INVESTIGATING HYPOGLYCAEMIC EFFECTS AND SAFETY OF THE HERBAL PRODUCT – *JT2016* IN VIVO STUDY

By

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KEYWORDS

1. Hyperglycemia
2. *Jiang Tang 2016*
3. Blood glucose
4. Diabetes
5. Insulin Resistance
6. African Traditional Medicine

ABSTRACT

Introduction

Diabetes has since been a global epidemic; an estimated 5.0 million deaths of diabetes in the world have been recorded; one in 11 adults have diabetes (415 million); and by 2040, one adult in 10 (642 million) will have diabetes. In Africa, more than two thirds of people with diabetes are undiagnosed, and 42 million have diabetes in the Sub-Saharan region with 324 877 adult deaths in South Africa (IDF, 2015). The global prevalence (age-standardized) of diabetes has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population. This reflects an increase associated with risk factors such as overweight or obese (WHO, 2016). Medicinal plants on the other hand, have played a significant role in the treatment and prevention of diabetes for centuries. In South Africa, indigenous medicinal plants have increasingly been used in the treatment of diabetes.

In this study, a new anti-diabetes herbal compound named *Jiang Tang* 2016 (JT2016), made of three well researched South African indigenous medicinal plants is investigated for its hypoglycemic effects in HFD/STZ induced diabetic SD rats. These plants have been used for centuries in the indigenous system of medicine against various ailments, they are easily accessible, they grow in abundance, and are economically sustainable.

Aim

The aim of this study was to investigate the hypoglycemic effects and safety of the anti- diabetes herbal compound, *Jiang Tang* 2016 (JT2016) in HFD/STZ induced diabetic SD rats.

Materials and Method

There were 60 male normal Sprague Dawley (SD) rats (210-250g) used for the study, provided by the Model Animal Research Centre of Nanjing University. All animals were housed at the

Beijing University of Chinese Medicine animal house at the temperature of 23 +/- 2^0C, and humidity of 55 +/- 10% with a 12-hour light/dark cycle. The animals were provided with food and water ad libitum.

The rats were divided into two main groups immediately after two weeks of adaptive feeding; the normal group fed with normal pellet diet (NPD) (n=10), and the experimental group fed with High Fat Diet (HFD) (n=50). After the two weeks of dietary manipulation, HFD-fed rats exhibited a significant increase in body weight, and basal plasma glucose as compared to NPD- fed rats. HFD-fed rats were then injected intraperitoneally with Streptozotocin (STZ) (35mg/kg). Three days later, the animals were fasted and tested for hyperglycemia and body weight: every rat tested positive for hyperglycemia was included in the study and randomly assigned into the following five groups; control group, positive drug group (pioglitazone), JT2016 compound A (JT2016A), JT2016 compound B (JT2016B), and JT2016 compound C (JT2016). All medicine was administered intragastrically. The JT2016 compounds were divided into different groups according to the different three medicinal plant ratios, to determine the most effective combination. Both body weight and random blood glucose were measured weekly for the period of six-weeks treatment. At the end of the six weeks, body weight was measured, serum was collected and the animals were tested for fasting blood glucose, plasma insulin/ insulin resistance, hepatic function, proteins, lipids, inflammatory cytokines etc. and all animals executed through euthanasia.

RESULTS AND DISCUSSION:

Jiang Tang 2016 significantly ($p < 0.05$) suppressed the blood glucose levels (FBG and RBG) in HFD/STZ induced diabetic SD rats and treated insulin resistance by the *Jiang Tang* 2016 compound C, decreased the low density lipoproteins cholesterol (LDL-C) levels and maintained elevated high density lipoproteins cholesterol (HDL-C) improving lipid

metabolism in HFD/STZ diabetic rats by the *Jiang Tang* 2016 compound A. The inflammatory cytokines levels (interleukin 6 (IL-6) and tumor necrosis factor - alpha (TNF-α)) were also significantly ($p < 0.05$) brought down and alleviating insulin resistance significantly by the *Jiang Tang* 2016 compound C. Moreover, the AST and the proteins (albumin, globulin, and total proteins) were unaffected indicating the safety of JT2016, and the body weight measurements showed no variation. Together, these results confirm the potential hypoglycemic effects of JT2016 and its safety in HFD/STZ induced diabetic SD rats. Furthermore, *Jiang Tang* 2016 compound A and C were found to be the most effective compounds in the study.

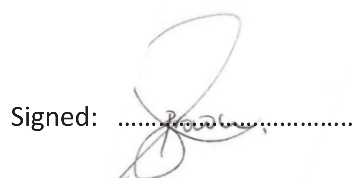
CONCLUSION

The results in this study establish the anti-diabetic effects of JT2016 in HFD/STZ induced diabetic SD rats, through their ability to suppress both fasting and random blood glucose levels; alleviating insulin resistance; reducing inflammatory levels and improving lipid metabolism by reducing LDL-C and maintaining elevated HDL-C levels in experimental rats. Therefore, this study confirms that JT2016 has potential antidiabetic properties and can be developed as an effective alternative/ complementary anti-diabetes herbal compound for T2DM management. However, further *in vivo* investigations are needed with an extended treatment period of +/-14 weeks, using different diabetic rodents e.g. use of genetically engineered diabetic mice ($Lepr^{db}$/ $Lepr^{db}$ ordb/db).

DECLARATION

I declare that: Investigating hypoglycemic effects and safety of *JIANG TANG* 2016 (anti- diabetic herbal compound) in HFD/STZ diabetic Sprague Dawley rats is my own work, that has not been submitted before for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged as complete references.

Mary, Nthabeleng Brown May 2020

Signed: …………………

DEDICATION

I dedicate this master's thesis to my Lord and Savior Jesus Christ.

My late parents, Ntate William Ntoa Brown and 'Me' Mathabang Lylian Brown, may the Lord bless you on the other side of life.

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CHAPTER 1

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder of the endocrine system, it is marked by hyperglycemia resulting from defects in insulin secretion and action (ADA, 2009). Patients with Diabetes mellitus have the option to turn to alternative remedies reported to improve hyperglycemic effects and the management of the disease or use allopathic remedies. The long-term complications of untreated or ineffectively treated diabetes include: retinopathy, diabetic neuropathy, nephropathy, macro-vascular complications etc. (WHO, 2016). Therefore, strong, safe and effective oral hypoglycemic agents with minimum side effects to provide clinicians with a wide range of options to prevent, treat and manage the disease is needed. There are three types of diabetes mellitus, namely: Type 1 diabetes mellitus, Type 2 diabetes mellitus, and Gestational diabetes mellitus (GDM) (WHO, 2016).

In this study, *Jiang Tang* 2016 an anti-diabetic herbal compound is investigated for its safety and hypoglycemic effects in HFD/STZ diabetic SD rats.

1.1 Epidemiology of Type 2 Diabetes Mellitus (T2DM)

It is estimated that 366 million people had DM in 2011; by 2030 this would have risen to 552 million. The number of people with T2DM is increasing in every country with close to 80% of people with T2DM living in low-income and middle-income countries. T2DM caused 4.6 million deaths in 2011 (Chamnan *et al.,* 2011). The prevalence of T2DM varies substantially from one geographical region to the other because of the environmental and lifestyle risk factors (Zimmet *et al.,* 2001).

Literature search has shown that majority of the diabetes mellitus burden in Africa appears to be T2DM, with less than 10% of diabetes mellitus cases being Type 1 diabetes mellitus (T1DM). According to the IDF Diabetes Atlas 8th Edition, an estimated 15.5 million adults aged 20-79 years were living with diabetes in the IDF Africa Region in 2017, representing a regional prevalence of 3.3%. The highest prevalence of diabetes in the African Region is found in adults aged 55 to 64. The region has the highest proportion of undiagnosed diabetes, with over two-thirds (69.2%) of adults currently living with diabetes unaware of the condition. More than half (55.3%) of adults living with diabetes in the Africa Region live in urban areas (IDF, 2018).

Global projections demonstrate an estimated 451 million people (age 18-99 years) people with diabetes worldwide with expected increase to 693 million by 2045 (figure. 1) (IDF, 2017). The increase of diabetic rates is largely driven by the development of the economy and the changes in lifestyle (Antony *et al.,* 2017; Kengne *et al.,* 2013). The prevalence of diabetes in South Africa between 2000 and 2009 creased from 5.5% to 9% (Bradshaw *et al.,* 2007; Bertram *et al.,* 2013). Approximately 2000 diabetes related amputations, 8000 cases of blindness, and over 73 000 disability-adjusted life were attributable to T2DM (Bertram *et al.,* 2013).

