working standard were prepared as well.

3.4.1.2. Serum and tissue malondialdehyde assay using the thiobarbituric acid method as described by Varshney and Kale (1990).

Reagent	Contents	Initial Concentration of Solutions
Solutions	Trichloroacetic acid (TCA) Thiobarbituric acid (TBA) Hydrochloric acid	15 g/100ml 0.375 g/100ml 0.25 N

Preparation of the TBA-TCA-HCl stock solution

PROCEDURE: 15 g of TCA and 0.375 g of TBA was measured into 100 ml of 0.25 N HCl, the solution was heated mildly to facilitate dissolution.

Tissue homogenate: Dilute 1 + 9 with distilled water

Assay Protocol

	Blank	Sample
TCA TBA-HCl Stock (ml)	2.0	2.0
Sample (ml)	---	1.0

Solution was heated for 15 minutes in boiling water and cooled, thereafter centrifuged at 3500rpm for 10 minutes. The supernatant was separated and sample absorbance read against blank at 535 nm.

Calculation

$$\text{MDA concentration in units/ml homogenate} = \frac{\text{Absorbance} \times \text{vol of rxn mixt}}{\text{Extinction coefficient} \times \text{vol of sample}}$$

Volume of reaction mixture =3mls
Volume of sample = 1ml

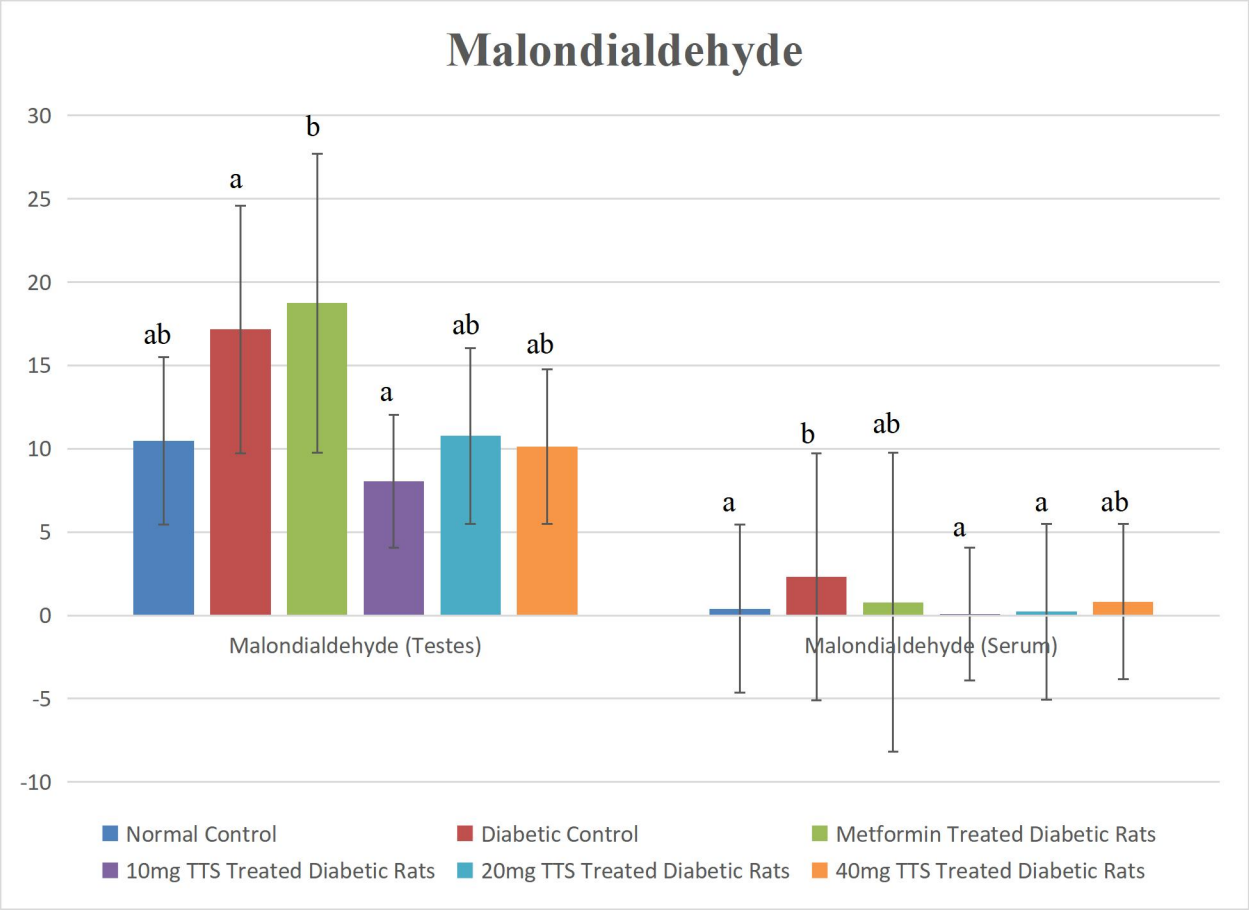
Extinction coefficient $= 1.56 \times 10^5$

