Cape Peninsula
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EFFECT OF AQUEOUS EXTRACT OF *ANCHOMANES DIFFORMIS* ON TESTES AND EPIDIDYMIS OF STREPTOZOTOCIN-INDUCED DIABETIC MALE WISTAR RATS

By

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A thesis submitted in fulfilment of the requirements for the degree Master of Science: *Biomedical Technology* in the Faculty of *Health and Wellness Sciences* at the Cape Peninsula University of Technology

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ABSTRACT

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia, and its prevalence continues to rise worldwide. Hyperglycemia is accompanied by oxidative stress, which consequently leads to the damage of macromolecules in the body. Due to oxidative stress, the male reproductive organs are compromised, which interferes with sperm production and storage, and leads to male infertility. Increase in the prevalence of DM is associated with the increase in the prevalence of male infertility. Synthetic drugs have been produced as treatment of DM complications. Most of these drugs cause side effects which include hypoglycemic in DM patients. Due to the side effects, cost of production and unaffordability of these synthetic DM drugs, scientists have found alternative and complementary medicine in plants / plant products. Oxidative stress is alleviated by antioxidants, both endogenous and exogenous. Some plants have been found to contain antioxidant compounds and this has led to the interest in using medicinal plants to increase antioxidant capacity where oxidative stress is increased in diabetic models. This has yielded findings that suggest the potential benefits of medicinal plants in ailments accompanying DM, including male infertility. *Anchomanes difformis* (A. *difformis*) is one of the plants found to contain antioxidant compounds and has been reported for its benefits in health. A. *difformis* has however, limited studies related to the treatment of male infertility. This study investigated the ameliorative effect of A. *difformis* extract on oxidative stress-induced testes and epididymis in a DM model, to find this extract's potential benefits on the treatment of male infertility.

Sixty-four (64) male Wistar rats weighing approximately 180±10g were randomly grouped into 7 groups namely, normal control (NC), control treated with 200 mg/kg dosage (C200), control treated with 400 mg/kg (C400), diabetic control (DC), diabetic treated with 200 mg/kg (D200), diabetic treated with 400 mg/kg (D400) and diabetic treated with glibenclamide (GLB), with 8 rats in each group of non-diabetic (normal) rats, and 10 rats per group of diabetic rats. Type 2 diabetes mellitus (T2DM) was induced after 14 days by 10% fructose administration and intraperitoneal injection with 40 mg/kg body weight dose of streptozotocin (STZ). Treatment with A. *difformis* aqueous extract commenced 5 days after the induction which lasted for 42 days. Both the testes and epididymis were weighed to assess weight relative to body weight and antioxidant and oxidative stress assays were performed on both organs to determine the effect of A. *difformis* aqueous extract. Oxidative stress was determined by measuring the level of malondialdehyde (MDA), a biomarker of lipid peroxidation. Catalase (CAT) and superoxide dismutase (SOD) activities, and reduced glutathione (GSH) concentration were measured to determine antioxidant status. Furthermore, antioxidant capacity of A. *difformis* against

free radical such as 2, 2-diphenyl-1-picrylhydrazyl and metal chelating property were also investigated by performing ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays. The relative testes weight was increased with DM induction while *A. difformis* extract showed no effect on the relative testes weight. However, relative epididymis weight was lower in the diabetic group, and increased with A. *difformis* extract treatment. MDA was higher in the diabetic control group compared to the normal control group. A. *difformis* extract lowered MDA levels in diabetic rats. Both CAT and SOD activities were reduced in the diabetic testes and restored back to normal with the administration of the extract. GSH level remained unchanged in the testes of all groups. All the antioxidants enzymes and GSH showed no significant difference in the epididymis with induced diabetes and treatment with the plant extract. Similarly, FRAP and DPPH showed no significant change in the epididymis. The testes also showed no change in DPPH. FRAP capacity was reduced in the testes of diabetic control compared to the control group, and the plant extract showed no effect in this reduction. T2DM model was established with observed symptoms such as elevated glucose levels and a decrease in body weight in diabetic control rats. Diabetes-mediated hyperglycemia caused oxidative stress in the testes, although it was not observed in the epididymis. A. *difformis* extract showed a protective effect against oxidative stress on the testes. With these in *vivo* experimental findings, A. *difformis* extract looks promising for the development of potential agent for the treatment of male infertility associated with DM. Thus, further studies on the isolation of the pure compounds and their mechanism of actions are worthy of investigation.

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ABBREVIATIONS AND ACRONYMS

6-OHD	6-hydroxydopamine
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAT	Catalase
DETAPAC	Diethylenetriaminepentaacetic acid
DM	Diabetes mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5'-Disulfanediylbis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
F6P	Fructose 6 phosphate
FRAP	Ferric Reducing Ability of Plasma
FSH	Follicle stimulating hormone
G6P	Glucose-6-Phosphate
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GLUT1	Glucose transporter type 1
GLUT2	Glucose transporter type 2
GLUT4	Glucose transporter type 4
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
IRS	Insulin receptor substrate
LH	Luteinizing hormone
MDA	Malondialdehyde
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NrF2	Nuclear factor E2-related factor 2
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinase
PKB/AKT	Protein kinase B
RAGEs	Receptor for advanced glycation end products
ROS	Reactive oxidative species
SOD	Superoxide dismutase
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive species
TNB	2-nitro-5-thiobenzoic acid
TPTZ	2,4,6-Tripyridyl-S-triazine

Vit C	Vitamin C
Vit E	Vitamin E

CHAPTER 1
INTRODUCTION

1.1. Background

Diabetes Mellitus (DM) is a metabolic disease first studied in the 21st century after a concern of trending symptoms such as constant excretion of urine containing glucose, and increased thirst (Chukwuemeka, 2015). The discovery of DM led to studies that were conducted to understand its pathogenesis and the treatment thereof (Iya *et al*., 2019; Roxo *et al*., 2019; Zaidun *et al*., 2019) . DM is caused by the complications in the secretion or action of insulin that lead to hyperglycemia, a characteristic of DM (Temidayo & Stefan, 2017; Johnson *et al*., 2019). Insulin is a peptide hormone synthesized in the islets of Langerhans of the pancreas by the beta cells (Figure 1.1 shows the arrangement of cells in the pancreas) and its primary function is to bind to its receptors on body cell membranes to activate the intake of glucose from the bloodstream into the cells (Wilson *et al*., 2017). Insulin is secreted in all vertebrate animals (Petersen & Shulman, 2018), and its receptors are found in all cells except hepatocytes, brain cells, gut cells, and beta cells of the pancreas (Yan, 2014). Chemical drugs such as metformin and glibenclamide were produced for the treatment of DM by lowering blood glucose levels (Rambiritch *et al*., 2014; Liu *et al*., 2018). However, side effects from the use of these synthetic drugs caused great concern and led to more studies of DM (Khaki
et al., 2014).

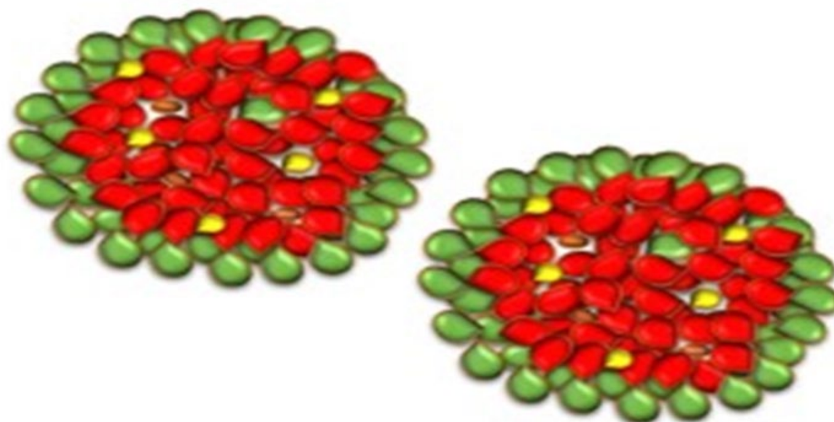

Figure 1.1: Arrangement of the cells of the islets of Langerhans in the pancreas. The red colour shows the population of the beta cells against the alpha (green) and delta (yellow) cells. Beta cells secrete insulin and make up the most population in the islets of Langerhans (Graus-Nunes & Souza-Mello, 2019).

Hyperglycemia causes an excessive production of free radicals (Silva *et al*., 2020; Tian *et al*., 2020). A new approach in the treatment of DM emerged after the discovery of oxidative stress as a major factor that contributes to the complications that are accompanied by DM (Nna *et al*., 2019). The antioxidant system in the body counteracts the effect caused by the elevation of ROS in the body (Pajares *et al*., 2018). Most of the non-enzymatic antioxidant compounds are major components of some plants (Alabi *et al*., 2020). Due to this knowledge, several studies have investigated the effect of certain medicinal plants on DM complications targeting the antioxidant pathway (Khaki *et al*., 2014; Ostovan *et al*., 2017; Nna *et al*., 2019). One of the major complications that can be caused by DM is male infertility (Alsenosy *et al*., 2019).

Oxidative stress leads to the destruction of the male gonads (testes and epididymis) which affects the production and storage of the spermatozoa, further leading to male infertility (Ostovan *et al*., 2017; Tian *et al*., 2020). Several studies have yielded positive results regarding the effect of medicinal plants in the treatment of male infertility as a complication caused by oxidative stress (Ghafari et al., 2011; Asadi et al., 2017; Oyenihi et al., 2020). Native Americans used Araceae species in the past for both food and medicine (Tchicaillat-Landou *et al.*, 2018). *A. difformis* is one of the medicinal plants investigated and found to have health promoting effects on diabetic conditions due to its antioxidant content (Alabi *et al.,* 2020).

Anchomanes difformis (A. *difformis*), also known as Blume, is an Araceae plant that grows in tropical areas and is mostly found in African countries such as Nigeria, Togo, and Ivory Coast (Ataman & Idu, 2015; Ahmed, 2018). It is a large green-stem plant with white color at the bottom of the stem (Ataman & Idu, 2015). Its prickly stem contains watery or milky latex that can grow long from up to 0.8 m to 2 m in height (Ataman & Idu, 2015) (Figure 1.2). This plant has been well known in traditional medicine for its use in treating different diseases (Oghale & Idu, 2016; Alabi *et al*., 2020). Several studies have reported that A. *difformis* alleviates symptoms brought by diabetes (Aliyu *et al*., 2013; Ataman & Idu, 2015; Alabi *et al*., 2020).

Figure 1.2: Representation of an *A. difformis* plant (Ataman & Idu, 2015).

1.2. Problem statement

Diabetes Mellitus is a worldwide concern and a possible cause of male infertility arising from oxidative stress which causes damage to the reproductive organs and germ cells consequently (Nna *et al*., 2017; Temidayo & Stefan, 2017). Medicinal plants play an important role in developing countries as therapeutic remedies for complications caused by diabetes (Ataman & Idu, 2015). The preference of traditional medicines over conventional 'Western' medicines by many patients in the management of diabetes, is evident (WHO, 2003). This is because most diabetic drugs are expensive and unaffordable (Temitope, 2015). Sustainable development and medical research continue with further research on the benefits that some plants have on diabetic complications, and the possibility of these plants to treat diabetes with fewer side effects (Tchicaillat-Landou *et al*., 2018). Although A. *difformis* has been found to be a potential therapeutic plant for several diabetic complications, not enough studies have been conducted to investigate its effect on male infertility. This study specifically focuses on the effects of *A. difformis* against oxidative stress, associated with hyperglycemia, on the male reproductive organs.

1.3. Aim

The study aimed to determine the ameliorative effects of A. *difformis* on the testes and epididymis of streptozotocin-induced diabetic male Wistar rats.

1.4. Objectives

- To measure the ameliorative effect of *A. difformis o*n antioxidant enzymes in the testes and epididymis: catalase (CAT) and superoxide dismutase (SOD) in diabetic and non-diabetic male Wistar rats.
- To determine the effect of *A. difformis* on antioxidant capacity by measuring ferric-reducing antioxidant Power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays in testes and epididymis of diabetic and non-diabetic male Wistar rats.
- To determine the effect of *A. difformis* on oxidative stress biomarkers: lipid peroxidation levels using TBARS measuring malondialdehyde (MDA)
- To determine possible weight changes in the testes and epididymis of diabetic and non-diabetic male Wistar rats cause by A. *difformis*.

1.5. Research questions

- What is the effect of A. *difformis* on the oxidative status of the testes and epididymis of diabetic and non-diabetic male Wistar rats?
- What effect does A. *difformis* have on the antioxidant capacity of the diabetic and non-diabetic male Wistar rats?
- What is the effect of A. *difformis* on the relative weights of the testis and epididymis of male Wistar rats?

1.6. Hypothesis

The study was premised on the hypothesis that an aqueous extract of A. *difformis* would ameliorate oxidative stress and reproductive dysfunction in a diabetic rat model.

1.7. Rationale

The prevalence of DM Continues to rise each year in almost all countries (Cho *et al*., 2018). According to Nna and colleagues (2017), there is a relationship between the percentage of diabetic men and the percentage of male infertility. Current modern treatment for DM is still not available to every diagnosed patient as some diabetic patients do not have access to the medication due to the high cost (Temitope, 2015). The discovery that diabetes is associated with oxidative stress paves a way to a new intervention to treat diabetes. The administration of antioxidant-rich medicinal plants to

reduce oxidative stress in diabetes mellitus is the focus of the present research especially that oxidative stress is associated with complications that arise in DM, including male infertility.

Studies have shown the availability of antioxidants and free radical scavenging properties of *A. difformis* (Ataman & Idu, 2015). Anti-inflammatory and anti-diabetic properties of *A. difformis* have been investigated (Adebayo *et al.*, 2014; Ataman & Idu, 2015). *A. difformis* has also been proven to have hypoglycemic properties (Adeyemi *et al*, 2015) and renal effects (Ataman & Idu, 2015). The effect of this plant extract on male infertility have not been explored in that depth (Nna *et al.*, 2017). This study will provide insight into the effects of *A. difformis* on the oxidative state of diabetic and non-diabetic male Wistar rats. Furthermore, the potential effects *A. difformis* on the testes and epididymis will be explored to determine if the plant could be a treatment option for infertility in diabetic males.

CHAPTER 2
LITERATURE REVIEW

Diabetes mellitus has been linked to oxidative stress (Hosseini *et al*., 2019) and the development of male reproductive dysfunction (Johnson *et al*., 2019). Diabetic studies have shown the relationship between hyperglycemia and an evident increase in free radicals (Shoorei *et al*., 2019; Fan *et al*., 2020). In some cases of male infertility, oxidative status of the male gonads was found to have been altered, with a decrease in antioxidant defense and a consequent increase in oxidative stress (Nna *et al*., 2019). This chapter explains the development of DM from the normal physiology, generation of free radicals as a result of hyperglycemia, and the effect of the resulting oxidative stress on male reproduction. This chapter also takes a closer look at the modern treatment of versus traditional medicine treatment of DM, concentrating more on the medicinal plant, *Anchomanes difformis*.