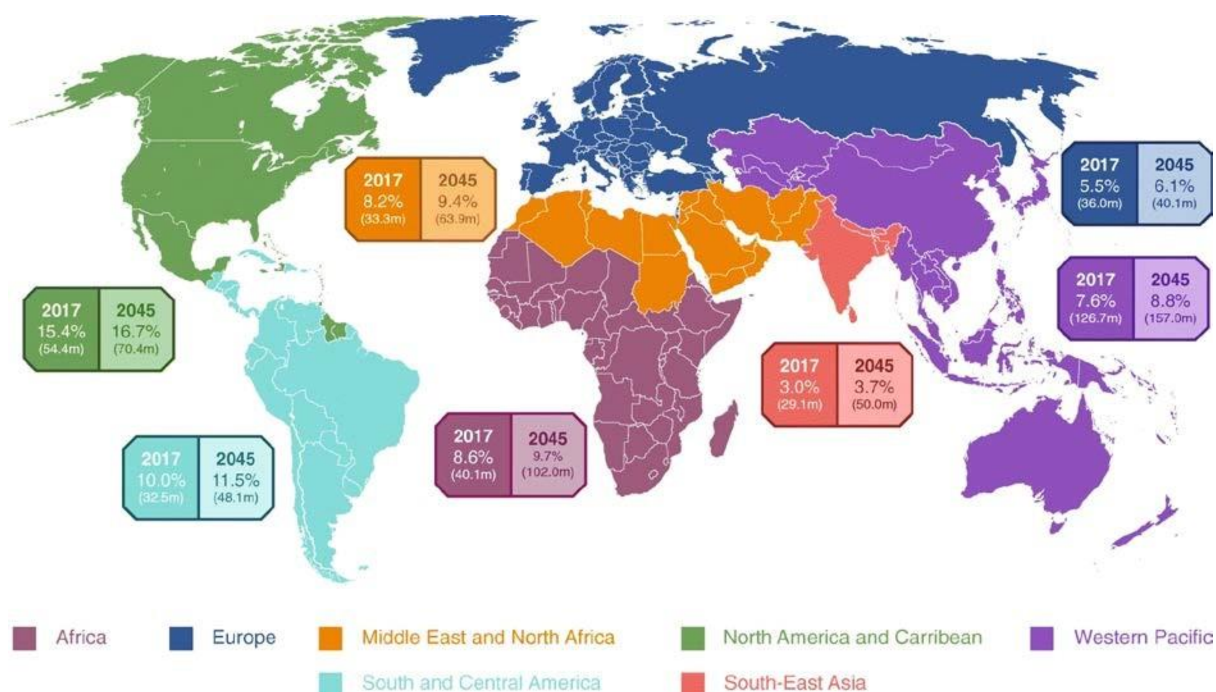

Figure 1: Global prevalence estimates of impaired glucose tolerance by International Diabetes Federation (IDF) region. Percentages represent unadjusted regional prevalence estimates. Numbers in parentheses denotes the estimated number of individuals affected by impaired glucose tolerance in each region. Prevalence estimates calculated by the IDF using a generalised linear regression model. A variety of country-level data sources were included (Data source: International diabetes federation: IDF diabetes atlas - 8th edition, 2017).

1.2 Rationale

Diabetes has been confirmed to pose a significant burden on the economic, the costs of hospitalization, medication, and disability grants. Governments and families also place a significant burden on the economy indirectly through e.g.: work absenteeism, time spent caring for sick families, leading to reduced production rates. Diabetes has claimed 76% of deaths in South Africa in people of the age of 60 or less, which is the most economically active

demographic segment of the population (Manyema *et al.,* 2015; Peer *et al.,* 2014). Total health expenditure for diabetes for adults in SA is projected to increase by approximately 50% between 2010 and 2030. In SA, these costs are projected to be between 1.1 to 2 billion USD in 2030 (Zhang *et al.,* 2010). The average costs of hospitalization per diabetic patient in South Africa were R27 000 as to R18 000 of non-diabetic patients in 2009 (Manyema *et al.,* 2015; Ncube-Zulu *et al.,* 2013).

Diabetes mellitus has been controlled and treated traditionally for centuries in India and China using herbal medicine. Medicinal plants or herbal medicine is also widespread in Africa, and has been in practice orally and ritually transmitted from generation to generation. A study was done in Morocco in the Fez-Boulemane region to determine the prevalence of herbal medicine in the diabetic population (T2DM). In this country, despite the development and availability of oral antidiabetics and insulin, a large fraction of Moroccan diabetic patients is still dependent on treatment with medicinal plants. The motivations behind this therapeutic choice has been the efficacy of medicinal plants in 90.6% of cases; of which 3% of these cases claim that conventional medical treatment is expensive (Diarra *et al.,* 2016).

Recently, there is an increasing interest in seeking the safety and effectiveness of herbal medicine to treat diabetes alongside conventional medicine hence the study "investigating hypoglycaemic effects and safety of JT2016 (herbal compound) in HFD/STZ induced SD rats."

There is currently a number of effective allopathic medicine available to treat and manage T2DM, but there is still an immense challenge finding medication with less side effects and with low costs. Allopathic medicine for the treatment of diabetes often has side effects, like

obesity, osteoporosis, cardiovascular diseases etc. (Prabhakar *et al.,* 2011). These side effects prevail even the more because of continual use of the drugs. Moreover, treatment is quite costly because T2DM is a chronic disease and has to be treated long-term with medication. Herbal medications are the closest alternative to supplement allopathic medicine (Li *et al.,* 2004; Prabhakar *et al.,* 2011).

Herbal medications used to treat T2DM are able to target multiple mechanisms e.g. they can increase insulin sensitivity, treat insulin resistance, stimulate insulin secretion, reduce carbohydrate absorption, lower blood glucose etc. all at the same time (Li *et al.,* 2004). Unlike allopathic medication that aim for a specific mechanism with a single active ingredient, herbal compounds comprise of several active ingredients/ constituents targeting many different mechanisms. This herbal (Phyto) medicine is built on the all-inclusive theory, putting emphasis on the complete body as to singular tissues, although allopathic medicine is more strong than Phytomedicine in suppressing the increase of blood glucose levels. However, Phyto compounds have been confirmed to treat diabetes and its complications (Jia *et al.,* 2003).

In this study, several pharmacological mechanisms found in different medicinal plants for the treatment of T2DM have been studied for the development of a new herbal compound *Jiang Tang* 2016 (JT2016).

1.3 Type 2 diabetes mellitus (T2DM)

Type 2 diabetes mellitus (T2DM), is an upshot of the body's ineffective use of insulin. The symptoms include excessive urination and thirst, but are every so often less marked or absent resulting to the disease going undiagnosed for years, until the upsurge of complications (WHO,

2016).

1.4 Pathophysiology of T2DM

T2DM is linked to different metabolic defects which include the reduction of production and secretion of insulin, insulin resistance and continued glucagon production. In healthy persons, insulin binds to insulin receptor which initiates phosphorylation based on signaling cascade that results in translocation of intracellular Glucose transporter type 4 (GLUT4) to membrane for uptake of glucose molecules; and in a diabetic state, insulin receptors become non- functional and cytoplasmic GLUT4 does not get translocated to membrane causing insulin resistance (figure 1.2) (Prasad *et al.,* 2018). Insulin resistance refers to body cells such as the fat cells, muscle cells, and liver cells failing to respond to insulin, even in its highest levels.

In the fat cells, triglycerides are broken down to produce free fatty acids for energy; but the muscle cells are deprived of this energy because the skeletal muscle oxidative capacity has been reduced and has impaired metabolic flexibility (Ashcroft *et al.*, 2017).

In the liver , the glucose production is markedly elevated, and the mechanisms of elevated basal Hepatic Glucose Production (HPG) lie in the course by which the hepatic gluconeogenesis is switched/ turned off. In particular, the deficiency in the production of glucagon that causes constant HPG reduction, leading to an overall rise of the postprandial blood glucose levels (figure 1.1).

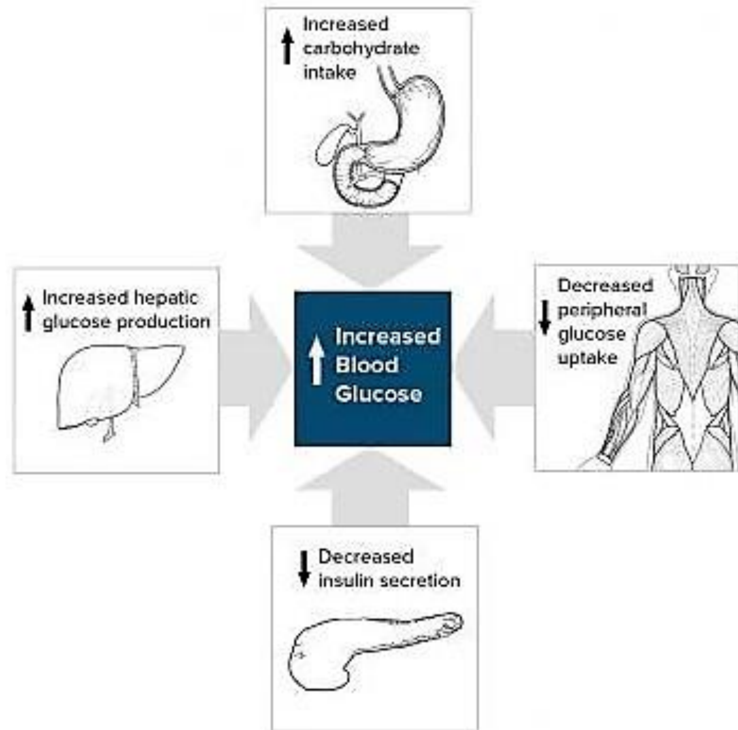

Figure 1.1: Summary of Pathophysiology of T2DM, T2DM consists of an array of dysfunctions characterized by hyperglycaemia and caused by the combination of resistance to insulin action, inadequate insulin secretion, and extreme or unsuitable glucagon secretion. (emedicine.medscape.com)