3.5 Statistical analysis

Results of experiments were expressed as mean ± standard error of mean (S.E.M). Analysis of variance (ANOVA) was carried out on the result data at 95% confidence level using SPSS statistical software package, version 26.

CHAPTER FOUR

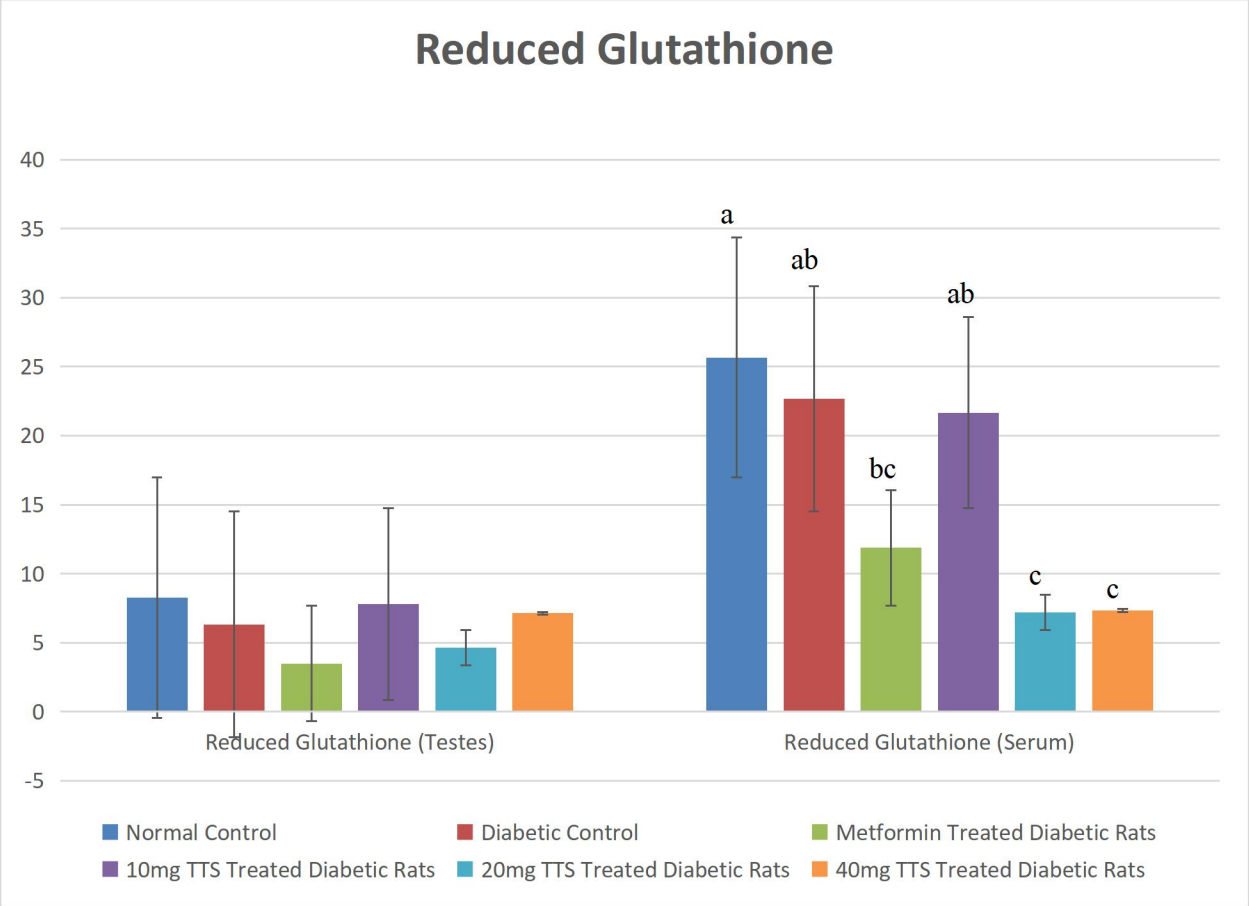
RESULTS



Values with different superscripts on their column are significantly different at P <0.05

Fig. 4.1: Effect of *Tetrapleura Tetraptera* saponins on Malondialdehyde (MDA) concentrations of Streptozotocin induced diabetic Wistar rats.

In the testes, there was a statistically significant ($p<0.05$) increase in the MDA levels of the untreated diabetic group compared to the normal control. This was significantly ($p<0.05$) reduced to the normal level in diabetic rats treated with different concentrations of TTS. Likewise in the serum, treatment with metformin and TTS fractions significantly ($p<0.05$) reduced the MDA levels.



Values with different superscripts on their column are significantly different at P <0.05

Fig. 4.2: Effect of *Tetrapleura Tetraptera* saponins on Reduced Glutathione (GSH) concentrations of Streptozotocin induced diabetic Wistar rats.

In the testes and serum, the reduced glutathione significantly ($p<0.05$) decreased in the metformin treated diabetic group compared to the diabetic untreated group. Similarly, the reduced glutathione significantly ($p<0.05$) decreased in the TTS treated groups (groups 5 and 6) compared to the diabetic untreated group.

CHAPTER FIVE

DISCUSSION

Streptozotocin induces diabetes in experimental animals by destruction of the pancreatic β-cells which results in the elevation of the blood glucose level in diabetic rats (Maiti *et al*., 2004). Evidence suggests that oxidative stress is increased in diabetes, because of excessive production of reactive oxygen species (ROS) and an impaired antioxidant defense mechanism (West, 2000). It has been suggested that ROS induces membrane lipid peroxidation and that the toxicity of the generated fatty acids peroxides are important causes of cell malfunction (Sanocka and Kurpisz, 2004). The most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MDA).