2.1. Glucose metabolism

Glucose is the main source of energy in the body. The mitochondria utilizes glucoses during the production of ATP in all body tissues. Blood glucose is normally regulated to keep its level balanced. This section explains how glucose is metabolized, the hormones involved, and the pathophysiology of glucose metabolism.

2.1.1. Insulin secretion and signaling

When blood glucose is increased after a meal, some of the glucose enters the beta cells of the pancreas through glucose transporter type 1 (GLUT1) (Zhang *et al.*, 2019). In the beta cells, glucose is phosphorylated at its sixth carbon by an enzyme, glucokinase, to produce glucose-6-phosphate (G6P) (Wilson *et al*., 2017). Oxidation of G6P leads to the production of adenosine triphosphate (ATP), which subsequently leads to the closure of ATP-sensitive potassium ion channels, cell membrane depolarization, and an influx of calcium ions through voltage-gated calcium ion channels (Wilson *et al*., 2017). This activates insulin vesicles in the cell to move to the cell membrane where insulin is released to the outside of the cell, into the bloodstream (Zhang *et al.*, 2019).

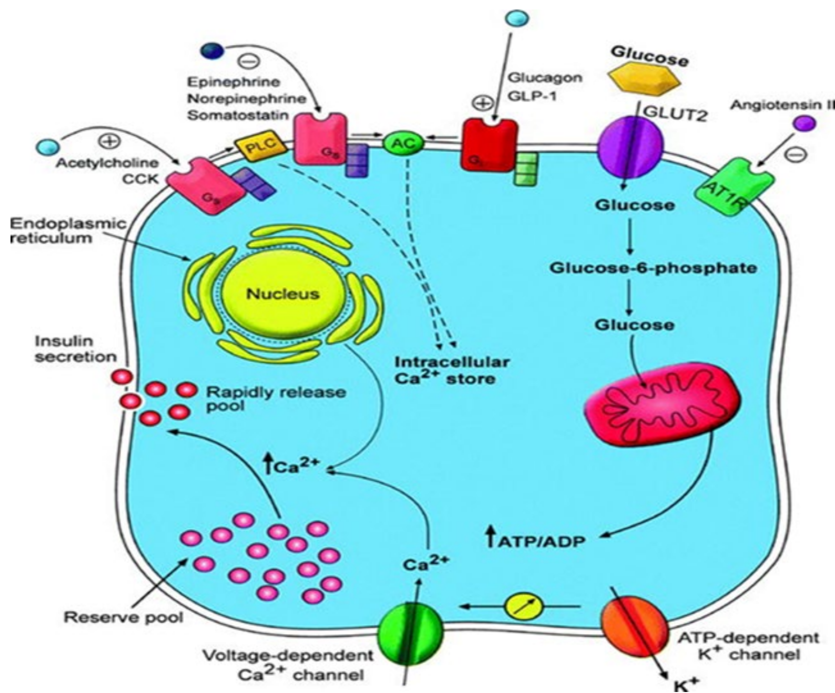

Figure 2.1: Insulin secretion pathway in the beta cell of the pancreas. Transportation of glucose into the beta cell of the islets of Langerhans leads to the production of ATP, which causes an influx of calcium, leading to the release of insulin into the blood stream (Leung, 2010).

A study by Zhang and colleagues (2019) confirms that depolarization of the beta cells occurs after a meal, leading to calcium ion influx. Insulin binds to insulin receptors on cells to initiate a pathway that leads to glucose metabolic homeostasis (Wilson *et al*., 2017; Haeusler *et al*., 2018). As an example, this pathway can lead to glycogen synthesis and inhibit gluconeogenesis in both hepatic tissue and skeletal muscle, reducing the increased blood glucose (Leung, 2010).

The insulin receptor is a glycoprotein embedded in the cell membranes of cells with its external receptor domain made of two alpha subunits, and an internal catalytic domain made of two beta subunits. Each subunit of the beta domain contains a tyrosine kinase domain (Petersen & Shulman, 2018). When insulin binds to the insulin receptor, a conformational change occurs on the glycoprotein causing activation of the tyrosine kinase domains on the beta subunits (Haeusler *et al*., 2018). As a result, autophosphorylation of tyrosine residues on the beta subunits occurs, which amplifies the activation of kinases and leads to activation of activators of phosphoinositide 3-kinase (PI3K), Insulin receptor substrate (IRS) proteins (Graus-Nunes & Souza-Mello, 2019) (Quan *et al*., 2020). Activated PI3K activates Protein kinase B (PKB(AKT), an important protein in glucose metabolism (Haeusler *et al*., 2018). PKB(AKT) causes glucose

transporter type 4 (GLUT4) translocation to the membrane, promoting glucose transportation from the outside into the cells (Petersen & Shulman, 2018). PKB(AKT) also promotes glycogen synthesis and inhibits the production of glucose in the liver, thereby decreasing blood glucose level (Petersen & Shulman, 2018). When this pathway fails, glucose metabolism complications occur (Gulseth *et al*., 2019).

2.1.2. Glucose usage

In the cell, as previously mentioned, glucose is converted to G6P by glucokinase. G6P is converted to fructose 6-phosphate (F6P) and pyruvate is produced downstream. Figure 2.1 shows the pathway through which glucose is consumed to produce pyruvate (Yan, 2014). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) is one of the important enzymes in this pathway because it converts G3P to 1, 3-bisphophoglycerate, an intermediate of pyruvate (Ghashghaeinia *et al*., 2019). Pyruvate enters the Krebs cycle where other enzymes are involved in the production of ATP, NADH and $FADH_2$ (Nolfi-Donegan *et al*., 2020). Both NADH and $FADH_2$ enter the mitochondria and are substrates of the electron transport chain (Ghashghaeinia *et al*., 2019). The electron transport chain (situated in the inner membrane of the mitochondria) contains five complexes (complex I, II, III, IV and V), Coenzyme Q and Cytochrome C (Zhao *et al*., 2019). It is the chain through which electrons are passed on from one complex to another to form a proton gradient that helps ATPase (complex V) to produce ATP (Nolfi-Donegan *et al*., 2020).

NADH is oxidized by complex I, releasing 2 electrons into the complex and charging the complex to pump a hydrogen proton to the inter-membrane space (Yan, 2014). FADH2 is a substrate for complex II and gives out one electron (Zhao *et al*., 2019). The electrons from both complexes I and II are passed to Coenzyme Q, to complex III, complex IV and finally to an oxygen ion, forming water (Nolfi-Donegan *et al*., 2020). Complex III and IV also pump hydrogen protons to the inter-membrane space, adding on to the proton gradient (Zhao *et al*., 2019). When the gradient is too high, the hydrogen protons pass through complex V from the inter-membrane space into the matrix, while complex V produces ATP from ADP (Yan, 2014).

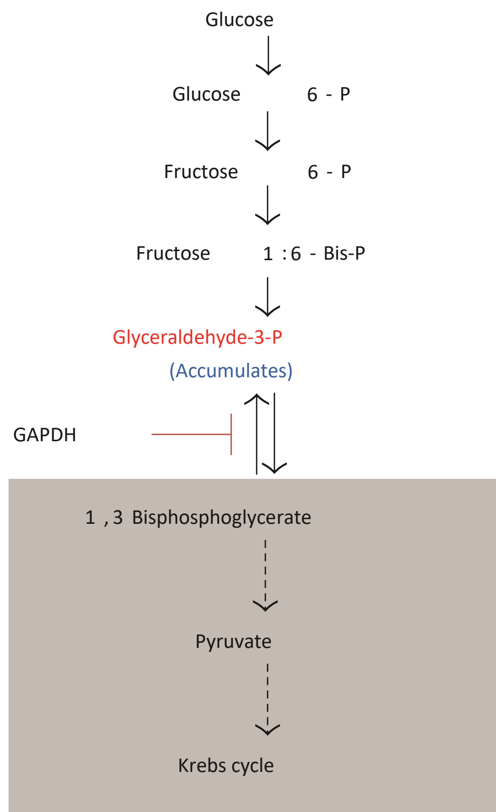

Figure 2.2: The glycolysis pathway. Intermediates such as glucose 6 phosphate and fructose 6 phosphate are formed during glycolysis and the product, pyruvate, is formed. Some important enzymes such as glucokinase and GAPDH catalyze these reactions (Yan, 2014).

2.1.3. Diabetes mellitus

Diabetes mellitus (DM) is a chronic disease that arises due to prolonged high blood glucose levels, caused by an imbalance in glucose metabolism homeostasis (Corrêa *et al*., 2019; Sefidgar *et al*., 2019). The high glucose level in the blood can be caused by either compromised insulin secretion or abnormality in insulin action (Temidayo & Stefan, 2017; Iya *et al*., 2019; Johnson *et al*., 2019; Roxo *et al*., 2019). Deficiency of insulin caused by inflammation of the pancreas (destruction of the beta cells) results in Type 1 diabetes mellitus (T1DM) (Ostovan *et al*., 2017; Temidayo & Stefan, 2017; Johnson *et al*., 2019). Type 2 DM (T2DM) is characterized by insulin resistance, which occurs when cells are insensitive to insulin which consequently leads to an abnormality in insulin action (Ostovan *et al*., 2017; Temidayo & Stefan, 2017; Johnson *et al*., 2019).

T2DM patients possess a high level of insulin in the blood and excessive secretion of insulin by the pancreas, which eventually causes dysfunction of the beta cells and subsequently, insufficient secretion of insulin (Gulseth *et al*., 2019). Pregnant women can also experience high levels of glucose (gestational diabetes) which can prolong

throughout the gestation period (Johnson *et al*., 2019; Kong *et al*., 2018). Risk factors that lead to the pathogenesis of DM include age, family history (genetics), ethnicity, obesity, and lifestyle behaviour (Iya *et al*., 2019). The prevalence of DM is 8.3% worldwide (Johnson *et al*., 2019). T1DM is mostly prevalent in children and affects a smaller percentage of people compared to T2DM (Ostovan *et al*., 2017). Amongst the percentage of people affected by DM, 10% is diagnosed with T1DM, and out of the 10%, 8.5% is diagnosed below the age of 20 years old (Temidayo & Stefan, 2017).The prevalence of DM rises by 60% each year, and it is estimated that 36 million people will be affected by the disease in 2030 with the number rising to up to 642 million in 2040, worldwide (Long *et al*., 2018). This has become a major concern.

DM is accompanied by complications which include renal failure, foot ulcers, cardiovascular diseases, neurodegenerative diseases, male infertility and others (Corrêa *et al*., 2019; Iya *et al*., 2019; Johnson *et al*., 2019). Due to increased public health concern, several studies have been carried out to investigate the aetiology of DM and possible treatment alternatives. Most studies use streptozotocin (STZ) drug to induce diabetes in animals because of its cytotoxic effect on the beta cells of the pancreas that causes less or no secretion of insulin leading to the development of diabetes (Omolaoye *et al*., 2018; Alabi *et al*., 2020). STZ is produced by a soil bacterium called *Streptomyces achromogenes* (Eleazu *et al*., 2013; Alabi *et al*., 2020). Its effect is usually observed between 1 to 3 days after injection (Eleazu *et al*., 2013; Alabi *et al*., 2020). A low dosage of STZ leads to partial toxicity in the beta cells and is used to induce T2DM while a higher dose causes total beta-cell death and a T1DM model (Alabi *et al*., 2020). Omolaoye *et al*. (2018) studied the use of STZ in inducing DM and its effect on male reproductive parameters. It was found that STZ dosage of as low as 30 mg/kg could still induce DM. T2DM can be induced by the combination of administration of 10% of fructose for two weeks, and STZ dosage of 40 mg/kg BW (Omolaoye *et al*., 2018). There is an evident link between DM and oxidative stress, which is a root to the underlying complications (Wang *et al*., 2011; Ostovan *et al*., 2017; Fishman *et al*., 2018; Liu *et al*., 2018; Corrêa *et al*., 2019).

2.2. Oxidative balance

This section brings into light the normal oxidative status of the body by explaining the findings on what free radicals are, their functions and how they are related to oxidative stress. The causes of oxidative stress are briefly explained. Due to the existence of the

antioxidant system, a homeostatic oxidative balance is kept. The antioxidant defense system is also explained in this section.

2.2.1. Free radicals and oxidative stress

Free radicals are produced during normal metabolism in the mitochondria by the electron transport chain (Aliyu *et al.*, 2013; Kurutas, 2016; Khorramabadi *et al*., 2018). They are useful for normal physiological systems in the body such as gene expression and signal transduction (Aliyu *et al*., 2013). Free radicals that have oxygen atom are called reactive oxidative species (ROS) and their examples are hydroxyl (-OH), superoxide(O_2^-), peroxyl (ROO-), peroxynitrite (OONO-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), nitrous acid (HNO_2), nitroxide (NO^-), (Aliyu *et al*., 2013; Kurutas, 2016; Perrone *et al*., 2018). When they contain nitrogen, they are called reactive nitrogen species (RNS). Free radicals containing oxygen (ROS) are the most important as they are the most reactive (Kurutas, 2016). Under normal conditions, 1-3% of the oxygen taken in during respiration is converted to ROS which is responsible for metabolic functions (signal transduction, expression of genes, and acting as signaling molecules) (Rahal *et al*., 2014).

Free radicals also act against infections. Superoxide is produced endogenously when oxygen receives an electron (Yan, 2014; Kurutas, 2016). This can occur through the electron transport chain where oxygen is reduced to water. Free radicals are formed as products of the electron transport chain but are bound to the complexes of the electron transport chain (Yan, 2016). In this process, some electrons can move into the matrix of mitochondria where they partially reduce oxygen ions, forming superoxide anions (Pajares *et al*., 2018; Perrone *et al*., 2018). Superoxide can also be produced in the vascular endothelium to neutralize nitric oxide (NO) in the regulation of relaxation of the vessels (Kurutas, 2016). Hydrogen peroxide can be produced from enzymatic reactions but is mainly produced by the dismutation of superoxide (Pajares *et al*., 2018). Hydrogen peroxide does not directly cause damage but is a precursor for the formation of hydroxyl radical, a final reactive radical that causes more tissue damage (Kurutas, 2016). Hydroxyl radical can also be formed when superoxide reacts with hydrogen peroxide (Kurutas, 2016).