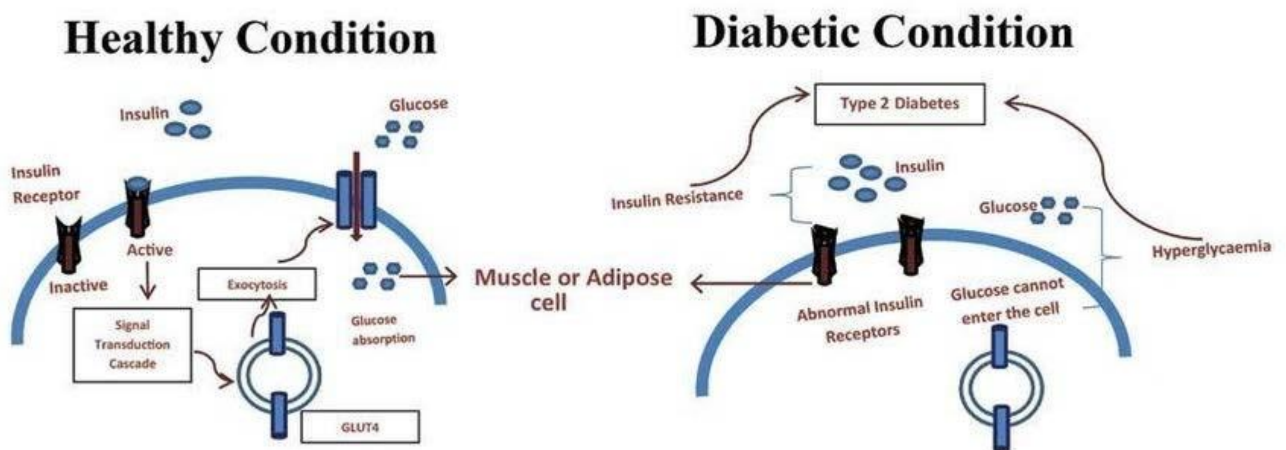

Figure 1.2: Illustrative of pathophysiology in T2DM: In healthy persons, insulin binds to insulin receptor

which initiates phosphorylation based signaling cascade that results in translocation of intracellular Glucose transporter type 4 (GLUT4) to membrane for uptake of glucose molecules; and in a diabetic state, insulin receptors become non-functional and cytoplasmic GLUT4 does not get translocated to membrane causing insulin resistance. Subsequently, glucose uptake does not take place, which leads to hyperglycaemia (Prasad *et al.,* 2018).

1.5 Glucose and Insulin metabolism in T2DM

1.5.1 Glucose metabolism

To maintain normal plasma glucose levels, accurate and equal glucose use with endogenous glucose production are required. Glucose which results from the digestion of dietary carbohydrates is transported into cells through different metabolic pathways i.e. as stored glycogen; glycolysis to pyruvate; and then released into the circulation by the liver and kidneys to be utilised by the body (Appel *et al.,* 2003).

In the state of fasting, plasma glucose concentrations are stable, which designates equal rates of glucose production and use, hence accurate blood glucose test preferably done after eight hours of fasting (Garber *et al.,* 1976). After a meal, glucose absorption marks rates of exogenous glucose delivery into the circulation, which can be twice the rate of post-absorptive endogenous glucose production, reliant on the carbohydrate content of the meal and the degree of glucose absorption. When glucose is adsorbed, endogenous glucose production is repressed, and the use of it by the liver, muscle, and fat tissues increases. When the exogenous glucose is integrated, the plasma glucose concentrations are able to return to their fasting state maintaining the balance (Radziuk *et al.,* 1978).

1.5.2 Insulin secretion and T2DM

Insulin as a peptide hormone that plays a significant role in energy metabolism. It was first discovered in 1921 for effective therapy for diabetes and the treatment of T1DM (Banting *et al.,* 1956). It is therefore (Insulin) produced by the pancreatic β-cells responding to hyperglycaemia or amino acids. The production of insulin is secreted into the blood circulation to facilitate absorption and use of glucose for energy substrate by body organs. The action of Insulin is important for tissue development, growth and for the maintenance of energy homeostasis (Sesti, 2006).

In the earliest stages of T2DM, the β-cell function in T2DM has shown a consistent pattern that points out a complex interplay between insulin secretion and insulin sensitivity. In people with impaired glucose tolerance (IGT) and mild diabetes, the fasting plasma insulin concentration is consistently increased as well as the basal insulin secretion (DeFronzo *et al.,* 1992). When fasting glucose is increased, fasting plasma insulin progressively upsurges as well, and when this fasting glucose exceeds a certain degree insulin secretion drops off swiftly. And when the pancreas fails to maintain a high rate of insulin secretion the hepatic glucose production increases in absolute terms and begins to contribute to the elevation in fasting plasma glucose (FPG) (DeFronzo *et al.,* 1992).

The progression from normal to IGT to T2DM with mild fasting hyperglycaemia is marked with an increase in both fasting and glucose-stimulated plasma insulin levels (Saad *et al.,* 1989). Overt fasting hyperglycaemia results from the inability of pancreatic beta cells to maintain high insulin secretion. And a similar pattern of insulin secretion occurs during the

development of diabetes in animal models. Therefore, pancreatic beta cells and insulin secretion are seen to play a crucial role in the development of T2DM (Hansen *et al.,* 1986).

1.5.3 Insulin resistance and T2DM

Insulin resistance is a result of muscle, fat, and liver cells not responding correctly to insulin. Therefore, insulin does not absorb glucose from the bloodstream and the body therefore demands more of insulin to help glucose enter into cells. The pancreatic beta cells will then work towards keeping up with the increased demand of insulin by producing more insulin. So long as the pancreatic beta cells are able to produce enough insulin to overcome the insulin resistance, blood glucose levels stay in a healthy range (Leibiger *et al.,* 2008).

Over time, insulin resistance leads to T2DM and prediabetes especially when the beta cells fail to upkeep with the body's high demand for insulin. Without sufficient insulin, excess glucose builds up in the bloodstream, leading to diabetes, prediabetes, and other metabolic syndrome disorders (Leibiger *et al.,* 2008).

1.5.4 Insulin resistance in skeletal muscle cells and liver cells

One of the characteristics of T2DM in patients are a decreased fat oxidative capacity and high levels of circulating free fatty acids (FFAs). The latter are known to cause insulin resistance, particularly in the skeletal muscle. They reduce insulin stimulated glucose uptake, especially via accumulation of lipids inside the muscle cell (Gonzalez-Freire *et al.,* 2018).

T2DM is associated with impaired metabolic flexibility, reduced fat oxidative capacity, and metabolic inflexibility. These are core components of insulin resistance found within the skeletal muscles (Gonzalez-Freire *et al.,* 2018). The cause of these derangements in skeletal muscle of T2DM patients remain to be expounded. An impaired mitochondrial function also found to contribute to insulin resistance even though it may not be direct. Evidence from both *in vivo* and *in vitro* studies supports the idea that an impaired skeletal muscle mitochondrial function is related to the development of insulin resistance and T2DM (Gonzalez-Freire *et al.,* 2018).

On the other hand, insulin resistance in hepatocytes is also found to be an integral underlying cause of the metabolic syndrome that manifests itself in T2DM. In hepatocytes the insulin resistant state is brought about by one or more of the following pathological alterations: hyperglycaemia and hyperinsulinemia, formation of advanced glycation end-products, increased free fatty acids and their metabolites, oxidative stress and altered profiles of adipocytokines. Insulin resistance in hepatocytes changes glucose metabolism directly, especially the control over glucose output into the circulation and interferes with cell survival and proliferation. Moreover, insulin resistance can be exacerbated in hepatocytes by increased oxidative stress and by secretion of cytokines such as TNF and IL-6 by the sinusoidal liver cells also called effector cells, which are target cells that respond to the pathological alterations occurring in the insulin resistant state (Jung *et al.,* 2014).

1.6 Blood lipid disorder and T2DM

Diabetes mellitus as mentioned is a metabolic disorder defined by deficiencies in insulin

secretion or insulin action (ADA, 2009). It is therefore linked with hyperglycaemia and disorders in different body metabolisms e.g. Lipid metabolism, glucose and protein metabolism (Abou-Seif *et al.,* 2004). In previous studies blood glucose levels with numerous factors have been found to have an effect on blood lipid levels, because of an association between sugars and lipid metabolism (Khan *et al.,* 2008).

Diabetic dyslipidaemia is a well-defined high concentration of triglycerides (TG), high concentration of low-density lipoprotein cholesterol (LDLC), and low concentration of high- density lipoprotein cholesterol (HDLC) (figure 1.3). Diabetic dyslipidaemia is prevalent in T2DM resulting from insulin resistance (Taskinen, 2002; Folli, 2011).

1.6.1 Total cholesterol and T2DM

Total cholesterol is the sum of all cholesterol measured in blood (figure 1.4). This total includes high-density lipoprotein (HDLC), and low-density lipoprotein (LDLC), and very low-density lipoprotein (VLDL), which is the triglyceride-carrying component of lipids in non-diabetic patients (Preiss *et al.,* 2011).

1.6.2 Triglycerides and T2DM

Elevated triglyceride levels are a common dyslipidaemia feature in T2DM and pre-diabetic states (Yusuf *et al.,* 2001). Hypertriglyceridemia is frequently seen in patients with metabolic syndrome, T2DM or familial combined hyperlipidaemia (FCHL) (Taskinen, 2003). The presence of insulin resistance in these clinical phenotypes serves as benchmark.