In this study, although the MDA levels were increased in the diabetic control group, treatments with TTS in diabetic rats at the dose of 10mg, 20mg and 40mg significantly decreased the levels of MDA in the testes and serum, even more than the metformin treated group (fig. 4.1). Several reports show that sexual behavior and reproductive tract function are markedly affected by diabetes mellitus and that increased oxidative stress leads to the impairment of spermatogenesis and increased level of MDA in rat testes (Nazıroglu, 2003). As constant hyperglycemic levels of NIDDM patients are exposed to an increased oxidative stress, the production of several reducing sugars (through glycolysis and polyol pathways) are enhanced. Thus, the lipid peroxide in the blood provides useful information for the prognosis of diabetes in which secondary disorders are often fatal (Nazıroglu, 2003). TTS may thus be protective against testes oxidative damage.

This study revealed the diabetic state induced more free radicals in excess of the antioxidant activities of the experimental animals, resulting in the decreased levels of glutathione as observed in the diabetic treated groups (groups 3-6). Antioxidants can be defined as substances whose presence inhibits the rate of oxidation of lipids, proteins, carbohydrates and DNA. Antioxidants such as

reduced glutathione (GSH) act as potent electron donors; they donate hydrogen atoms to pair up with unpaired electrons on free radicals. Thus, they convert reactive free radicals into inactive substances (Bagchi and Puri, 1998).

CONCLUSION

In this study, administration of TTS significantly reduced lipid peroxidation in the experimental rats as measured by malondialdehyde. However, there was an imbalance in the oxidant-antioxidant state as observed in the decreased levels of reduced glutathione. It can therefore be concluded that diabetes may induce oxidative stress in the testes of streptozotocin-induced diabetic rats.

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