NO, produced by nitric oxide synthase (NOS) is responsible for the relaxation of blood vessels for endothelial balance (Strijdom *et al*., 2009). Superoxide is produced during the incomplete reduction of oxygen in the electron transport chain (Rahal *et al*., 2014). Both NO and superoxide can react to produce ROS and RNS. Free radicals are unstable, with

an electron missing from a pair of electrons in their outermost orbital. Free radicals are reductants and can oxidize other molecules (Kurutas, 2016). This implies that more free radicals could lead to more oxidation of biomolecules (Johnson *et al*., 2019). The level of free radicals is controlled because they are produced by enzymes that are well regulated (Johnson *et al.,* 2019). However, the electron transport chain is an unregulated source of free radicals (Kurutas, 2016).

When the production of free radicals exceeds the level of antioxidants, due to environmental or metabolic factors, oxidative stress results (Aliyu *et al*., 2013; Kurutas, 2016; Khorramabadi *et al*., 2018; Ferreira *et al*., 2019). This condition occurs due to either excessive production of free radicals, or when the antioxidant system is compromised (Kurutas, 2016; Perrone *et al*., 2018). Previous studies have shown a significant increases in hydrogen peroxide together with decreases in some antioxidants, suggesting that in most cases both of these factors are involved (Ostovan *et al*., 2017; Nna *et al*., 2019).

During oxidative stress, biological molecules such as proteins, lipids, and DNA are damaged (Aliyu *et al*., 2013; Kurutas, 2016; Oghale & Idu, 2016; Khorramabadi *et al.*, 2018). Damage of lipids can lead to the destruction of the cells membrane or induce cell death (Kurutas, 2016). Oxidative stress plays a role in the progression of diseases (Khaki *et al*., 2014; Kurutas, 2016). Terminal illnesses such as heart disease, cancer, neurodegenerative diseases, ageing and insulin resistance can result (Aliyu *et al*., 2013; Oghale & Idu, 2016; Zahran *et al*., 2019). Polyunsaturated fatty acids are targets of lipid peroxidation, a process caused by oxidants, which leads to the production of reactive aldehydes/ thiobarbituric acid reactive species (malondialdehyde (MDA), trans-4-hydroxy-2-nonenal and isoprostanes) (Liguori *et al*., 2018). Lipid peroxidation in homogenates of tissues can be determined by measuring TBARS (byproducts of lipid peroxidation). MDA is an example of decomposed TBARS and its level is measured when determining lipid peroxidation using the TBARS assay (Nna *et al*., 2019). MDA reacts with TBA in a 1:2 ratio at 90-100°C in an acidic environment. Therefore, the level of MDA can be detected by measuring how much MDA-TBA adduct is formed at an absorbance of 530 nm. Figure 2.3 below shows the reaction between MDA and TBA in the TBARS assay. Testicular and epididymis tissues contain a mixture of TBARS which include lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of antioxidants. These products are indicators of oxidative stress (Liguori *et al*., 2018). These products of lipid peroxidation can also react with biomolecules leading to the pathogenesis of diseases (Kurutas, 2016). MDA can also

be taken in from food, which raises the question of whether measuring levels of MDA can be reliable as an indicator of lipid peroxidation (Kurutas, 2016). However, when the diet is controlled in the subjects, MDA measured is assumed to be as a result of lipid peroxidation (Kurutas, 2016). A study conducted by Ostovan and colleagues (2017) showed an increase in MDA of the diabetic testis and epididymis of rats compared to normal rats. This was supported by similar studies (Oguntibeju *et al*., 2020; Oyenihi *et al*., 2020).

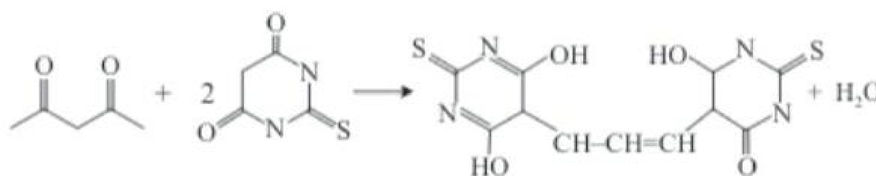

***Figure 2.3: Reaction between MDA and TBA to form MDA-TBA adduct* (Manzocco *et al*., 2016).**

Oxidative stress can be marked by a higher than normal level of ROS, mobilization of antioxidants, and by measuring lipid peroxidation as a marker for damage (Ferreira *et al*., 2019). Oxidative stress can also be assessed by considering its indicators such as the ratio of glutathione (GSH) to glutathione disulfide (GSSG), and the balance between oxidized and reduced thioredoxin (Mulholland *et al*., 2011). GSH to GSSG ratio is the most common indicator of oxidative status since it is the most abundant system in mammalian species (Mulholland *et al*., 2011).

2.2.2. The antioxidant system

The level of free radicals in the body is normally balanced by the presence of the antioxidant system (Rahal *et al*., 2014; Khorramabadi *et al*., 2018). The antioxidant system consists of enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic (vitamin E and C, carotenoids, lipoic acid, selenium, etc.) molecules that can scavenge free radicals or/and inhibit the oxidation of biological molecules by free radicals (Kurutas, 2016; Khorramabadi *et al*., 2018; Zahran *et al*., 2019). This is the first line of defense in eradicating ROS (Zahran *et al*., 2019). Antioxidants are produced by the body and can also be taken in from food and beverages (Oghale & Idu, 2016). Some antioxidants do not directly scavenge or neutralize free radicals, but they cause the production of antioxidants in the body that can neutralize free radicals (Kurutas, 2016). Diseases or complications caused by oxidative stress can be treated or prevented by the introduction of antioxidants (Pajares *et al*., 2018).

During ROS increase, antioxidant enzymes are increased through the nuclear factor E2-related factor 2 (Nrf2) pathway to counteract the action caused by the oxidants (Kurutas, 2016; Pajares *et al*., 2018; Alabi *et al*., 2020). Nrf2 is only active for a limited time before it is degraded by Keap1 so that the level of antioxidants is decreased back to normal after the reactions (Nna *et al*., 2019). However, when oxidative stress is too high, the activity of Keap1 is decreased and Nrf2 stays high, thereby causing continuous expression of antioxidant enzymes (Alabi *et al*., 2020). This was supported by a study done by (Zahran *et al*., 2019). In this study, the increase in the antioxidant enzyme, GPx was evident when oxidative stress was increased. (Zahran *et al*., 2019). In another study, however, antioxidant enzymes were rather compromised/decreased with an increase in oxidative stress (Nna *et al*., 2019; Fan *et al*., 2020). Although the level of antioxidants can be increased by antioxidant supplementation, the activation of antioxidant enzymes is enhanced through the activation of NrF2 (Kurutas, 2016; Pajares *et al*., 2018).

Antioxidants contain compounds such as phenols, polyphenols, flavonols, quercetin, catechins and anthocyanins (Aliyu *et al*., 2013). These compounds have similar structures that enable them to neutralize free radicals by donating an electron creating oxidative stability, or by delaying oxidation of the biomolecules (Aliyu *et al*., 2013). Most of these compounds are also found in fruits, vegetables, and beverages. When ROS is increased, antioxidants are mobilized to the site of high ROS for neutralization.

In a study carried out by Ferreira and colleagues (2019), mobilization of antioxidants was determined by measuring the change in the level of some examples of antioxidants such as reduced glutathione (an antioxidant that neutralizes peroxides), the activities of antioxidant enzymes such as superoxide dismutase (enzyme that protects body tissue from lipid peroxidation) and non-enzymatic antioxidants such as vitamin E (a non-enzymatic antioxidant that inhibits lipid peroxidation) (Khorramabadi *et al*., 2018; Ferreira *et al*., 2019). Eukaryotic organisms naturally produce antioxidants that convert ROS to more stable molecules. Examples of antioxidant enzymes produced naturally by the body (endogenous antioxidants) are catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) (Aboonabi & Singh, 2016; Kurutas, 2016).

CAT is a ubiquitous enzyme that lowers oxidative stress by lowering the levels of hydrogen peroxide (H_2O_2). This enzyme catalyzes the reaction that involves the conversion of two molecules of H_2O_2 into non-harmful compounds, water and oxygen.

GPx contains selenium and catalyses the reduction of hydroperoxide coupled by the conversion of reduced glutathione (GSH) to oxidised glutathione (GSSG). SOD, with its forms (Mn-SOD found in the mitochondria and Cu/Zn SOD in the cytosol), is an enzyme that catalyzes the dismutation of superoxide radical (O_2^-) into less harmful H_2O_2 and oxygen. Figure 2.3 shows a diagram that represents the action of the above-mentioned antioxidant enzymes. In the diagram, either Cu/Zn SOD or Mn-SOD depending on where the reaction is taking place dismutates O_2^- producing H_2O_2. H_2O_2 becomes harmful when it undergoes the Fenton reaction forming OH. Both catalase and glutathione peroxidase can further break down H_2O_2 into water and oxygen (Kurutas, 2016). Glutathione peroxidase also catalyzes the reaction, which leads to the conversion of GSH to GSSG as previously mentioned. GSH-reductase reduces GSSG to GSH with the conversion of NADPH to NADP (Kurutas, 2016). Other non-enzymatic antioxidants are shown in Figure 2.4 (vitamin C and vitamin E). Vitamin C also converts harmful dihydrolipoic acid to a less harmful lipoic acid (Kurutas, 2016). In a diabetic model, free radicals are higher than the antioxidant capacity (Nna *et al.*, 2019).

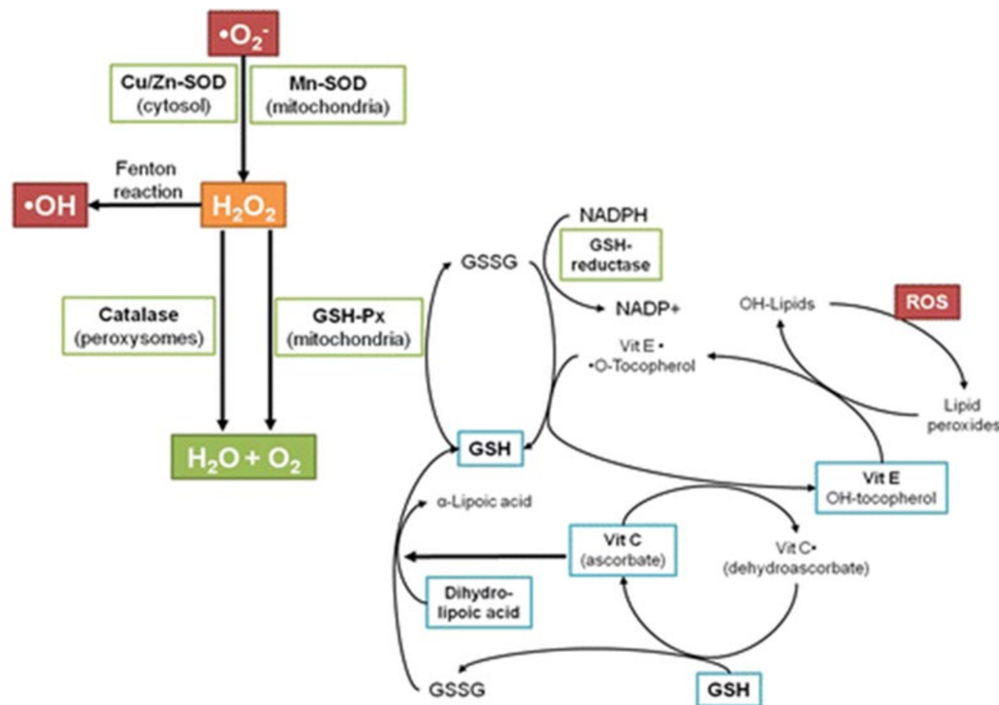

Figure 2.4: Antioxidant defence mechanism against oxidative stress by targeting free radical molecules (Kurutas, 2016).

2.3. Link between diabetes and oxidative stress

The continuous increase in blood glucose level over time increases in the production of ROS (Alsenosy *et al*., 2019; Sefidgar *et al*., 2019). Section 2.1.2 explains the use of glucose by the body as a source of energy and the pathways through which it happens in the cells. Section 2.1.2 also explains the normal production of free radicals through the electron transport chain, the machinery through which energy is mainly produced. In both types of diabetes (T1DM and T2DM), glucose cannot be utilized by the body cells, due to insulin shortage or insulin resistance. However, hepatocytes, brain cells, cells of the gut and pancreatic cells do not have insulin receptors and therefore can take in glucose with the absence of insulin action (Yan, 2014).

Due to hyperglycemia, excess NADH is produced in these cells (Yan, 2014). This excess NADH is oxidized by complex I of the electron transport chain, to form NAD in compensation to reduce the level of NADH (Zhao *et al*., 2019). Some of the electrons leak while complex I pump protons during the oxidation of NADH (Nolfi-Donegan *et al*., 2020).These electrons are a lot more than normal as NADH production is increased and NAD level is low. The electrons leak and partially reduce oxygen molecules leading to the formation of superoxide radicals in excess. Some of these superoxide radicals are converted to hydrogen peroxide, which can be further converted to hydroxyl ions (Yan, 2014). Due to the continuous production of ROS, the antioxidant system is compromised and oxidative balance is lost (Ostovan *et al*., 2017).

Production of ROS by the electron transport chain leads to further production of more free radicals, leading to oxidative stress (Zhao *et al*., 2019). Increased ROS by the mitochondria impairs the enzyme, GAPDH (Figure 2.2), and leads to pathways that branch off from the accumulated G3P and other intermediates above it (Hillion *et al*., 2017). These pathways are insignificant during normal glycaemic conditions. Advanced glycation end products (AGEs) production is one of the pathways and causes major production of ROS (together with the polyol pathway) compared to the other pathways (Yan, 2014; Ostovan *et al*., 2017; Alabi *et al*., 2020).

AGEs are produced when reducing glucose reacts with amino groups of biomolecules like proteins and nucleic acids in a process called Maillard reaction (Singh *et al*., 2001; Fishman *et al*., 2018; Iya *et al*., 2019). Formation of AGEs leads to activation of transcription factors responsible for the generation of free radicals (Ostovan *et al*., 2017; Khorramabadi *et al*., 2018; Liguori *et al*., 2018; Roxo *et al*., 2019). In the branch-off

pathway explained above, AGEs are produced when G3P forms methylglyoxal (a reducing sugar) which reacts with proteins (Fishman *et al*., 2018).