Therefore, a response to insulin is decreased in the state of insulin resistant. This attenuated

response leads to irregularities in lipid metabolism within the adipose tissues, the liver and the skeletal muscles, which are said to be chiefly sensitive to insulin and its metabolism (Adiels *et al.,* 2008).

Triglycerides are lipids found in the blood, which are converted calories unwanted or not in use. The triglycerides are stored in the fat cells and later hormones release them for energy between meals. When food is consumed regularly and more calories are stored as to being burned especially from eating high carbohydrate foods, triglycerides are increased (hypertriglyceridemia). Therefore, poor lifestyle and diet, side effects of medication, poor management of diabetes etc. will cause an increase in triglycerides (Adiels *et al.,* 2008).

1.6.3 Low density lipid cholesterol (LDLC) and T2DM

Janghorban et al. (2018) research study done in Iran, indicate that high levels of LDLC predict T2DM, independent of age, gender, fasting plasma glucose etc. in most high-risk individuals. LDLC is considered as "bad" cholesterol, because of its contribution towards atherosclerosis and other cardiovascular diseases (Janghorban *et al.,* 2018).

1.6.4 High density lipid cholesterol (HDLC) and T2DM

High-density lipoprotein cholesterol (HDLC), referred to as "good" cholesterol takes up excess cholesterol in the blood and transports it to the liver to be broken down and excreted from the body. HDLC is called good cholesterol because HDLC acts as a scavenger, carrying LDLC away from the arteries and back to the liver, where the LDLC is broken down and passed out from the body. However, HDLC does not completely eliminate LDLC because it carries only one-third to one-fourth of blood cholesterol. Healthy HDLC levels may protect against heart attack and

stroke. Studies show that low levels of HDLC increase the risk of heart disease (Yusuf *et al.,* 2001).

When HDLC levels are low, epidemiological studies confirm that T2DM is at increased risk (Schmidt *et al.,* 2018). HDLC low levels together with high levels of triglycerides and diabetic dyslipidaemia are said to be well interrelated and confirmed as T2DM marker (De Silva *et al.,* 2011).

1.7 T2DM and inflammation factors

T2DM is commonly associated with classical factors and chronic low-grade inflammation. Epidemiological studies demonstrate an increase in plasma levels of inflammatory markers such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) in patients with clinically overt T2DM and metabolic syndrome (Pickup *et al.,* 2012).

1.7.1 Interleukin-6 in T2DM

Interleukin-6 (IL-6) is a proinflammatory cytokine that decisively induces the development of insulin resistance and pathogenesis of T2DM. The existence of IL-6 in tissues is normal, but long-term unbalanced production of IL-6 leads to inflammation, inducing insulin resistance and T2DM. Therefore IL-6 induces insulin resistance by damaging the insulin receptor substrate-1 together with phosphorylation of insulin receptor, causing expression of suppressor of cytokine signaling 3 (potential inhibitor of insulin signaling) (Rehman *et al.,* 2017).

1.7.2 Tumour Necrosis Factor-alpha in T2DM

Tumour necrosis factor alpha (TNF-α) is an adipocytokine associated with systemic inflammation (Moller, 2000). TNF-α contributes its decisive role to induce tissue-specific inflammation which ultimately leads towards the pathogenesis of T2DM (Rehman *et al.,* 2017). TNF-α activates the endothelial production of adhesion molecules such as intracellular adhesion molecule-1 which promotes the development of insulin resistance (Hu *et al.,* 2004). TNF-α also plays a role in the development of insulin resistance in reducing the expression of glucose transporter type 4 (GLUT4) (an insulin-regulated glucose transporter, mainly located in adipocytes, skeletal, and cardiac muscles) (Huang *et al.,* 2007).

1.8 T2DM diagnosis and tests

1.8.1 T2DM Diagnosis

Tests for screening and diagnosis of T2DM are readily available. The tests recommended for screening are the same as those for making diagnosis, with the result that a positive screen is equivalent to a diagnosis of pre-diabetes or DM e.g. random blood glucose (RBG) test, fasting plasma glucose (FPG) test, oral glucose tolerance test (OGTT) etc. (Holst *et al.*, 2011).

About 25% of patients with T2DM already have microvascular complications at the time of diagnosis suggesting that they have had the disease for more than five years at the time of diagnosis. The American Diabetic Association (ADA) guidelines of 1997 and the World Health Organization (WHO) National diabetic group criteria of 2006 considers T2DM diagnosis when there is a single raised glucose reading with the following symptoms: polyuria, polydipsia,

polyphagia and weight loss; or raised values on two occasions of either fasting plasma glucose (FPG) (≥7.0 mmol/L (126 mg/dL)) or with an oral glucose tolerance test (OGTT) of two hours after the oral dose of plasma glucose (≥11.1 mmol/L (200 mg/dL). The glycated haemoglobin (HbA1c) and fructosamine are also useful tools in determining blood sugar control over time (WHO, 2006).

1.8.2 Random Blood Glucose Tests

When a person does not make insulin correctly, glucose remains in the blood. Hyperglycaemia occurs when glucose levels remain consistently high. Random glucose testing as one of the ways of checking the levels of glucose in the blood is carried out any time of the day. If the result indicates that a person has higher than expected glucose levels, a follow-up test to confirm the diagnosis is done, including either of the fasting blood glucose test (FBG), oral glucose tolerance test (OGTT) etc.

For a random glucose test, a result of 200 mg/dL (11.1 mmol/L) or above indicates diabetes and levels below are considered normal. However, for a more reliable diagnosis, a repeat test on another day is carried out together with FBG or OGTT.

1.8.3 Fasting Blood Glucose Test and T2DM

Fasting blood glucose (FBG) test is a tool used to diagnose diabetes and impaired fasting glucose (IFG). *In vivo*, FBG of less than 100 mg/dl is considered as normal (Hsueh *et al.,* 2015). According to the American Diabetes Association (ADA), fasting plasma glucose (FPG) of greater than >100 mg/dl (5.5 mmol/L) but less than <126 mg/dl (7.0 mmol/L) to be a diagnostic of impaired fasting glucose (IFG). Blood glucose at two hours post-glucose load

>140 mg/dl (7.8 mmol/L) but <200 mg/dl (11.1 mmol/L) on the 75g oral glucose tolerance test are diagnostic tools for impaired glucose tolerance (IGT), and fasting plasma glucose (FPG)
>140 mg/dl (7.8 mmol/L) diagnostic for diabetes (ADA, 2009).

1.8.4 Body weight and T2DM

When people who are inclined to diabetes to get the disease, have excess weight, the cells in their bodies are said to become less sensitive to the insulin released from their pancreas. Some research studies confirm that fat cells become more resistant to insulin, and individuals affected by excess weight, particularly obesity and severe obesity, are more likely to develop T2DM as a related condition of their excess weight (Yang *et al.,* 2016). There are many forms of measurements used to evaluate individual's excess weight; though, the most commonly and generally used method is calculating the body mass index (BMI).

There is approximately 90% of patients with T2DM who are overweight, and this influences the existing physiological and metabolic disorders associated with the disease negatively. In particular, hyperlipidaemia and hypertension which can greatly increase the risk of early death (Mulnier *et al.,* 2006). The development of obesity promotes insulin resistance and impaired glucose tolerance (IGT), which are major factors in the pathophysiology of T2DM (Mulnier *et al.,* 2006).

1.8.5 Insulin resistance evaluation and T2DM

Insulin resistance plays a crucial role in the pathogenesis of T2DM (Alberti *et al.,* 2009). Over

the years, there has been a number of methods to assess insulin resistance. The euglycemic– hyper-insulinemic clamp is the gold standard technique for evaluating insulin sensitivity in humans (Ohn *et al.,* 2016) but its complexity limits its use in the clinical practice. Therefore, various methods have been proposed as alternative measures of insulin resistance and among these, is the Homeostatic Model Assessment (HOMA) (Matthews *et al.,* 1985). The homeostasis model assessment of insulin resistance (HOMA-IR), provides a convenient and inexpensive means of estimating insulin resistance, and the estimates derived have been shown to correlate well with those derived from the euglycemic clamp. Evaluation of insulin resistance is useful in clinical practice, and even more importantly, in diabetes and metabolic research (Ohn *et al.,* 2016).

1.9 T2DM treatment

1.9.1 Conventional Medicine treatment

Blood glucose levels is the primary concern when treating diabetes and preventing further complications is also of absolute importance (Polonsky, 2012). T2DM treatments comprise of non-insulin oral antidiabetic medications, inclusive of sulfonylureas, thiazolidinediones, biguanides, and α-glucosidase inhibitors. This class of medication have the following distinctive mechanism of action:

1. Sulfonylureas increase insulin production by the pancreatic β-cells.
2. Pioglitazone (thiazolidinediones) increase insulin sensitivity in peripheral tissues, such as the muscles and fat cells. Their primary action is the nuclear regulation of genes involved in glucose & lipid metabolism and adipocyte differentiation.

3. Metformin (biguanide) increases the liver's insulin sensitivity and restores its glucose metabolism.
4. α-glucosidase inhibitors delay the absorption of carbohydrates from the gastro-intestinal tract.

1.9.2 Anti-diabetic medicinal plants as antidiabetic treatments

Over centuries, medicinal plants have served as major sources of medicine in the treatment and prevention of diseases inclusive of diabetes mellitus in many countries. About +200 species of plants globally present with hypoglycemic properties. To date, there are hundreds of medicinal plants reported to have been used for the treatment of diabetes mellitus (Choudhury *et al.*, 2017).

In South African, indigenous medicinal plants have retained remarkable attention in-terms of hypoglycemia activities e.g.: *Aspalathus linearis* (Rooibos), *Olea Africana* (Olea), *Centella asiatica* etc.

1.9.2.1 *Aspalathus linearis* (Rooibos)

Rooibos (*Aspalathus linearis*) (Brum f) Dahlg. (Family Fabaceae; Tribe: Crotolarieae) is a shrubby legume indigenous to the Clanwilliam mountain area in the Western Cape Province, South Africa (figure 2.6). Traditionally, rooibos is used to make herbal beverages free of caffeine, rich in polyphenolic antioxidants, and low tannin content. Rooibos is rich with exceptional antioxidants and medicinal properties which have gained attention worldwide.

1.9.2.1.1 Botanical description and distribution of *Aspalathus linearis*

Rooibos is an erect to spreading, highly variable shrub up to 2m high (figure 1.3 (a)). Its young branches are often reddish. The leaves are green and needle-like, 15-60 mm long and up to about 1mm thick (figure 1.3 (b)). They are without stalks and stipules and may be densely clustered. Rooibos has yellow flowers (figure 1.3 (c)), which appear in spring to early summer, are solitary or arranged in dense groups at the tips of branches. Its fruit is a small lance-shaped pod usually containing one or two hard seeds (figure 1.3 (d)) (Theresae, 2002).

Rooibos is naturally distributed in the winter rainfall area from about Vanrhynsdorp in the north to the Cape Peninsula and the Betty's Bay area in the south. The area experiences cold wet winters and hot dry summers with about 300-350 mm of rain per annum (figure 1.4). Generally, farmers plant seeds in February and March and transfer the seedlings to farmsteads. The shrub takes approximately 18 months to be ready for harvest (from December through to April). The rooibos shrubs can be harvested for a period of five years, pulled out and new plants planted for the next five-year period (Dahlgren, 1988).

Figure 1.3: *Aspalathus linearis* a) Rooibos shrubs, b) rooibos leaves, c) rooibos flowers (http://pza.sanbi.org).

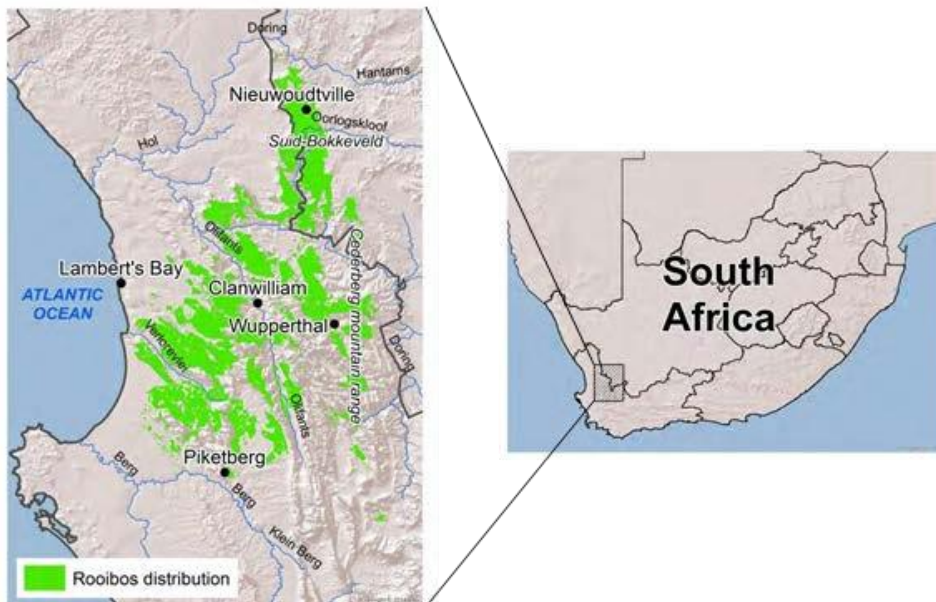

Figure 1.4: The map of the Cederberg region, showing the natural distribution of rooibos in the north western part of the Western Cape Province, South Africa (map adapted from Lötter, 2015).

1.9.2.1.2 Traditional and contemporary uses of Rooibos

Rooibos was first used by native South Africans; its leaves and fine stems were equally used for fermented and unfermented tea. Studies report that both fermented and unfermented rooibos tea have been used traditionally for calming digestive disorders, reducing nervous tension, alleviating allergies etc. (Morton, 1983).

One of the most famous breakthroughs of rooibos is the infusion which was administered to a colicky baby, which managed to cure the chronic restlessness, vomiting, and stomach cramps of the baby. This results lead to a broader consumer base, and since then many babies have been nurtured with rooibos, either added to their milk or given as a weak brew (Mahomoodally, 2013).

In the contemporary age, rooibos extracts usually combined with other ingredients are made available in pill form as dietary supplements. Research has emphasized the potential of aspalathin (aspalathin-enriched green rooibos extract) and selected rooibos extracts as antidiabetic agents (Mahomoodally, 2013). Today, rooibos amongst other medicinal plants, has been used to treat diabetes mellitus by traditional healers, and more scientific research on anti-diabetic effects of rooibos has been done on the latter basis.

1.9.2.1.3 Phytochemistry and pharmacological activities of Rooibos in T2DM

The phytochemistry of rooibos include its chemical constituents; phenolic compounds, flavonoids and non-flavonoids, polyphenols etc. There are about 85% main flavonoids found in unfermented aqueous rooibos extract called aspalathin. Rooibos known to be the only

known natural source of aspalathin and has the following pharmacological activities: anti- diabetic, anti-oxidation, hepatoprotective, anti-viral etc. Sae-Kwang et al. (2015) found Asp or Not to suppress the development of reactive oxygen species together with the activation of NF-κB. Thus, showing important of treating diabetic complications (Sae-Kwang *et al.,* 2015).

1.9.2.1.4 Antidiabetic effects of Rooibos: *in vivo* and *in vitro* Studies

Several studies show the effects of rooibos (*Aspalathus linearis*) and aspalathin (ASP) the main polyphenol in diabetics. To validate the latter, Kamakura et al., (2015) investigated the hypoglycaemic effects of green rooibos extract (GRE) on the protective ability of pancreatic β- cell from reactive oxygen species (ROS) in RIN-5F cells and on blood sugar uptake in L6 myotubes (Kamakura *et al.,* 2015).

The *in vivo* effect of GRE was also investigated in obese diabetic KK-A^y mice. GRE was found to improve the glucose uptake in the absence of insulin and has managed to induce phosphorylation of 5′-adenosine monophosphate-activated protein kinase (AMPK) in L6 myotubes shown for aspalathin. Aspalathin significantly suppresses fasting blood glucose (FBG) levels and improves glucose intolerance. Moreover, aspalathin decreases expression of hepatic genes related to gluconeogenesis and lipogenesis (Son *et al.,* 2013). These *in vitro* and *in vivo* results propose antidiabetic GRE effects via manifold modes of action and pathways (Kamakura *et al.*, 2015).

Najafian, et al. (2016), studied the inhibitory effects of aspalathin as a flavonoid on alpha amylase activity, on sugar levels and lipids in streptozotocin-induced diabetic rats and normal rats. In their results aspalathin was reported as a competitive inhibitor for alpha amylase with

Ki = 37.0 µM, and in both diabetic and normal rats and in all doses, aspalathin decreased blood glucose levels and reduced high urine volume and water intake. Aspalathin also indicated weight loss-inductive effects, and reduced food intake (Najafian *et al.,* 2016).

Marnewick et al. (2003) examined a range of indices for safety of both green and red rooibos following a 10-week *in vivo* study. Results indicate that neither green nor red rooibos tea adversely affected the liver and kidney parameters (Marnewick *et al.,* 2003).

1.9.2.2 *Olea africana* (Wild Olive)

Olea europaea subsp. *africana* (Family name: Oleaceae), the origin of the cultivated olive widespread in Europe, Asia, and Africa. There are many subspecies of Olea that are globally recognized, one of the subspecies is *Olea africana*. It is widespread in a variety of habitats, from forest and riverside bushes to mountain kloofs throughout South Africa (figure 1.6).

1.9.2.2.1 Botanical description and distribution of *Olea africana*

The olive tree (figure 2.9 (a)) is thick and short, of 10 m in height. The trunk has a diameter that is twisted and bent, with lots of reedy branch-lets and branches. The leaves are short-stalked, lanceolate, oval, narrow, leathery, etc. The color of these leaves is pale green and has a silver to white color below. The petiole is 5mm width, 4–10cm in length, 1–3cm wide, has 5–11 primary veins on each side of the midrib and has ad axially (figure 2.9 (b)) (Reipubl *et al.,* 1996). The flowers are bisexual, small, sub-sessile, off-white, and fuzzy (figure 1.5 (c)). The calyx is shortened/ truncate and corolla short with four lobes of 1-2 mm long. The olive fruit is small with its skin surrounding a shell of hardened kernel. The fruit is oval, black-violet when ripe,

normally 1–2.5 cm long, and small in wild plants (figure 1.5 (d)) (Reipubl *et al.,* 1996). The bark is pale grey in color (figure 1.5 (e)). The medicinal plant parts used in Olea are dried leaves, the fruit, sometimes the roots, the seeds, and stem bark (Parvaiz *et al.*, 2013).