Production of excess AGEs increases oxidative stress, contributing to diabetic complications (Tian *et al*., 2020). This suggests that there is a relationship between AGEs, oxidative stress, and the development of diabetic complications. However, fewer studies have focused on the level of AGEs in the diabetic models as the method used to detect AGEs is not universally accepted (Singh *et al*., 2001). It is therefore unclear whether AGEs are the main cause of diabetic complications, or these complications are mainly as a result of the oxidative stress caused downstream. Besides causing oxidative stress, AGEs can react and change the function and structure of other biomolecules (Fishman *et al*., 2018).They can also cause diabetic complications directly by causing upregulation of RAGEs, binding to them and causing the production of inflammatory cytokines, consequently leading to chronic inflammation and more ROS (Liguori *et al*., 2018; Yan, 2014). The formation of AGEs relies on the availability of reducing sugars (Fishman *et al*., 2018). Therefore, hyperglycaemic conditions possibly leads to excess formation of AGEs and ROS. Hyperglycemia can also cause inflammation by activating macrophages, further causing ROS production (Sefidgar *et al*., 2019).

2.4. Male reproductive organs and oxidative balance

Free radicals are physiologically produced in the male gonads for important functions in reproduction. These functions are explained in this section, together with the existence of antioxidants in the male gonads. This section explains the link between oxidative imbalance and the development of male reproductive dysfunction and male infertility.

2.4.1. Testes and epididymis structure and environment

The male human reproductive system consists of two testes, each with compartments made of coiled seminiferous tubules where sperm cells are produced. The seminiferous tubules are lined by a layer of germinal epithelium made of germ cells which differentiate to form spermatids that mature to spermatozoa (Oliver & Stukenborg, 2019). The interstitial space between seminiferous tubules contains somatic cells, including the Leydig cells, where testosterone is produced and secreted (Oliver & Stukenborg, 2019). The epididymis is a long tube-shaped organ that joins the testes through the rete testes connected to the seminiferous tubules (James *et al*., 2020). Spermatozoa produced in the seminiferous tubules are transported to the epididymis where they are stored while they

mature before being ejaculated (James *et al*., 2020). Figure 2.5 shows the structure of the human testes with compartments of seminiferous tubules, where sperms are produced, and the different parts of the epididymis.

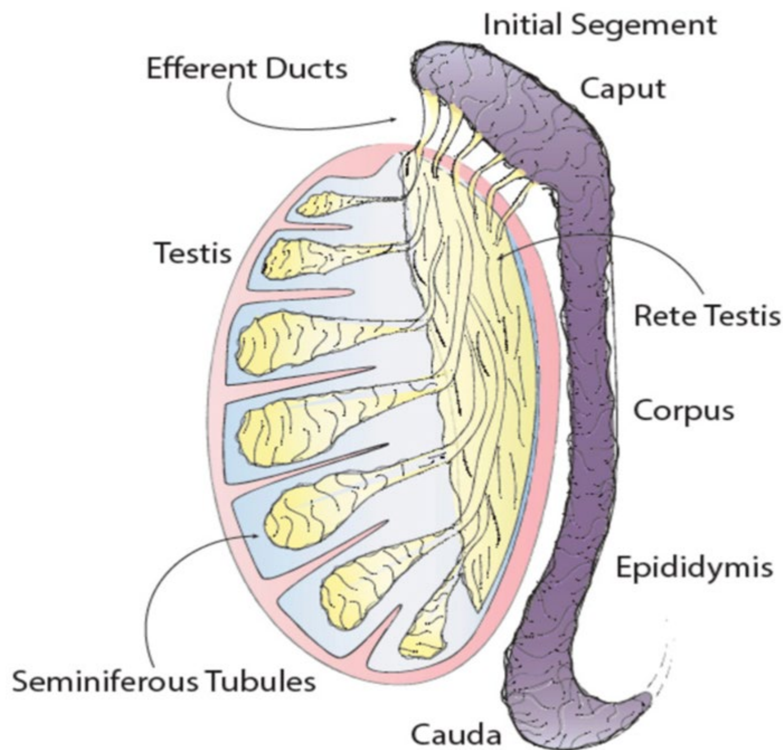

Figure 2.5: Anatomy of the human testes and epididymis. The spermatozoa are produced in the seminiferous tubules coiled in compartments in the testes. The initial segment of the epididymis receives spermatozoa from the rete testes, for storage in the epididymis. When sperms are released from the epididymis, they move in the direction from the initial segment to the cauda and out (James *et al*., 2020)**.**

2.4.2. Normal oxidative status of testes and epididymis

A low level of ROS is essential in the testes and epididymis of the male reproductive system for the normal function of the sperms (Tian *et al*., 2020). Sperms are contained in semen, a liquid that consists of macromolecules and enzymes that provide a proper environment for them to survive (Oliver & Stukenborg, 2019). Some free radicals such as superoxide anions, hydrogen peroxide, and hydroxyl radicals are produced in the human semen during oxygen metabolism and are important for sperm maturation and function (Khorramabadi *et al*., 2018). In the testes, a normal amount of free radicals is produced by germ cells and somatic cells (Tian *et al*., 2020). The testes and epididymis tissue contain antioxidants such as vitamin C, vitamin E, superoxide dismutase, glutathione and thioredoxin that lower the effect caused by free radicals in the semen to protect the sperm

from being damaged (Alsenosy *et al*., 2019). Any increase in ROS is neutralised by antioxidants produced by the cells in the reproductive tissue (Khorramabadi *et al*., 2018; Iya *et al*., 2019). The epididymis consists majorly of the epithelial cells which have a high rate of metabolism, forming more ROS (James *et al*., 2020). However, the epididymis epithelial cells also produce antioxidant enzymes to protect sperms from the free radicals (James *et al*., 2020).

2.4.3. Male infertility

Recent studies have shown the rise in prevalence of male infertility from approximately 7% to almost 10% with more than 50% decrease in sperm parameters over the past 50 years (Oliver & Stukenborg, 2019; Ravitsky & Kimmins, 2019). Infertility is defined as the inability of a couple to achieve pregnancy after a year of unprotected sexual intercourse (Silva *et al*., 2020). A male individual that fails to impregnate a female while having no history of impregnating before, suffers from primary infertility (Öztekin *et al*., 2019). Secondary infertility is when an individual suffers from infertility but has a history of conceiving before (Öztekin *et al*., 2019). A percentage of 10-25% of couples are reported with a concern of infertility (Temidayo & Stefan, 2017), and more than half of the cases are caused by males (Öztekin *et al*., 2019).

The rise in male infertility has caused emotional and financial stress to 1 in 7 people over the years (Ravitsky & Kimmins, 2019; Silva *et al*., 2020). Some factors associated with male infertility are endocrine failure, oxidative stress, and genetic factors (Khorramabadi *et al*., 2018; Öztekin *et al*., 2019). Obesity and exposure to environmental chemicals that can disrupt the endocrine system can also lead to male infertility (Ravitsky & Kimmins, 2019). DM has also been linked to male infertility (Khaki *et al*., 2014; Tian *et al*., 2020). Up to 35.1% of type 2 diabetic men are experiencing male infertility (Iya *et al*., 2019). Male infertility is either caused by defects that lead to less or no production of sperm cells, or destruction in the mobilisation of the sperms from the testes or epididymis (Oliver & Stukenborg, 2019).

2.5. Testicular and epididymis oxidative stress and its link to male infertility

A reduction in the male fertility rate has been observed in diabetic men. Most cases of male infertility in diabetic men have been reported to be due to damage in testicular tissue caused by hyperglycemia (Tian *et al*., 2020). Hyperglycemia causes excessive production of ROS in the testes by somatic and germ cells which interferes with sperm production (Tian *et al*., 2020). Oxidative stress in the testes and epididymis is one of the factors

associated with this decrease in fertility rate (Sefidgar *et al*., 2019; Silva *et al*., 2020). Khorramabadi *et al*. (2018) reported high levels of testicular and epididymis ROS in 25–40% of infertile men. NrF2/ARE system was found to be down regulated in diabetic rat testes suggesting a decrease in antioxidant production and action. In another study conducted by Nna *et al*. (2019), testicular and epididymis SOD, CAT, GSH and GR were reduced in diabetic control male rats compared to normal rats. Similarly, an increase in testicular and epididymis MDA was observed in diabetic rats compared to normal rats (Ostovan *et al*., 2017).

Oxidative stress leads to damage in macromolecules in the gonads of the male reproductive system (Khorramabadi *et al*., 2018). Oxidative stress leads to disruption in the process of spermatogenesis which later leads to male infertility (Shoorei *et al*., 2019). Exposure of sperms to high level of free radicals in the testes and epididymis causes damage to the DNA of the sperm cells and death of the sperms (Korejo *et al*., 2016; Johnson *et al*., 2019). Damage in testicular tissue in diabetic rats suggests the relationship between hyperglycemia and male infertility (Khaki *et al*., 2014). This damage was linked to oxidative stress as described by some studies documented where testicular structure and function were altered due to lipid peroxidation of the testicular cells caused by oxidative stress (Alsenosy *et al*., 2019; Corrêa *et al*., 2019) .

A study carried out by Johnson and colleagues (2019) documented the effect of oxidative stress on the structure of the testes by comparing the histological structure of diabetic rat testes to the control rat testes. This revealed bigger lumens with smaller germinal epithelium and loosened seminiferous tubules in rats with oxidative stress caused by diabetes (Corrêa *et al*., 2019; Johnson *et al*., 2019). This was supported by another study by Nna *et al*. (2019) which showed a decrease in the diameter of seminiferous tubules and increased spaces between seminiferous tubules. DM-induced oxidative stress further causes a reduction in germ cells and Leydig cells population because of apoptosis (Khorramabadi *et al*., 2018; Corrêa *et al*., 2019; Nna *et al*., 2019). Sperm count was decreased in the epididymis of diabetic rats (Nna *et al*., 2019). This is caused by exposure of sperms to the epididymis oxidative stress, which causes a peroxidation of polyunsaturated fatty acids in the membrane of the sperms that consequently leads to sperm death (Khaki *et al*., 2014). Oxidative stress was also found to cause seminiferous tubule thickness, a decrease in volume of testis and semen, and reduced testosterone (Khaki *et al*., 2014; Ostovan *et al.,* 2017; Khorramabadi *et al*., 2018; Iya *et al*., 2019).

2.6. Treatment of diabetes mellitus

DM is a concern worldwide and its cure is currently unknown (Quan *et al*., 2020). However, different methods of treatment have been found, targeting the complications that accompany DM. Synthetic (chemical) medication has been produced and studied to reduce blood glucose and to treat the related complications. Similarly, traditional medicine has paved a way into discovery of treatment for DM. Both the modern and traditional medicine treatment of DM have been explained in this section.

2.6.1. Modern medicine

Synthetic drugs have been produced as agents of treating DM. However, most synthetic drugs have been linked with hypoglycaemia and toxicity to organs (Khaki *et al*., 2014). Glibenclamide is a type of sulfonylurea drug that alleviates diabetic complications by causing increased production of insulin by the beta cells of the pancreas (Liu *et al*., 2018; Rambiritch *et al*., 2014). Metformin is also a chemically synthesized drug that is given to T2DM patients (Roxo *et al*., 2019). It increases the binding of insulin hormone to its receptors in the cell, thereby stimulating the downstream effects (Roxo *et al*., 2019). Metformin has also been reported to ameliorate oxidative stress in the testes, thereby improving fertility (Silva *et al*., 2020). Both Glibenclamide and Metformin manage hyperglycemia in diabetic patients by decreasing glucose levels in the blood, which decreases the underlying effects of glycation and ROS production (Roxo *et al*., 2019). It has been reported that Glibenclamide causes ATP-dependant potassium channels in the mitochondria to close leading to cell apoptosis (Liu *et al*., 2018). Side effects of diabetic drugs have led to further studies in search of more treatment solutions to DM with less to almost no side effects.

2.6.2. Traditional medicine

Traditional medicine is the use of plants to treat diseases and ailments. Traditional medicine is used by 80% of people in developing countries as treatment for illnesses (WHO, 2003). The weak health systems in developing countries led to the preference of traditional medicine over modern medicine (Tchicaillat-Landou *et al*., 2018). Many studies have shown that medical plants contain bioactive compounds which have beneficial effects on the human body (Sefidgar *et al*., 2019). The benefits of medicinal plants is dependent on the phytochemicals they produce (Temitope, 2015). They can be found in all parts of plants (roots, stems, leaves and roots) (Oghale & Idu, 2016). Less documentation of medicinal plants confirms the need for research on the potential benefits of some plants in treating diseases of concern. Research has shown the benefits of some

of the popular medicinal plants such as buchu, devils' claw, African potato, rooibos tea, Cape aloe amongst others, in the treatment of DM (Tchicaillat-Landou *et al*., 2018).

Traditional medicine efficiency is further confirmed by the benefits of vegetables and fruits which suggested beneficial constituents in plants (Rahal *et al*., 2014) . Phenols and polyphenols, the main classes of antioxidants, were found as constituents of fruits and vegetables (Aliyu *et al*., 2013). Phytochemicals can be classified into primary and secondary phytochemicals, with the secondary phytochemicals being the most important chemicals in the treatment of ailments (Temitope, 2015). Some secondary phytochemicals also found to be beneficial in plants are flavonoids, tannins and alkaloids (Oghale & Idu, 2016). Developing countries practice traditional medicine because it is much cheaper to make and to afford compared to modern synthetic drugs (Ataman & Idu, 2015). The idea of using the most natural way to treat diseases and ailments was derived from the concern that synthetic drugs have negative side effects, and some patients develop drug resistance. Treatment of diabetes by modern synthetic drugs mostly focuses on the management of hyperglycemia than the underlying complications, while the use of traditional medicine is a new perspective of managing both hyperglycemia and oxidative stress, by increasing antioxidants in the system (Roxo *et al*., 2019). Several studies have reported the benefits of different plants in the improvement of male fertility caused by DM (Oguntibeju *et al*., 2020; Oyenihi *et al*., 2020). A. *difformis* is also a medicinal plant with beneficial effects in the treatment of DM.

2.7. *Anchomanes difformis*

A. *difformis* is also a medicinal plant recorded for its benefits in traditional medicine. Chapter 1 provides an overview of the plant, its structure and its place of origin. This section explains the importance of A. *difformis* in the treatment of several ailments, its benefits in DM treatment and toxicity is also considered. The constituents of this plant are also mentioned in relation to the effect of the plant.

2.7.1 Composition and benefits

A. *difformis* contains nutritional components together with some compounds such as alkaloids and phenolic compounds, which are components of antioxidants (Alabi *et al*., 2021). A. *difformis* was found to contain macromolecules such as carbohydrates, minerals, proteins, minerals, and amino acids (Ataman & Idu, 2015). Studies have investigated the potential effects of A. *difformis* in the treatment of DM complications, yielding positive results. Some of these studies reported the benefit of the A. *difformis* in

the improvement of renal function in a diabetic model (Ataman & Idu, 2015; Alabi *et al*., 2021). A. *difformis* roots were used in Benin republic to treat anal and oral wounds and for the treatment of DM and its complications (Aliyu *et al*., 2013). The roots of the plant were also reported for treating dysentery in Nigeria (Aliyu *et al*., 2013). Other diseases and ailments such as asthma (Oghale & Idu, 2016; Alabi *et al*., 2020), malaria (Olanlokun *et al*., 2017), cough and throat related issues (Ataman & Idu, 2015), which were treated by extracts from the plant were recorded. These studies have used either the ethanol or water extract of the plant to investigate its beneficial constituents. The beneficial effect of A. *difformis* has however not been clearly understood.