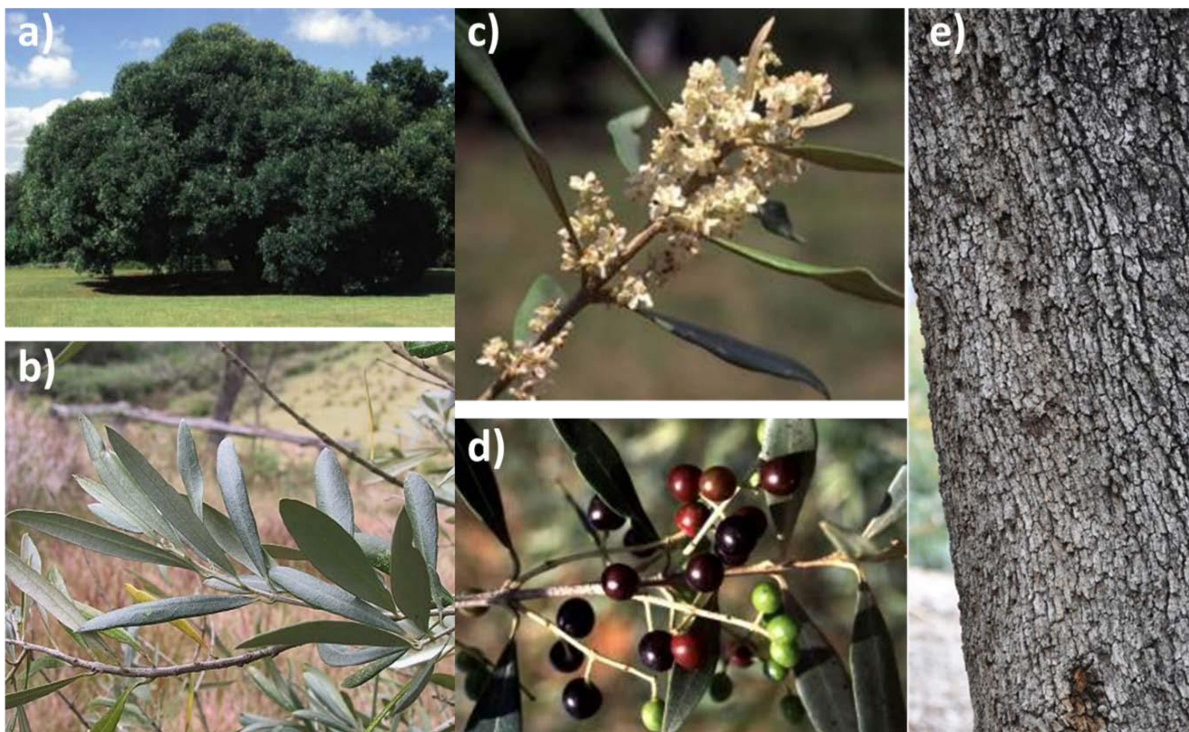

Figure 1.5: *Olea africana* a) Olive tree, b) *Olea africana* leaves, c) *Olea africana* flowers, d) *Olea africana* fruits, e) *Olea africana* bark (http://pza.sanbi.org).

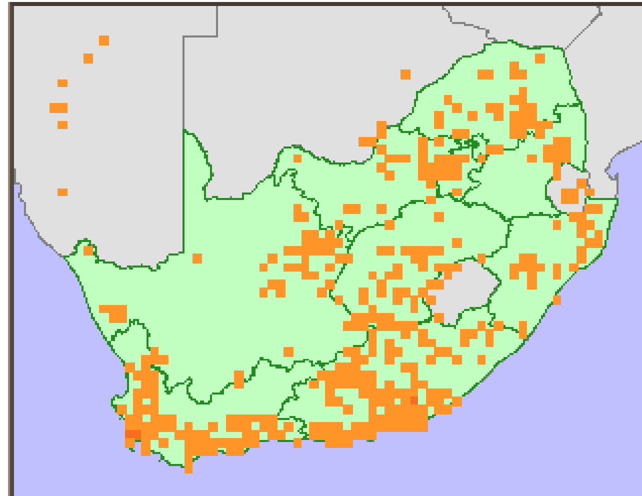

Figure 1.6: The map showing *Olea africana* widespread in a variety of habitats, from forest and riverside bush to open grass veld, stony flats, mountain kloofs and rocky ledges throughout Southern Africa and northwards through east Tropical Africa into Eritrea (Map adapted from http://pza.sanbi.org)

1.9.2.2.2 Traditional and contemporary use of *Olea africana* L.

Olea has folk and modern use in medicine. Olea is widely used in traditional medicine for various diseases in many different countries. The following plant parts are used for medicinal purposes: Olea's bark, wood, seeds, leaves, fruits, leaves etc. 1) Seed oil from Olives (fruit) is used as a laxative taken orally and can be applied externally for inflammation (Al-Khalil, 1995).

2) Decoctions of dried leaves and fruit can be used to treat infected urinary system, stomach and intestinal diseases (Ouarghidi *et al.*, 2013). 3) Olive oil can be used to treat dry scalp and stop hair loss. 4) Infusions from olive leaves can be taken orally to treat hypertension (Hashmi *et al.*, 2015).

5) In East-Africa the infusion of the bark of olive tree soaked for a night, is taken for tapeworm infestation (Kokwaro, 2009). 6) In Greece, hot infusion of olive leaves is taken orally to treat

high blood pressure and diabetes (Lawrendiadis, 1961). 7) In Italy, the essential oil from the olives is taken orally for the treatment of renal lithiasis, it can also be applied externally to promote circulation (De Feo *et al.,* 1996). 8) Fresh leaves infusion can also be taken to treat inflammation (Pieroni *et al.,* 1996). 9) In Italy as well, tincture made from olive leaves are taken orally as febrifuge (Rev *et al.*, 2007) and applied externally to restore the epithelium (De Natale *et al.,* 2009). 10) In Japan olive leave infusion is used for stomach and intestinal diseases and the essential oil for liver pains (Bellakhdar *et al.*, 1991).

1.9.2.2.3 Phytochemistry and pharmacological activities of *Olea Africana* in T2DM

Active ingredients found in leaves are secoiridoids, triterpenoids (oleanolic acid, ursolic acid, and uvaol) and the lignans isolated from the bark. The research studies done on *Olea* have found out many other ingredients of flavonoids, flavone glycosides, flavanones, iridoids, iridane glycosides, secoiridoids, secoiridoid glycosides, triterpenes, biophenols etc. (Obied, 2013). The main active compounds found in the leaves are two secoiridoids, oleuropein and oleacein with the following pharmacological activities; 1. Antihyperglycemic activity, 2. Antimicrobial activity, 3. Antihypertensive and antiarrhythmic activity, 4. Anti-inflammatory.

1.9.2.2.4 Anti-diabetic activities: *in vivo* and *in vitro* study of *Olea africana* leaf extracts

Omar (2010) study shows positive hypoglycemic effects of 500mg/kg per day of Olive leaf extracts administered intragastrical (IG) to both normal and alloxan-induced diabetic male rats. The aqueous decoctions of 32.0mg/kg Olea leaves, administered intragastrical to both normal and the alloxan diabetic rats presented with positive hypoglycemic activities (Omar, 2010).

Al-Azzawie and Alhamdani (2006), in their study on diabetic rabbits were treated with oleuropein (20 mg/Kg body weight) for up to 16 weeks. After treatment, the blood glucose levels along with antioxidants were restored to values near normal in control rabbits. The study proved oleuropein as an anti-hyperglycaemic and anti-oxidative agent (Al-Azzawie *et al.,* 2006).

A research study was carried out to test the anti-diabetic effects of olive leaf extracts in both the diabetic and normal rats. To induce diabetes, the rats were injected with Streptozotocin. Following inducing diabetes with the rats, an aqueous olive leaf extract was orally administered at different doses of 100, 250, and 500 mg/kg per body weight. Moreover, the reference drug, glibenclamide, was given for 14 days at a dose of 600 μg/kg. The results therefore showed the anti-diabetic effects of the aqueous Olive leaf extract as more effective when compared to the glibenclamide. Moreover, the olive leaf extract, lowered the serum glucose levels, the TC levels, the TG levels, and also managed to increase serum insulin levels in both the normal rats and the rats induced with diabetes (Cruz-Vega *et al.*, 2009).

Therefore, these studies confirm that *Olea europaea subsp. africana* has anti-diabetic effects and can be used in the treatment of diabetes mellitus.

1.9.2.3 *Centella asciatica* (Centella/ Gotu kola)

Centella asiatica (L.) is a medicinal plant from *Umbellifere* (Apiaceae) family indigenous to the Southeast Asian countries and Southern Africa (figure 1.8) (Orhan, 2012; Vaidya, 1997). *Centella asiatica*, has also been used for medicinal importance in other countries with a long

history of application in Chinese traditional medicines and ayurvedic medicine (Orhan, 2012; Dahanukar *et al.,* 2000).

1.9.2.3.1 Botanical description of *Centella asiatica*

Centella asiatica (CA), a clonal, perennial herbaceous creeper is found growing throughout regions of moist places of up to an altitude 1800m. There are about 20 species related to Centella that grow in most parts of the tropic or wet pantropical areas such as rice paddies, and rocky, higher elevations (Bown, 1995). Centella is a tasteless, odourless plant that thrives in and around water. It has small fan-shaped green leaves which are edible, they are sometimes yellowish-green in colour, thin, others have long petioles, and typically reniform, orbicular in shape with several veins (Stafford *et al.*, 2008) (figure 1.7 (a)). Centella has white or light purple-to-pink or white flowers (figure 1.7 (b)) and bears small oval fruit. Mostly, the whole plant is useful for medicinal purposes (Singh *et al.,* 2002). The plant grows flat through its green to red stolon’s and syndicate through roots underground (figure 1.7 (c)) (Orhan, 2012; Bown, 1995).

Figure 1.7: *Centella asciatica*, a) Centella leaves, b) Centella flowers, c) plant growing horizontally and roots underground (https://www.sanbi.org).

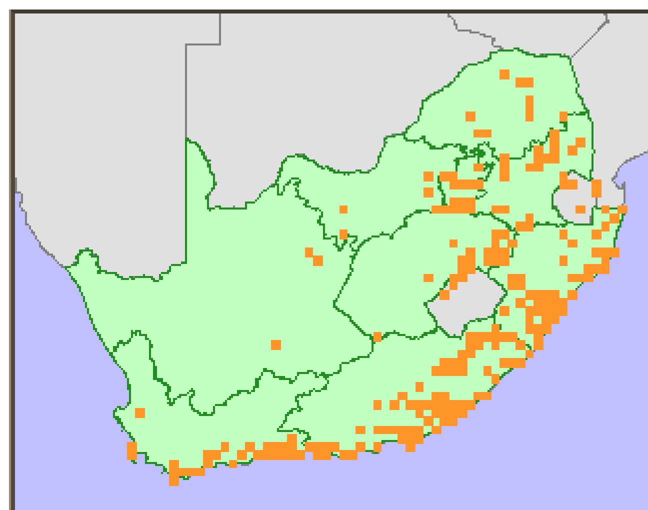

Figure 1.8: The distribution map of *Centella asiatica* in South Africa throughout the wetlands (map adapted from http://redlist.sanbi.org)

1.9.2.3.2 Traditional and contemporary use of *Centella asiatica*

Centella asiatica is a medicinal plant traditionally known to re-vitalize the brain and nervous system, to increase attention span and concentration and combat aging. The effect of an aqueous Centella extracts (100, 200 and 300 mg/kg for 21 days) was evaluated in intracerebroventricular (ICV) streptozotocin (STZ)-induced cognitive impairment and oxidative stress in rats. The rats treated with Centella showed a dose-dependent increase in cognitive behaviour, in passive avoidance, and elevated plus-maze paradigms (Kumar *et al.,* 2002).

Centella asciatica, has also been used traditionally to treat T2DM for decades. Previous studies show that Centella exhibits anti-diabetic and anti-oxidative functions in experimental diabetic rats induced by a high fat diet and Streptozotocin (Chauhan *et al.,* 2010). Investigations on the therapeutic effects of Centella on T2DM KKAy mice and the possible associations with skeletal muscle, results showed Centella's ability to significantly reduce food intake and body weight in T2DM KKAy mice. Treatment with Centella decreases the blood glucose and HbA1c levels and also increases the insulin sensitivity. Moreover, Centella ameliorated hyperlipidaemia and induced a lower free fatty acid level, displaying an effect on disorders of lipid metabolism. Centella significantly increased the expression of insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase (PI3K), protein kinase B (PKB/Akt) and glucose transporter 4 (Glut4) and decreased the expression of glycogen synthase kinase 3β (GSK3β). Therefore, these studies emphasised *Centella asiatica's* effective anti-diabetic properties by regulating the PI3K/Akt signalling pathway in skeletal muscle (Gohil *et al.,* 2010).

In summary, folk medicinal effects of Centella have indicated different biological activities

anticipated for human health such as revitalizing the brain and the nervous system, treating diabetes, Alzheimer's disease, rheumatoid arthritis etc. (Sharma *et al.,* 2016)

1.9.2.3.3 Phytochemistry and pharmacological activities of *Centella asiatica* in T2DM

Centella asiatica is a medicinal plant with primary active constituents called saponins (also named triterpenoids), this include asiaticosides, where a trisaccharide moiety is linked to the aglycone asiatic acid, madecassoside and madasiatic acid, and ursolic acid and rosmarinic acid etc. (Singh *et al.,* 1969). These triterpene saponins and sapogenins are responsible for a range of health benefits including anti-hyperglycaemic effects anti-inflammatory effects, the wound healing and vascular effects which inhibit the production of collagen at the wound site, and for treatment of rheumatoid arthritis etc. (Kumar *et al.,* 2002).

1.9.2.3.4 Anti-diabetic activities of Centella asiatica: *in vivo* and *in vitro* studies

A study designed to evaluate the glucose and cholesterol lowering effects of the *Centella asiatica* leaf aqueous extract, using the alloxan-induced diabetic rats and compared the activity with diabetic control and antidiabetic drug (Glibenclamide) was performed by Emran et. al., (2015). The leaf extract of 50 mg/kg Centella and Glibenclamide were administered to normal and experimental diabetic rats for the duration of ten days. In the alloxan-induced diabetic rat group, Centella extract 50 mg/kg significantly ($p < 0.05$) lowered the fasting blood glucose levels as well as the total cholesterol levels. Serum insulin levels were not stimulated in the animals treated with the extract. Moreover, changes in body weight, serum lipid profiles and liver glycogen levels assessed in the Centella extract that treated the diabetic rats were

compared with diabetic control and normal rats and significant results ($p < 0.05$) were observed (Emran, 2015).

Chauhan, Pandey and Kumar Dhatwalia (2010) carried a study on the ethanolic and methanolic extracts of the *Centella asiatica* leaves. The anti-diabetic activity by glucose tolerance test in normal rats and alloxan induced diabetic rats were tested. Ethanolic and methanolic extracts of Centella in the results showed significant protection and lowered the blood glucose levels to normal in glucose tolerance test. In alloxan induced diabetic rats the maximum reduction in blood glucose was observed after three hours at a dose level of 250 mg/kg of body weight. The percentage protections by ethanolic and methanolic extracts were 30% and 48% respectively. In long term treatment of alloxan induced diabetic rats, the degree of protection was determined by measuring blood glucose, triglycerides, cholesterol and urea levels on five different days. Both the extracts ethanolic and methanolic showed a significant anti-diabetic activity comparable with that of Glibenclamide (Chauhan *et al.,* 2010; Sasikala *et al.,* 2015)

Centella leaves were investigated for their anti-diabetic properties in a study by Sasikala et al. (2015). Rats were randomly divided into six groups containing six rats in each group, two groups served as positive–normal control and negative-diabetic control, and the other four groups were diabetic rats treated with the methanol extract of *Centella asiatica* leaves at 100, 200, 300 and 400 mg /kg body weight respectively. The extract dose of 300 mg/kg body weight and 400mg/kg body weight caused a substantial decrease in blood glucose levels (Sasikala *et al.*, 2015).

1.10 Aims

The aim of this study is to investigate the hypoglycemic effects and safety of JT2016 (herbal compound) in HFD-STZ induced diabetic SD rats.

1.11 Objectives

- Modelling T2DM using HFD-STZ induced diabetic rats
- Investigating anti-diabetic effects of JT2016 in HFD-STZ induced diabetic rats
- Investigating the safety of JT2016 in T2DM rats

1.12 Hypothesis

JT2016 is an effective and safe anti-diabetes herbal compound, that can be used for the treatment and management ofT2DM.

CHAPTER 2

2. MATERIAL AND METHODS

2.1 Ethical Clearance

The experimental protocols were approved by the Animal Care and Ethics Committee of Beijing University of Chinese Medicine (Beijing, China). Is there an approval ethics number? Qoute it

2.2 Chemicals and reagents

1. Lyophilized powder of the recombinant mouse IL-6 was purchased from Chengdu PuRuiFa Technology Development Co. Ltd (Chengdu, China).
2. Pioglitazone hydrochloride batch no. Zhunzi H2004066 was bought from Jiangsu Hengrui Medicine Co. Ltd. (Jiangsu, China).
3. The high-fat diet (HFD) which contains 2.5% cholesterol, 20% sucrose, 10% lard, 0.3% sodium cholic acid, and the 66.5% (w/w) in standard/ normal diet was provided by Ke'ao xieli feed Co., Ltd. (Beijing, China).
4. Streptozotocin (STZ, Cat number S0-130) was dissolved into 0.1 mol/L sodium citrate-hydrochloric acid buffer when used and was purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, USA).

5. Insulin ELISA assay kits were bought from Beijing North Biotechnology Research Institute (Beijing, China).

6. An immune-histochemical kit and insulin receptor (InsR) antibody were bought from Boosen Biological Technology Co. Ltd. (Beijing, China).

7. Blood glucose kit and triglyceride (TG) kit were bought from Beijing Leadman Biochemical Co., Ltd. (Beijing, China).

8. The total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) etc. assay kits were obtained from Beijing North Biotechnology Research Institute (Beijing, China).