2.7.2. Toxicity

The knowledge that free radicals are useful for signaling has raised concern on whether the introduction of antioxidants can interfere with signaling (Kurutas, 2016). The introduction of antioxidants to the body should be conducted with the appropriate dose and duration (Kurutas, 2016). The toxicity of A. *difformis* was investigated in several studies to find out if, regardless of its benefits, it is safe to be ingested (Aliyu *et al*., 2013). The Araceae family species are generally toxic, hence, the importance of studies that investigated the toxicity of A. *difformis* (Aliyu *et al*., 2013).

In the study by Ataman & Idu (2015), rats that were fed with a higher dose of the crude extract (150 g extract and 50 g feed mash) of the plant lost more weight and developed hemorrhage and interstitial fibrosis compared to rats that were fed with a lower dose of the same extract (150 g feed mash and 50 g extract), which had an increase in weight and did not develop the above conditions (Ataman & Idu, 2015). However, the kidneys of the rats were affected negatively with all doses used in the study (Ataman & Idu, 2015). It was concluded in this study that the toxicity of the plant depends on the dosage used and that treatment requires caution (Ataman & Idu, 2015). There are fewer studies on the antidiabetic potential of *A. difformis* in the literature (Adeyemi *et al*., 2015; Aderonke & Ezinwanne, 2015; Ovuakporie-Uvo & Idu, 2015). Furthermore, treatment of DM and reproductive dysfunction by the plant extract, A. *difformis* needs to be explored, and hence, this justify the need purpose of this study.

CHAPTER 3
MATERIALS AND METHODS

3.1. Chemicals

Streptozotocin (STZ) was supplied by Biocom Africa, Cape Town, South Africa. The following chemicals were purchased from Sigma-Aldrich, USA: $Na_2HPO_4.7H_2O$ and $NaH_2PO_4.H_2O$, sodium acetate and acetic acid, iron chloride ($FeCl_3$), 2,4,6- tripyridyl-S-triazine (TPTZ), 2,2-diphenyl-1-picrylhidrazyl (DPPH), trolox, ascorbic acid, sodium chloride, diethylenetriaminepentaacetic acid (DETAPAC), while the following chemicals were purchased from Merck, MA, USA: hydrogen chloride (HCl), hydrogen Peroxide (H_2O_2), butylated hydroxytoluene (BHT), phosphoric acid, thiobarbituric acid, butanol and ethanol. Pechloric acid, 5, 5'-disulfanediylbis (2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2-nitro-5-thiobenzoic acid (TNB), glutathione reductase (GR) and thiobarbituric acid (TBA). The bicinchoninic acid Protein Assay kit was purchased from Thermo Fisher Scientific, South Africa.

3.2. Preparation of *Anchomanes difformis* extract

Anchomanes *difformis* leaves were harvested from Abeokuta, Ogun state in Nigeria. The plant parts were harvested and authenticated (LUH6623) and a specimen was kept at the herbarium in the University of Lagos in Nigeria. The difference in the output of phytochemicals between ethanol and aqueous extracts was assessed, and more Phytochemicals were yielded by the aqueous extract compared to the ethanol extract, hence, the use of the aqueous extract in this study. Water extracts of A. *difformis* were prepared from the leaves of the plant via cold extraction method (2-8°C) for the study. The leaves were first dried in the shade for fourteen days at 28±2° and then blended with a blender. The powder obtained was soaked into distilled water at a ratio of 1:10. The solution was filtered using vacuum filtration method and the extract was dried by evaporation using a lyophilizer. The final extracts were stored at -20°C until the commencement of the study where it was given to rats orally.

3.3. Ethical considerations

Ethical clearance (CPUT/HW-REC 2016/A4) for this study was obtained from the University's Research Ethics Committee. In addition, animal ethical clearance was obtained (REF. 04/17) from the Ethics Committee for Research on Animals from South

African Medical Research Council were animal study was carried out, and the guidelines for animals study were duly followed. This study made use of the reproductive organs namely; the testes and the epididymis, and this was approved by the Faculty of Health and Wellness Sciences Research Ethics committee (REC) (Ethics Number: CPUT/AEC 2019/04) before commencement of the study.

3.3. Animals

Male Wistar rats (8 weeks old) weighing 180±10 g were procured at the animal facility in Stellenbosch University (Tygerberg campus) and housed at the Primate Unit & Delft Animal Centre (PUDAC), South African Medical Research Council (SAMRC), South Africa. The rats were maintained under standard laboratory conditions, an ambient temperature of 22°C to 28°C and humidity of 45% to 55% and housed 5 rats per cage to allow for free movement of the rats in the cage. The cages were made of a plastic base, stainless steel roof and a feeder, which also held the water bottle. The beds were made of ground sterilized maize cob and were changed every day. The rats were fed *ad libitum* with standard rat chow (SRC) and water at a 12-hour dark/12- hour light routine. Before the commencement of the experiment, the rats were acclimatized for 3-4 weeks. The study included feeding (orally through a flexible feeding tube) and administration of the treatment with strict adherence to compliance and animal care guidelines.

The animals were handled with humane care and abnormal behaviors were observed during treatment. This was carried out according to the operating procedure of SAMRC PUDAC (SOP No: 2016-R01) and conforms to the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

3.4. Treatment and Induction of diabetes

Insulin resistance was induced by 10% fructose (Wilson and Islam, 2012). Rats were placed on 10% fructose *ad libitum* for 2 weeks. After 18 h of overnight fasting (Sharma *et al.,* 1997), the animals were injected once intraperitoneally with freshly prepared 40 mg/kg body weight STZ (Jaiswal *et al*., 2013). The STZ was dissolved in 0.1 M cold citrate buffer, pH 4.5 (Jaiswal *et al*., 2013). Blood was collected from the tails of the rats to measure blood glucose levels using a glucometer (Accu-check, Roche, Germany). Rats with blood glucose value ≥ 324 mg/dl three days STZ injection were considered diabetic. Treatment with A. *difformis* and the standard drug commenced after confirmation of diabetes, and that was considered day 1 of treatment. Rats were weighed every week and percentage

change was calculated between the initial body weight and the final body weight measured after 10 weeks.

3.5. Study design

Sixty-four (64) male Wistar rats were procured for this study. The rats were randomly divided into 7 groups with 8 rats in each group of non-diabetic (normal) rats, and 10 rats per group of diabetic rats. Water was used as a vehicle to dissolve *A. difformis* and fructose, while citrate dissolved STZ. Induction was carried out after 14 days of 10% fructose administration (for the diabetic rats) and treatment followed 5 days after the induction, lasting for 42 days. Rats were grouped according to the following design as illustrated in Figure 6: Group 1, the normal control group (NC), received SRC and the vehicle, water. Group 2, the normal treated group (C200), was treated with an aqueous extract of *A. difformis* at a 200 mg/kg dose. Group 3, the normal treated group (C400) was treated with a 400 mg/kg dose of aqueous extract of AD. Group 4, the diabetic control (DC), was treated with water, the vehicle for dissolving extract and standard drug. Group 5, diabetic treated group (D200), was treated with a 200 mg/kg dose of aqueous extract of *A. difformis*. Group 6, diabetic treated group (D400), was treated with a 400 mg/kg dose of AD. Group 7, diabetic glibenclamide group (GLB), was treated with the standard anti-diabetic drug, glibenclamide (5 mg/kg). Figure 6 represents the study design by which the study was conducted.

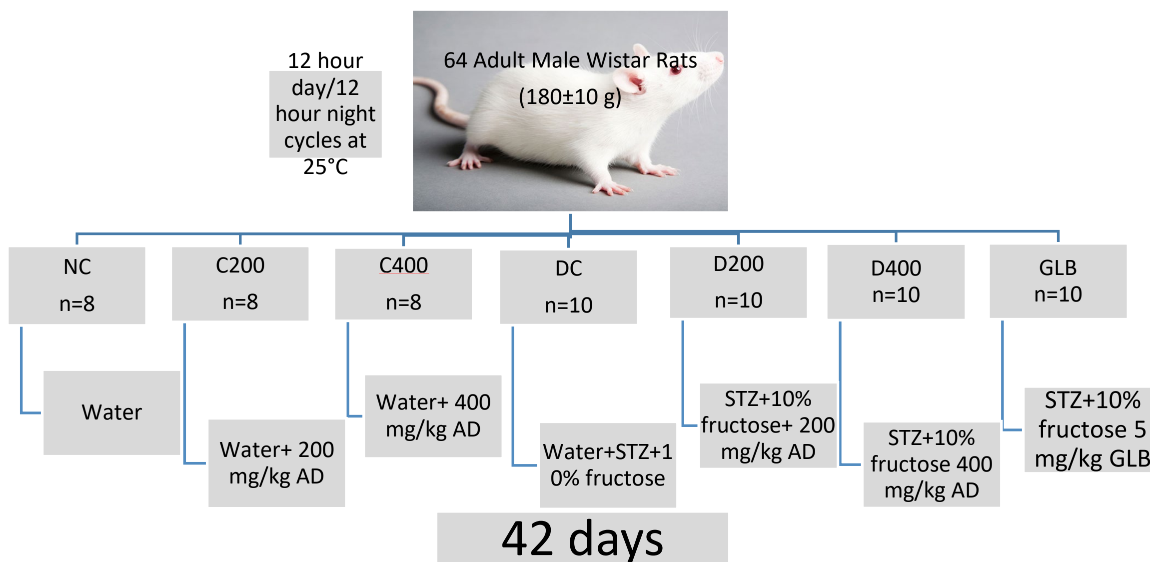

Figure 3.1: Induction and treatment of the different groups over 42 days.

3.6. Tissue preparation

After 10 weeks, all animals were euthanized with the inhalation of 2% isoflurane per oxygen (1 L/min flow rate) followed by cardiac puncture (Heerden *et al*., 2000). On dissection, the testes and epididymis were carefully removed from each animal and immediately weighed. The rest of the testes and epididymis was stored in the freezer for further biochemical analysis. Testes and epididymis tissue from individual rats were weighed (100 mg) in a 1 ml Eppendorf tube and 1 ml of Phosphate-buffered saline (PBS) was added onto the tissue. The tissue and buffer were transferred to a glass cylinder and homogenized by a homogenizer for 15 sec. Homogenates were transferred to the Eppendorf tubes and stored in a -80°C freezer for further assays.

3.7. Oxidative stress detection

Enzymatic and non-enzymatic antioxidant indices; lipid peroxidation (TBARS), ferric reducing antioxidant power assay (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), superoxide dismutase (SOD), catalase (CAT) and total glutathione (GSH) were measured in the testes and epididymis homogenates and the methods will be outlined below.

3.7.1. Thiobarbituric acid reactive species

Thiobarbituric acid reactive species (TBARS), a measure of lipid peroxidation, was determined according to the methods outlined by Buege & Aust (1978) and Esterbauer & Cheesemans (1990). Lipid peroxidation was measured in testes and epididymis by adding 12 µl of TBA (prepared by adding 0.11 TBA in 0.1 NaOH), 12 µl of BHT (prepared by adding 4 mM BHT in 10 ml absolute ethanol) followed by 100 µl of phosphoric acid (684 µl phosphoric acid in 50 ml distilled water) into 100 µl of each homogenate sample. This was placed in a water-bath at 90°C for 45 min. An aliquot of 1000 µl butanol and 100 µl of saturated salt were added to the same Eppendorf tubes after 15 min of cooling. Butanol was used as a blank. A pipette was used to load 300 µl of butanol and the testes and epididymis samples was loaded in the wells of a 96-well plate in triplicates, with the first three well containing only butanol and the rest containing the testes and epididymis samples. The plate was read in a Multiskan Spectrum plate (Thermo Fisher Scientific, Waltham, MA, USA) reader at 532 nm.

3.7.2. Ferric reducing antioxidant power assay

FRAP assay was performed to calculate the amount of reduced Fe^{2+} formed by the donation of electrons by antioxidants in the testes and epididymis tissue, from oxidized Fe^{3+} according to the method outlined by Esterbauer & Cheesemans (1990). The

antioxidant, L-Ascorbic acid was used as the standard reference. A volume of 50 ml of the FRAP reagent was prepared by adding 30 ml of the acetate buffer (pH 3.6), 3 ml of $FeCl_3$ reagent, 3 ml of 2, 4, 6-tripyridyl-s-triazine (TPTZ) reagent, and 6 ml of distilled water. $FeCl_3$ reagent was prepared by mixing 0.053 g of $FeCl_3$ with 10 ml of distilled water in a 15 ml plastic tube, and TPTZ was prepared by mixing 0.0093 g of TPTZ in 15 ml of 0.1 M HCl in a 15 ml plastic tube. A stock solution of ascorbic acid (100 mg/L) was diluted into a series of dilutions. Ten microliters (10 µl) of each sample (testes and epididymis) and the different concentrations of the vitamin C standard were loaded in triplicates in a 96-well plate, and 300 µl of FRAP reagent was added in each well. The plate was incubated for 30 min to allow the reaction to occur. The plate was read in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 593 nm.

3.7.3. 2, 2-diphenyl-1-picrylhydrazyl assay

The antioxidant activity of the sample was compared to the activity of Trolox as a standard in this study, following the method of Esterbauer & Cheesemans (1990). A dilution series was prepared from a Trolox stock solution of 1000 mg/L. A pipette was used to load 25 µl of both the testes and epididymis samples and standard in triplicates in the well of a 96-well plate and 275 µl of DPPH (0.4 mg/mL) was added in the wells. The plate was incubated for 30 minutes to allow the reaction to occur. The plate was read at 593 nm after 30 minutes in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.7.4. Protein determination

The testes and epididymis protein content was measured, and antioxidant enzyme activities (CAT and SOD) and GSH content was calculated relative to the protein concentration. A dilution series was prepared from the original concentration of 2000 mg/L of bovine serum albumin (BSA). This was used to set up a standard curve for the determination of protein concentration in the samples. Using a pipette, 25 µl of the standard and testes and epididymis sample homogenates were loaded in a 96-well plate in triplicates. Reagent A/B was prepared by mixing bicinchoninic acid solution (Reagent A) and copper sulphate pentahydrate 4% solution (Reagent B) at a 50:1 ratio. A micropipette was used to load 200 µl of Reagent A/B into each well loaded with the samples and the standards. The plate was incubated for 30 min and read in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 562 nm.

3.7.5. Superoxide dismutase activity determination

SOD activity was estimated by calculating the percentage inhibition of auto-oxidation of 6-hydroxydopamine (6-OHD) by superoxide free radicals. SOD activity was determined following the method of Brannan and colleagues (1981). DETAPAC was used to inhibit any cycle formation of other free radicals. A 6-OHD solution was freshly prepared by adding 4 mg of 6-OHD in 10 ml of distilled water and 50 µl of perchloric acid in a 15 ml plastic tube. DETAPAC was prepared by adding 2 mg of DETAPAC in 50 ml of the PBS buffer. A mixture of oxidised 6-OHD and DETAPAC forms a pink/orange colour. A volume of 10 µl of each sample (testes and epididymis) was loaded in wells in triplicates and 15 µl of 6-OHD was added in each well. A volume of 170 µl of DETAPAC was then added to each well and the plate was read at 490 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.7.6. Catalase activity determination

The dissociation of H_2O_2 due to the activity of catalase enzyme was determined by performing a catalase assay according to the method by Brannan *et al.*, (1981). H_2O_2 reagent was prepared by adding 34 µl of H_2O_2 to 10 ml of the PBS buffer. PBS buffer (170 µl) was added to each well of the 96-well plate, and 10 µl of each sample (testes and epididymis) was added to each well containing the PBS buffer. H_2O_2 reagent (75 µl) was added to each well and the plate was read immediately, in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 532 nm.

3.7.7. Glutathione assay

GSH was used as a standard in this assay and performed according to the method outlined by Ellerby & Bredesden (2000). PBS buffer, EDTA, pH 7.5, was used for this assay. NADPH solution was prepared by adding 12 ml of buffer in NADPH (0.3mM). DTNB (0.3 mM) was prepared by adding 0.006 g DTNB in 50 ml of the PBS buffer. A GR solution was freshly prepared by adding 80 µl of GR and 5 ml of the PBS buffer in a 15 ml plastic tube. GSH (standard) of 3 mM was prepared by adding 0.046 g GSH in 50 ml of the PBS buffer. 50 µl of the testes and epididymis samples and standard were plated and 50 µl of DTNB was added to the wells. A volume of 50 µl of GR solution was also added to the wells. 50 µl of NADPH solution was added just before the reading of the plate. The plate was read in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 412 nm.

3.8. Statistical analysis

The results were analyzed using Graph Pad Prism version 5 software. All values were expressed as mean ± SD. Normality and equality of variance in the data were tested using Levene's test. The differences in the means between the groups were estimated by using one-way analysis of variance (ANOVA). Group differences were tested by using the Kruskal-Wallis test. A probability of $P<0.05$ was considered significant.

CHAPTER 4
RESULTS

The following results were obtained and analysed. Each section of the results includes both the findings and the supporting figures below each section. Body weight, relative tissue weight and blood glucose change (common endpoints to confirm T2DM) will be outlined in the first two sections below. Section 4.3 will represent the level of oxidative stress (amount of MDA) in all the groups. Antioxidant capacity of all groups, recorded after FRAP and DPPH assays were performed, will be presented in sections 4.4 and 4.5, followed by antioxidant enzyme activities of SOD, CAT and GSH in all groups (section 4.6-4.8). The results are expressed as mean ± standard error of mean (SEM). A probability of $p < 0.05$ is considered significant.

4.1. Body weights and relative testes and epididymis weights

A decline in body weight was observed at the end of 10 weeks in diabetic rats (Figure 4.1A). A significant 24% weight loss was evident in the diabetic control rats ($p<0.05$) compared to the normal control group. The diabetic rats treated with a 200 mg/kg dosage of *A. difformis* had a significantly ($p<0.05$) decreased weight loss (8.6%) compared to the diabetic untreated group. Rats treated with a 400 mg/kg dose of *A. difformis* significantly ($p<0.05$) exhibited a reduced weight loss of 6.4% compared to the diabetic untreated group while rats treated with glibenclamide presented a significant 11.2% weight loss ($p<0.05$) compared to the diabetic untreated group. Relative testes weight significantly ($p<0.05$) increased in the diabetic control group compared to the control group (Figure 4.1B). The relative testes weight of the diabetic groups treated with both doses of *A. difformis* (200 mg/kg and 400 mg/kg) remained significantly higher compared to the diabetic control group. Similarly, there was no significant change in the relative testes weight of diabetic rats treated with glibenclamide compared to the diabetic control rats. Induction of diabetes caused a significant decrease in relative epididymis weight ($p<0.05$) of the diabetic control rats compared to the normal control rats, which was significantly ($p<0.05$) increased with both doses of *A. difformis* extract treatment (Figure 4.1C). Similarly, glibenclamide significantly ($p<0.05$) increased the relative weight of the epididymis of diabetic rats.

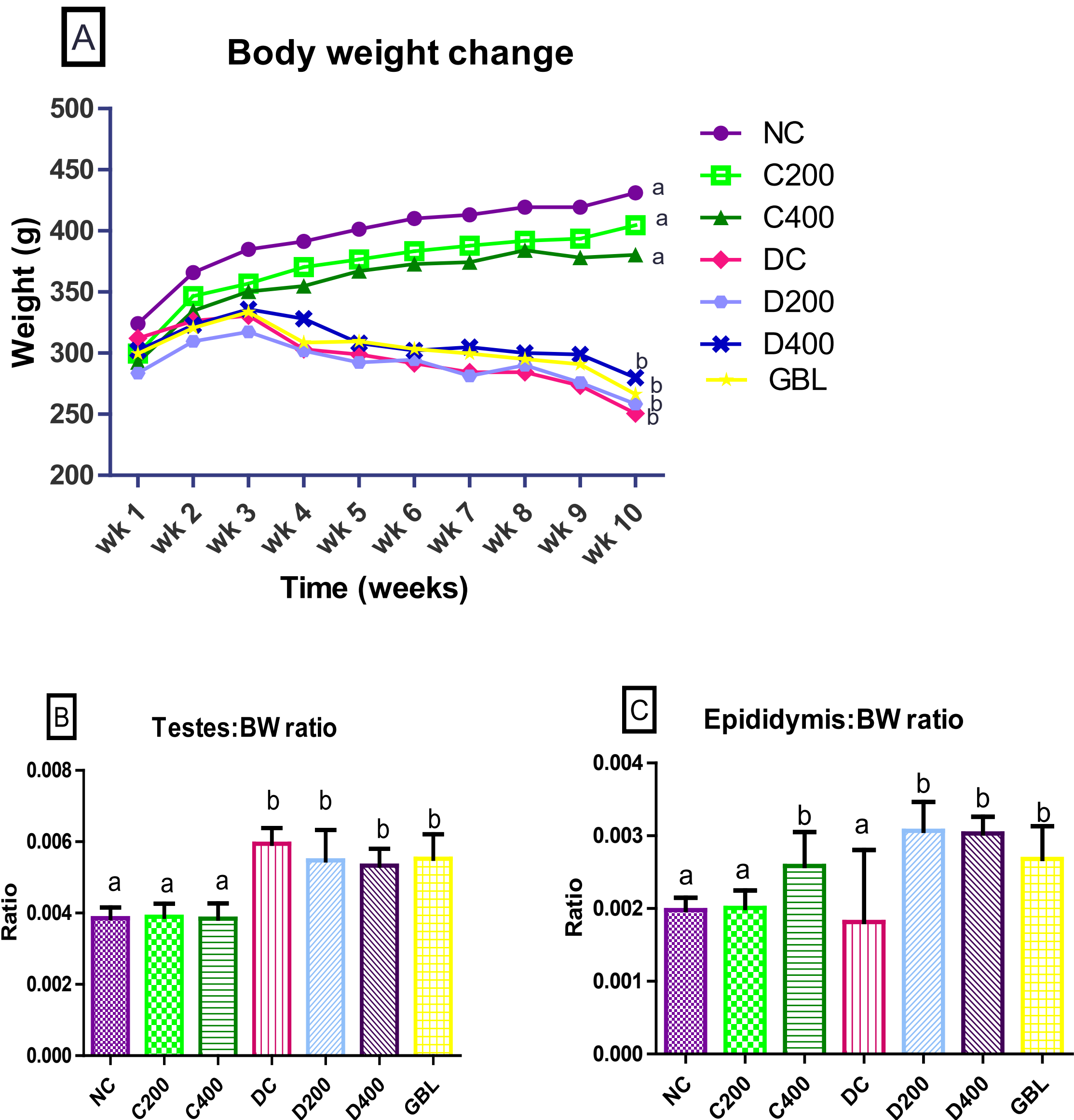

Figure 4.1: Effect of *A. difformis on* A. body weight of the diabetic rats, B. relative testes weight and C. relative epididymis weight. Mean values ±SD of body weight change are indicated by points and that of relative organ weights by bars. The difference in letters on bars indicate significance ($p<0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.2. Blood glucose levels

Blood glucose increased throughout the 10 weeks in the diabetic groups, while those of control groups remained largely unchanged as illustrated in Figure 4.2. The diabetic control group showed a significantly ($p<0.05$) higher blood glucose increase compared to the normal control groups. The diabetic groups treated with A. *difformis* extract showed a significant ($p<0.05$) decrease in blood glucose compared to the untreated diabetic group.

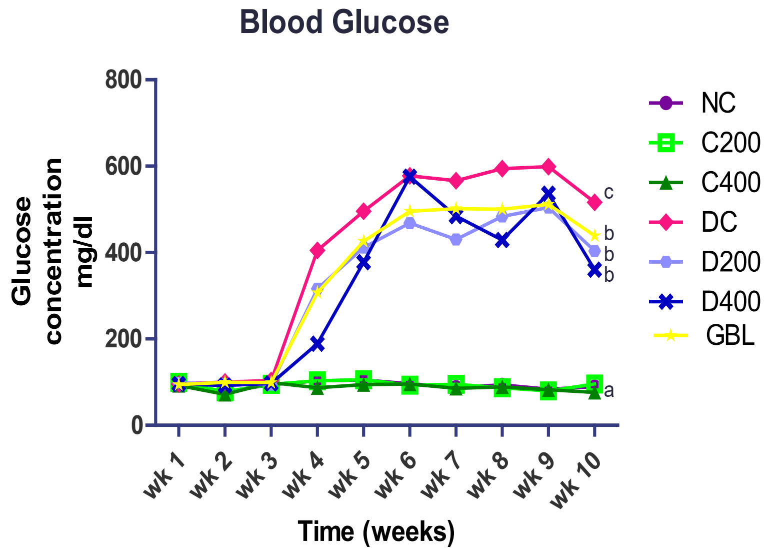

Figure 4.2: Blood glucose levels over 10 weeks of treatment. The effect of *A. difformis* extract on blood glucose of diabetic rats is illustrated. Mean values ±SD of body weight change are indicated by points and that of relative organ weights by bars. The difference in letters on bars indicate significance ($p<0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.3. Thiobarbituric acid reactive substances determination

There was a significant (p<0.05) increase in the amount of MDA produced in the testes of the diabetic rats compared to that of the control groups. As depicted in Figure 4.3, a significant (p<0.05) decrease in MDA production in the testes of the diabetic groups treated with 200 mg/kg and 400 mg/kg A. *difformis* compared to the untreated diabetic group was evident. However, MDA production in the 400 mg/kg A. *difformis* treated group was significantly higher compared to that of the group treated with the lower 200 mg/kg dosage of A. *difformis* (p<0.05). In the testes, no significant difference in the production of MDA was observed between the normal control groups and the group that was treated with 200 mg/kg of A. *difformis*. The rats treated with glibenclamide had a significantly (p<0.05) lower MDA production in the testes compared to that of the untreated diabetic rat testes. Figure 4.3B illustrates that there was no significant (p>0.05) difference in the epididymis production of MDA amongst the normal control groups and between the normal control groups and the untreated diabetic group. There was also no significant difference in MDA levels between the untreated diabetic group and the treated diabetic groups in the epididymis (p>0.05). In the epididymis, the levels of MDA were not significantly (p>0.05) different compared amongst all the diabetic treatment groups.

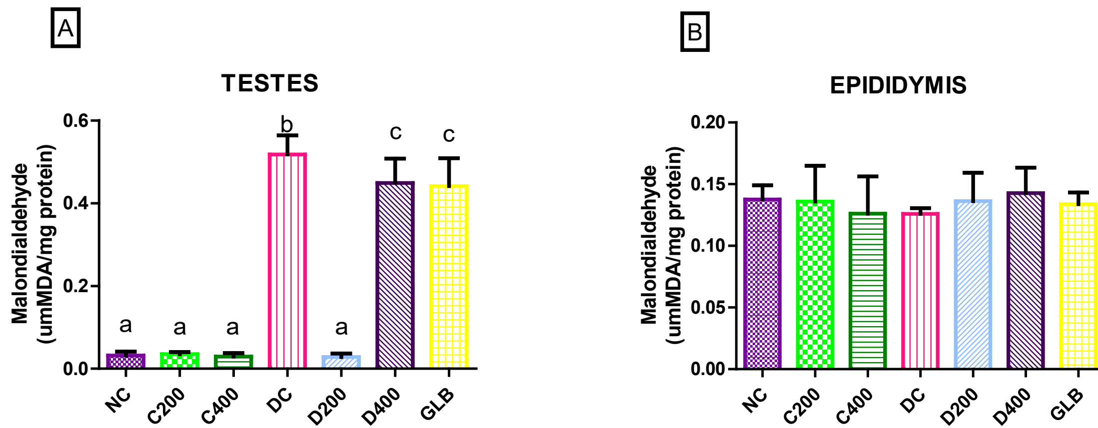

Figure 4.3: The effect of administration of *A. difformis* on oxidative stress. MDA level in the testes compared within groups (A. representing testes and B. epididymis). The difference in letters on bars indicate significance ($p<0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.4. Ferric reducing antioxidant power assay

The antioxidant capacity measured using FRAP in the rat testes and epididymis is illustrated in Figure 4.4A and 4.4B respectively. There was no significant ($p>0.05$) differences in antioxidant capacity between the different normal control groups of both the testes and epididymis. There was a significant ($p<0.05$) decrease in the reduction of Fe^{2+} in the testes of diabetic untreated rats compared to the normal control groups. Similarly, the testes of the diabetic group treated with 200 mg/kg and 400 mg/kg respectively, showed a significantly ($p<0.05$) lower antioxidant capacity compared to that of the normal control groups. There were no significant ($p>0.05$) differences in antioxidant capacity between the untreated diabetic group and both groups treated with doses of A. *difformis* (200 mg/kg and 400 mg/kg respectively) in the testes. However, there was a significant ($p<0.05$) elevation of Fe^{2+} reduction capacity in the testes of the rats treated with glibenclamide compared to the diabetic untreated group. There were no significant ($p>0.05$) differences in the Fe^{2+} reduction capacity amongst the normal control groups, and between the normal control groups and the untreated diabetic group in the rat epididymis. No significant ($p>0.05$) differences in antioxidant capacity were evident between the untreated diabetic group and all the treated diabetic groups of the rat epididymis.

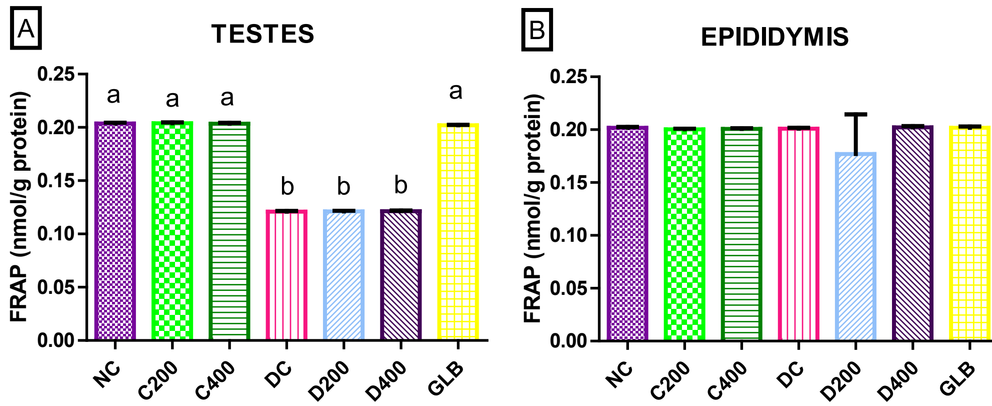

Figure 4.4: The effect of administration of *A. difformis* on FRAP scavenging capacity in the A. testes and B. epididymis compared within groups. The difference in letters on bars indicate significance levels ($p<0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.5. 2, 2-diphenyl-1-picrylhydrazyl assay

Figures 4.5A and B depict the anti-oxidant activity using DPPH in rat testes and epididymis respectively. There was no significant ($p>0.05$) change in the tissue radical scavenging capacity amongst the normal control groups of both the testes and epididymis. No significant change in antioxidant capacity was evident in the untreated diabetic group compared to the normal control groups of the testes, as depicted in Figure 4.5A. Similarly, the antioxidant capacity of the epididymis tissue in the untreated diabetic group showed no significant ($p>0.05$) difference when compared to the normal control groups. *A. difformis* extract administration of both doses (200 mg/kg and 400 mg/kg) had no significant ($p>0.05$) effect on radical scavenging capacity of the untreated diabetic groups of both the testes and epididymis. This was depicted in Figure 4.5A and B, where no significant differences were evident in the diabetic groups treated with A. *difformis* compared to the untreated diabetic groups in the testes and epididymis. In addition, there were no significant ($p>0.05$) differences in the radical scavenging capacity between the diabetic control group and that of the diabetic group treated with glibenclamide, in both the testes and the epididymis. The antioxidant capacity was not significantly ($p>0.05$) different compared amongst all the diabetic treatment groups in both the testes and the epididymis.

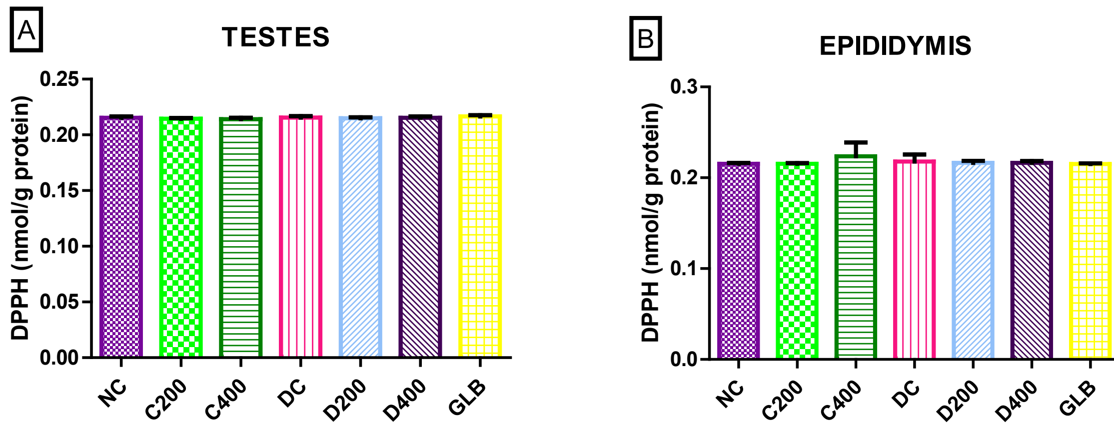

Figure 4.5: The effect of administration of *A. difformis* extract on scavenging capacity (DPPH) in A. testes and B. epididymis. Scavenging capacity in the organs compared within groups. The absence of letters on bars indicates insignificance (p>0.05). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.6. Superoxide dismutase assay

Figure 4.6A and B illustrates SOD activity in rat testes and epididymis respectively. No significant difference was evident amongst the normal control groups. The activity of SOD in the testes was significantly ($p>0.05$) decreased in the untreated diabetic group compared to that of the normal control rats. The activity of SOD was significantly higher in both the diabetic groups treated with 200 mg/kg and 400 mg/kg doses of *A. difformis*, compared to the untreated diabetic group ($p>0.05$) in the testes. The group treated with 200 mg/kg of A. *difformis* showed no significant ($p>0.05$) difference in SOD activity when compared to the treatment group treated with 400 mg/kg of A. *difformis* in the testes. SOD activity was significantly ($p<0.05$) higher in the diabetic group treated with glibenclamide compared to the untreated diabetic group in the testes. Figure 4.6B below shows there was no significant ($p>0.05$) difference in the SOD activity amongst the normal control groups in the epididymis. Similarly, there was no significant ($p>0.05$) difference between the SOD activity in the epididymis of the normal control groups and that of the untreated diabetic group. There was also no significant difference between the untreated diabetic group and all the treatment groups treated with A. *difformis* or glibenclamide ($p>0.05$). In both the testes and the epididymis, the activity of SOD was not significantly ($p>0.05$) different compared amongst all the diabetic treatment groups.

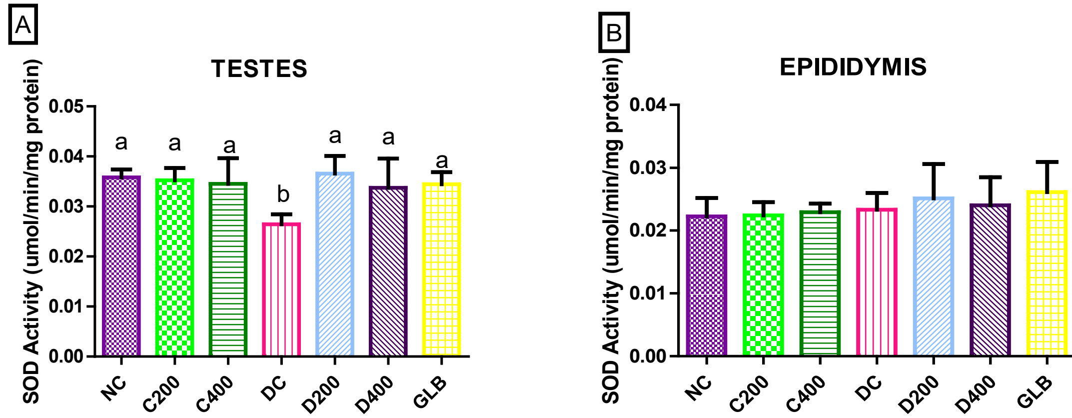

Figure 4.6: SOD activity in A. testes and B. epididymis compared within groups. The effect of administration of *A. difformis* and diabetic drug on SOD activity. Difference in letters on the bars represents significance ($p<0.05$), and the absence of letters shows insignificance ($p>0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.7. Catalase activity assay

Figures 4.7A and B illustrates the effect of diabetes and administration of A. *difformis* on catalase in rat testes and epididymis of normal and diabetic rats. There were no significant ($p>0.05$) differences in CAT activity in rat testes of normal control and A. *difformis* treated control groups. Diabetic control rats had significantly ($p<0.05$) lower CAT activity in comparison to CAT activity in the testes of the normal control groups. The administration of A. *difformis* extract caused a significant increase in CAT activity in the testes of diabetic rats, as depicted by Figure 4.7A, showing a significantly higher CAT activity in both A. *difformis* treatment groups compared to the untreated diabetic group. The CAT activity was significantly ($p<0.05$) higher in the testes of the group treated with glibenclamide compared to the untreated diabetic group. The CAT activity in testes was significantly ($p<0.05$) higher in the 200 mg/kg *A. difformis* dose treated group compared to the 400 mg/kg dose group. No significant ($p>0.05$) difference in CAT activity was evident between the testes of the 400 mg/kg A. *difformis* treatment group and the glibenclamide treatment group. In addition, the CAT activity in the testes of the group treated with glibenclamide was significantly ($p<0.05$) lower compared to that of the group treated with 200 mg/kg A. *difformis*. In the epididymis, there was no significant difference in CAT activity of the normal group compared to the normal A. *difformis* treatment groups. There was no significant difference in CAT activity between the normal control groups and untreated diabetic group in the epididymis tissue ($p>0.05$). There were also no significant ($p>0.05$) differences evident between the untreated diabetic group and all the diabetic treatment groups in the epididymis. The activity of CAT was not significantly ($p>0.05$) different compared amongst all the diabetic treatment groups in the epididymis.

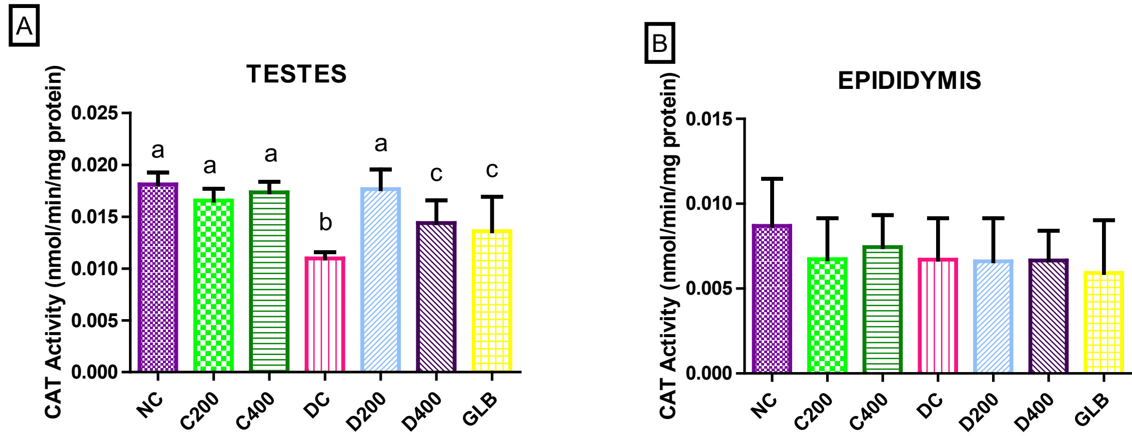

Figure 4.7: CAT activity in A. testes and B. epididymis compared within groups. The effect of *A. difformis* extract on CAT activity of a diabetic model. Difference in letters on the bars represents significance ($p<0.05$), and the absence of letters shows insignificance ($p>0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

4.8. Reduced glutathione assay

The quantification of glutathione in rat testes and epididymis is illustrated in Figures 4.8A and B below. In the testes, there were no significant ($p>0.05$) differences in GSH concentration amongst the normal control groups. Similarly, there were no significant ($p>0.05$) differences in GSH concentration amongst the normal control groups in the epididymis. In both the testes and the epididymis, the untreated diabetic groups were not significantly ($p>0.05$) different in GSH concentration compared to the normal control groups. *A. difformis* extract evidently had no effect on GSH concentration of both the testes and epididymis. This was illustrated in Figure 4.8A and B which shows no significant ($p>0.05$) differences in GSH concentration between the untreated diabetic groups and the groups treated with A. *difformis* for both the testes and the epididymis. There was also no significant ($p>0.05$) difference in the concentration of GSH between the untreated diabetic group and the groups treated with glibenclamide in both the testes and the epididymis. In both the testes and the epididymis, the amount of GSH was not significantly ($p>0.05$) different compared amongst all the diabetic treatment groups.

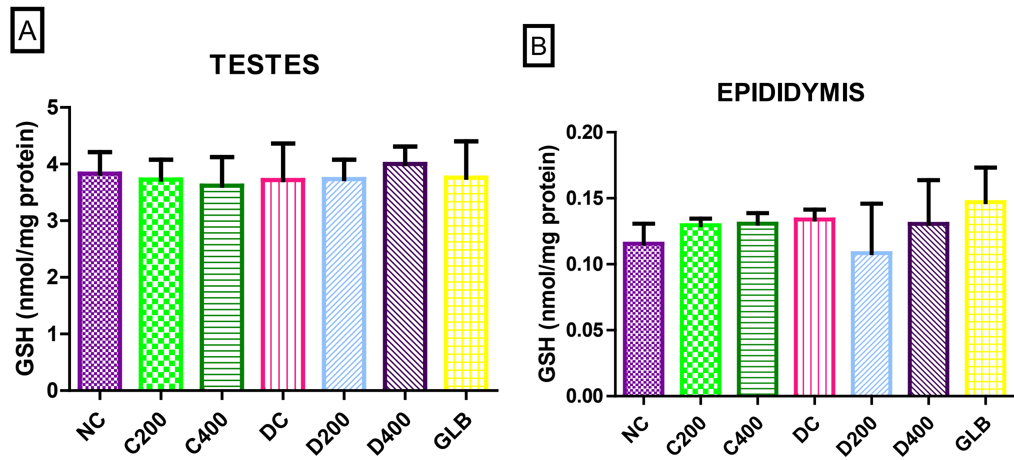

Figure 4.8: The effect of A. *difformis* extract on GSH concentration in A. testes and B. epididymis. GSH concentration compared within groups. The absence of letters on bars indicates insignificance ($p>0.05$). Group names abbreviated above are, Normal control (NC), Control-treated with 200 mg/kg dosage (C200), Control-treated with 400 mg/kg (C400), Diabetic control (DC), Diabetic-treated with 200 mg/kg (D200), Diabetic-treated with 400 mg/kg (D400) and Diabetic-treated with Glibenclamide (GLB).

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1. Discussion and final conclusion

Partial destruction of the pancreatic beta cells by STZ coupled with insulin resistance, induced by 10% fructose dose has been reported to cause symptoms and complications associated with T2DM (Goboza *et al*., 2019; Zaidun *et al*., 2019; Oyenihi *et al*., 2020). Symptoms such as frequent urination, polyphagia, increased thirst, elevated blood glucose levels and weight loss were exhibited in this study. Several studies have recorded evident weight loss in a diabetic model (Olson *et al*., 2016; Oyenihi, 2016; Yang *et al.*, 2016; Long *et al*., 2018). Similarly, a significant increase in body weight loss was observed in diabetic rats compared to the control rats in this study. Due to the body's inability to utilise glucose, protein and fats are broken down as a source of energy, leading to weight loss (Olson *et al*., 2016; Long *et al*., 2018).

T2DM has been reported to be associated with obesity in high-fat diet diabetic models (Sung *et al*., 2018; Rodríguez-Correa *et al*., 2020). Although weight loss between 5% and 10% have been stated to be one of the interventions to curb complications associated with T2DM (Wilding, 2014; Van Gaal & Scheen, 2015; Feldman *et al*., 2017), extreme weight loss may implicate compromised metabolism (Ige *et al*., 2011) and may lead to further complications (Olson *et al*., 2016; Yang *et al*., 2016). Olson *et al*. (2016) also regarded ≥10% weight loss as unusual and implicative of the development of diabetes. The potential benefits of A. *difformis* can be studied by observing the change in body weight caused by the extract. There was no significant change in weight loss between the diabetic groups treated with the extract and the untreated diabetic group. The diabetic group treated with glibenclamide also showed no significant change in weight loss compared to the untreated diabetic group. This suggests that A. *difformis* may not have a beneficial effect on body weight of a diabetic model. This finding supports that of a diabetic study conducted by Ovuakporie-uvo & Idu, (2015), where A. *difformis* showed no effect on body weight.

Relative organ weight can be used as a parameter to investigate the effect of diabetes following plant supplementation (Ovuakporie-uvo & Idu, 2015). Several studies have shown significantly lower relative testis and epididymis weights in diabetic rats compared to control rats (Korejo *et al*., 2016; Long *et al*., 2018). This change in relative organ weight

suggests the effect of elevated glucose levels on the reproductive organs. In contrast, other studies (Ricci *et al*., 2009; Ghafari *et al*., 2011) reported diabetic-induced increase in relative testes weight. In the study conducted by Ricci and colleagues (2009), histological analysis showed an increase in the interstitial compartment in the testes, which was an indication of possible hypertrophy. The result on the relative testes weight obtained in this study is in agreement with that reported by Ricci and colleagues, and this was not affected by both the plant extract and glibenclamide treatments. Similar to this, Ovuakporie-uvo & Idu, (2015) reported that A. *difformis* had no effect on organ-to-body weight ratio. In addition, the relative epididymis weight was significantly lower in untreated diabetic rats compared to the control. This is in support of other studies that reported atrophy observed in the epididymis due to hyperglycemia (Korejo *et al*., 2016; Long *et al*., 2018). Atrophy of the epididymis can lead to its constriction, causing apoptosis and consequently, a reduction in the stored sperm cells (Korejo *et al*., 2016).

DM is characterised by hyperglycemia (Quan *et al*., 2020). In this study, blood glucose ≥ 324 mg/dl was indicative of diabetes. The increase in blood glucose (final glucose level ≥ 324 mg/dl) of the diabetic rats after 10 weeks, confirmed the establishment of a diabetic model in this study. This suggests both the toxicity of STZ in the beta cells of the pancreas and insulin resistance, which may have led to the impaired glucose metabolism and consequently elevated blood glucose levels (Omolaoye *et al*., 2018). The significantly lower final blood glucose level of the 200 mg/kg A. *difformis* treated diabetic rats compared to the blood glucose level of the untreated diabetic rats suggests the hypoglycemic effects of A. *difformis*. This finding correlates with other studies that reported the probable effect of A. *difformis* in reducing the level of blood glucose (Ovuakporie-uvo & Idu, 2015; Alabi *et al*., 2021). In support to this, the significantly lower final blood glucose level of rats treated with a higher dose (400 mg/kg) of A. *difformis* compared to that of rats treated with 200 mg/kg A. *difformis* shows that the hypoglycemic effect of A. *difformis* is dependent on the dosage (Alabi *et al*., 2020). The evident decrease in final blood glucose level in rats treated with glibenclamide compared to that of the untreated diabetic group supports the finding that glibenclamide increases the secretion of insulin by the pancreas, decreasing blood glucose level (Rambiritch *et al*., 2014; Liu *et al*., 2018). However, this study shows a possibly higher hypoglycemic effect of A. *difformis* compared to glibenclamide.

Hyperglycemia has been associated with oxidative stress that consequently leads to further complications (Nna *et al*., 2019; Oguntibeju *et al*., 2020; Oyenihi *et al*., 2020). Male infertility is one of the complications that arise from oxidative stress (Long *et al*., 2018). The products of lipid peroxidation (TBARS), e.g. MDA, were indicative of oxidative stress

in this study. An increase in oxidative stress can be caused by excessive production of ROS by the mitochondrial due to hyperglycemia (Nna *et al*., 2019), or by the release of ROS caused by glycation (Liguori *et al*., 2018). According to previous studies, the amount of MDA increases in diabetic rats, implying an increase in oxidative stress with hyperglycemia (Ayeleso *et al*., 2014; Sankaranarayanan & Kalaivani, 2020).

There was a significant increase in the amount of MDA produced in the testes of the diabetic rat compared to that of the normal control groups. This increase in oxidative stress could have been caused by both the production of ROS that accompanied the established hyperglycemia, and also the decrease in antioxidant activity (Kurutas, 2016; Asadi *et al*., 2017; Alsenosy *et al*., 2019; Sefidgar *et al*., 2019)). These findings support the other studies that have reported the association between DM and oxidative stress (Nna *et al*., 2019; Oguntibeju *et al*., 2020; Oyenihi *et al*., 2020). Alabi and colleagues (2020) reported the possible significance of A. *difformis* extract in ameliorating oxidative stress through the Nrf2 pathway. In their study, it was reported that NrF2 was increased with diabetes and normalised when A. *difformis* was used as treatment, reducing oxidative stress. In support of these findings, this study depicted that the level of MDA in testes of rats treated with 200 mg/kg extract was significantly reduced, suggesting the effect of the extract in ameliorating oxidative stress in the testes, and possibly its positive effect in the improvement of male fertility.

Interestingly, the increase in the dose of A. *difformis* extract to 400 mg/kg had a lower ameliorative effect on the testes of diabetic rats compared to that of the control. Ataman & Idu (2015) concluded from their study, where toxicity of A. *difformis* extract was determined, that the positive effect of the plant extract is dependent on the dose. In their study, treatment with 300 mg/kg of the extract resulted in toxicity. In our study, 400 mg/kg could have possibly induced toxicity leading to the higher oxidative stress. There was a similar decrease in MDA in the testes of diabetic rats treated with glibenclamide compared to the normal control groups. Glibenclamide has also been associated with elevated oxidative stress by other studies (Wang *et al*., 2011; Liu *et al*., 2018), possibly elucidating the high MDA level compared to the group treated with 200 mg/kg, although still lower than the normal control group.

A previous study reported an increase in oxidative stress in the epididymis of untreated diabetic rats in comparison to that of the normal control rats (Ostovan *et al*., 2017; Nna *et*

al., 2019). This elevation of oxidative stress is caused by excessive production of ROS by the epithelial cells of the epididymis and compromised antioxidant system as a result of hyperglycemia (Nna *et al*., 2019; James *et al*., 2020). Incongruous to these findings, there was no significant difference in the level of MDA between the epididymis tissues of the diabetic control group compared to the normal control groups. In this study, the antioxidants in the epididymis were evidently not compromised, and could possibly be responsible for the scavenged free radicals and the non-significant change in MDA production. In addition to this, atrophy of the epididymis was evident in this study, suggesting the possible death of some cells of the epididymis, reducing the amount of free radicals produced.

Several previous studies have revealed the antioxidant capacity and the availability of phytochemicals such as saponins, tannins and flavonoids in *A. difformis* (Aliyu *et al*., 2013; Oghale & Idu, 2016; Alabi *et al*., 2020). The significant decrease in the reduction of Fe^{2+} in the testes of diabetic rats compared to the control groups in this study corroborates with the report of Oyenihi *et al*. (2020) that measured the FRAP capacity of the testes and deduced that antioxidant power was reduced when DM was induced. The antioxidant power may be compromised by oxidative stress (Pieme *et al*., 2017; Erukainure, 2019), further confirmed in the present study. This study showed a significant increase in FRAP in the testes of the diabetic rats treated with glibenclamide. The direct action of glibeclamide in scavenging free radicals was reported by Oguntibeju and colleagues (2020). This may suggest that the mechanism of scavenging free radicals by glibenclamide in the testes targets the reduction of Fe^{2+} in free radicals. Due to the unchanged oxidative status in the epididymis, there was no significant difference in Fe^{2+} reduction capacity between all groups in the epididymis.

There was also no significance in the DPPH antioxidant capacity between all groups in both the testes and epididymis. This shows that the induction of diabetes did not affect DPPH capacity. It is depicted in this study that the mechanism of action in free radical scavenging in the testes is by reduction of Fe^{2+}, hence the elevation of free radicals when FRAP was lower, and no change in DPPH capacity. Furthermore, the insignificant change in antioxidant power after the determination of FRAP and DPPH in the testes in the diabetic group treated with *A. difformis* extract implies that the antioxidant system's mechanism of action after treatment may not be by directly scavenging free radicals, but by other oxidative pathways such as activation of antioxidant enzymes (Asadi *et al*., 2017; Oyenihi *et al*., 2020).

ROS is produced in the testes to aid in the production of sperms (Khorramabadi *et al*., 2018). To counteract the continuous production of ROS, the testes contain antioxidant enzymes (Tian *et al*., 2020). Findings from this present study revealed that both CAT and SOD activities were decreased in the testes of diabetic rats compared to the CAT and SOD activities in the testes of the normal control groups. These findings correlate with the results In several studies where oxidative stress was induced in the testes of rats and all these parameters were reduced (Nna *et al*., 2019; Shoorei *et al*., 2019; Fan *et al*., 2020). Nna and colleagues (2019) observed the down regulation of NrF2 and linked this with the reduction in these antioxidant enzymes. This could imply that oxidative stress causes down regulation of NrF2.

In other studies reported, reduction in antioxidant enzymes and oxidative stress was associated with protein glycation (Ghelani *et al*., 2018; Dzięgielewska-Gęsiak *et al*., 2019). Protein glycation was reported to cause the inactivation of antioxidant enzymes due to the alteration of amino acids present in the active sites of these antioxidant enzymes (Mohasseb *et al*., 2011; Tavares *et al*., 2019). This phenomenon decreases the activities of CAT and SOD; it also contributes to the further elevation of oxidative stress (Pieme *et al*., 2017; Tavares *et al*., 2019). However, GSH concentration remained significantly unaffected throughout the different groups of the testes in this study. This could imply that the level of GSH was not affected by glycation or the increase in oxidative stress. It could also suggest that the antioxidant enzymes were affected by oxidative stress independently, opposing the idea of focussing on NrF2 as the main factor affected.

Expression of CAT, SOD and GSH in the epididymis of the diabetic group was not altered. This however, could possibly be due to the production of MDA which remained unaffected in the epididymis of the untreated diabetic group. This further suggests the association between oxidative stress and antioxidant enzyme activation (Pieme *et al*., 2017). In the study documented by Nna and colleagues (2019), reduction of antioxidant enzymes and increase in TBARS in the epididymis implied that germ cells were still exposed to oxidative stress even in storage. This study however suggests that damage of the germ cells could occur mainly in the testes, possibly due to the weight increase in the testes which could imply an increase in cell number or size, producing even more ROS. Literature has revealed the effect of *A. difformis* on the activities of antioxidant enzymes of other organs such as the liver, kidneys and heart (Alabi *et al*., 2020). However, studies revealing the effect of the extract in testes and epididymis are scarce. Treatment with 200 mg/kg and 400 mg/kg dosage of aqueous extract of *A. difformis* in the present study showed a

significant increase in both CAT and SOD in the testes of diabetic rats. This could imply that the plant alleviates the deactivation of these enzymes caused by oxidative stress in the testes. The extract could also possibly have ROS scavengers (Oghale & Idu, 2016), which possibly reduced oxidative stress, thereby leaving the antioxidant system uncompromised.

In the study by Mohasseb and colleagues (2011), a marker of protein glycation (HbA1c) was decreased with the administration of antioxidants in the reproductive organs of rats, and it was concluded that antioxidants could ameliorate glycation. Similarly, other studies conducted concluded that phytochemicals in medicinal plants can cause reduction of protein glycation (Sri Harsha & Lavelli, 2019; Martha *et al*., 2020). With the finding that *A. difformis* contains phytochemicals with antioxidant potential, it is fair to suggest that deactivation of some antioxidant enzymes could have been ameliorated with the administration of the extract, leading to an increase in these antioxidant enzymes. CAT and SOD activities was also increased in the testes of the group treated with glibenclamide compared to the untreated diabetic group, revealing the benefits of this drug in increasing the activation of these enzymes. Interestingly, CAT activity is higher in the 200 mg/kg dosage of A. *difformis* group compared to the 400 mg/kg dose group, supporting the dependency of the effect of A. *difformis* on its dosage (Olanlokun *et al*., 2017).

The diabetic model demonstrated in this study successfully mimicked the characteristics of T2DM, that is, reduced body weight gain, hyperglycemia, tissue oxidative stress, insulin dysfunction and resistance. Hyperglycemia and oxidative stress both synergistically lead to the impairment of the testes and epididymis. Results from this study have shown the benefits of the extract *A. difformis* in ameliorating oxidative stress. This study has successfully answered the research question and has concluded that treatment with *A. difformis* boosted the antioxidant system and reduced diabetes-induced free radicals in testes. The plant extract does not however increase antioxidant capacity. The above findings suggest *A. difformis* extract as a therapeutic agent against male infertility associated with DM, although more studies to elucidate molecular mechanisms of the A. *difformis* in male infertility are necessary.

5.2. Limitations and further directions

Other methods of determining antioxidant status (ORAC and GPx) in the testes and epididymis were unsuccessful because of the low protein content in both the organs. New

methods of performing these assays can be considered in future. Future analysis of the NrF2 factor as the main regulator of the antioxidant system is also necessary to further understand how DM leads to oxidative stress, and whether A. *difformis* extract targets the upstream factor or the antioxidant enzymes individually. Analysis of NrF2 factor by methods like Western blotting could also be necessary to investigate where any changes are evident in the epididymis, and where the duration of treatment after diabetes induction can be increased to observe any changes downstream in the epididymis.

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