2.3 Collection and preparation of plant samples of JT2016

Jiang Tang 2016 (JT2016) is an antidiabetic herbal compound/ polyherbal formulation made of three Southern African and Chinese indigenous medicinal plants; *Olea africana* (Olea) (figure 2.1), *Aspalathus linearis* (Rooibos) (figure 2.2), and *Centella asiatic* (Centella) (figure 2.3). The medicinal plants were collected and prepared by different groups: Olea leaves were collected by the author from the olive trees at the Nature Reserve University of the Western Cape, Robert Sobukwe Road, Bellville, Cape Town, 7535, in autumn/ winter (during harvest and pruning season) and were identified by supervisor Dr Xuesheng Ma (Chinese Medicine Doctor and UWC Senior Lecturer, Cape Town, South Africa) and Dr Caren Hauptfleisch (Phytotherapist, Somerset West, Cape Town, South Africa). The leaves were washed using normal water from the tap and were then washed with distilled water,

subsequently air-dried the leaves in a cool dry area. When dry, they were finely grinded, weighed and kept in a closed dry glass bottle in a dark cool area. The green rooibos loose tealeaves and Centella leaves were readily purchased from different companies. *Aspalathus linearis* was purchased from the Rooibos Ltd. in Clanwilliam, Western Cape, South Africa, and *Centella asiatica* was purchased from Xi'an Rainbow Bio-Tech Co., Ltd. Shaanxi, China.

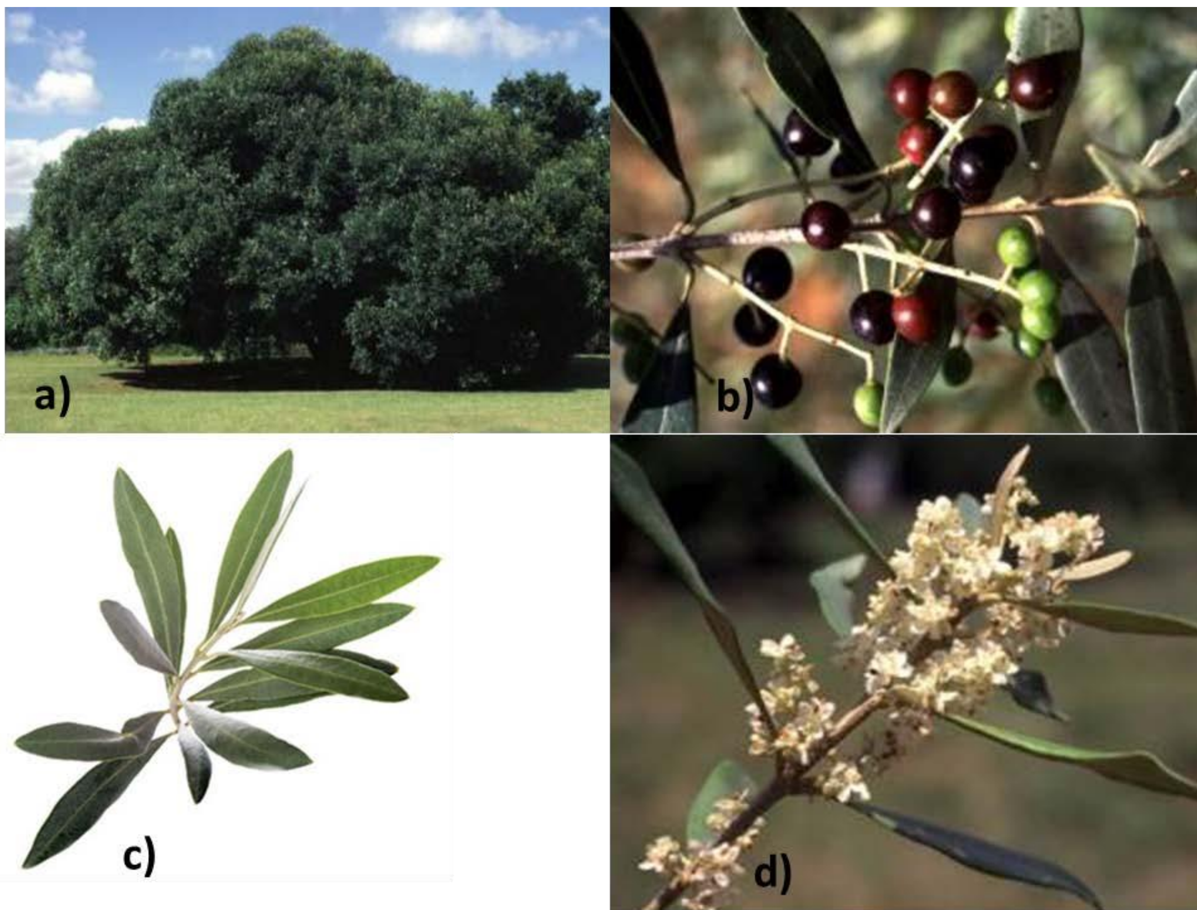

Figure: 2.1: a) *Olea europaea* subsp. *africana* tree (a neatly shaped evergreen tree with a dense spreading crown (9 x 12 m) of glossy grey-green to dark-green foliage), **b)** *Olea africana* fruits (Olea

fruits are small, spherical, thinly fleshy fruits (either sweet or sour) which ripen purple-black in March to July), **c)** *Olea africana* leaves (Olea leaves are grey-green to dark-green above and greyish below, the rough, grey bark sometimes peels off in strips), and **d)** *Olea africana* flowers (Olea sprays of tiny, lightly scented white to greenish flowers in October to February). Pictures were taken from the South African National Biodiversity Institute (SANBI) webpage http://pza.sanbi.org/olea-europaea-subsp-africana.

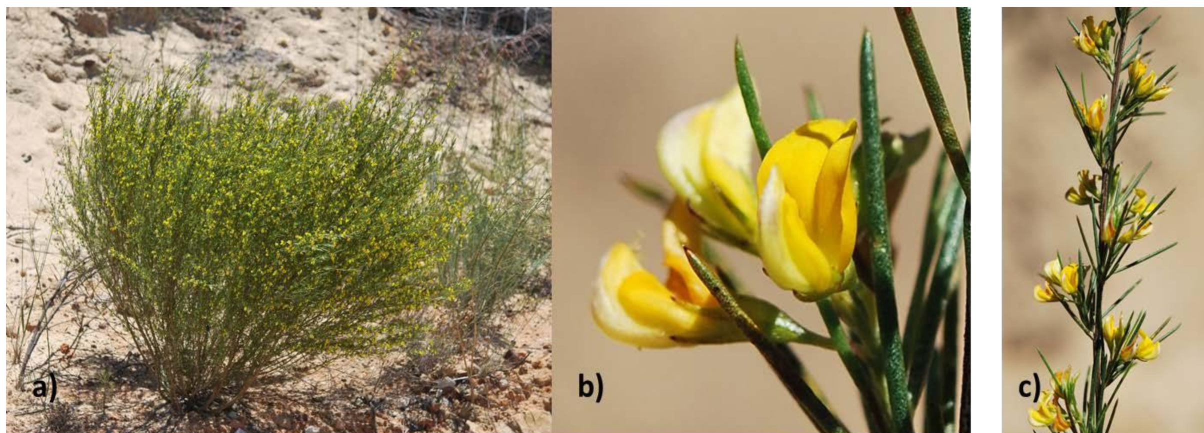

Figure 2.2: a) *Aspalathus linearis* plant (*Aspalathus linearis* is an erect to spreading, highly variable shrub up to two-meter-high and its young branches are often reddish), **b)** *Aspalathus linearis* flowers and fruit (Rooibos has yellow flowers, which appear in spring to early summer, are solitary or arranged in dense groups at the tips of branches. Their fruit is a small lance-shaped pod usually containing one or two hard seeds), **c)** *Aspalathus linearis* leaves (The rooibos leaves are green and needle-like, 15-60 mm long and up to about 1 mm thick. They are without stalks and stipules and may be densely clustered).

The pictures were taken from the Southern Africa iSpot nature webpage: https://www.ispotnature.org/communities/southernafrica/view/observation/374021/aspalathus-linearis .

Figure: 2.3: a) *Centella asiatica* leaves (Centella has long-stalked, green, reniform leaves with rounded apices which have smooth texture with palmate netted veins), **b)** *Centella asiatica* fruits (The fruits are smooth, warty and ribbed), **d)** *Centella asiatica* flowers (The flowers are white or pinkish to red in colour, born in small, rounded bunches (umbels) near the surface of the soil. Each flower is partly enclosed in two green bracts). The pictures were taken from the Southern Africa iSpot nature webpage:

https://www.ispotnature.org/communities/southern-africa/species-dictionary/25437/centella-asiatica

2.4 Extract and Compound preparation

The aqueous extracts of Olive leaves, Rooibos, and Centella leaves were prepared following the conventional protocols (Azwanida, 2015). For each plant (finely grinded leaves) (separately), 100g plant material were extracted with 1000ml boiling water using a Soxhlet apparatus (figure 2.4) at 75°C. Thereafter, the solution filtered through 125µm mesh cloth supplied by Tianjin Yabao pharmacy Co., Ltd. (Tianjin, China), followed by suction filtration (figure 2.5) using Whatman No.4 filter paper supplied by Tianjin Yabao pharmacy Co., Ltd. (Tianjin, China). The filtrate was then heavily concentrated using the vacuum rotary evaporator (Xingyang Kori Instrument Factory, Henan, China (Mainland)) (figure 2.6) with the vacuum maintained at 170 mbar, the temperature of the water bath kept at 60°C and solvent evaporated at 15^{0}C. The latter processes' outcome produced dense aqueous extracts, which were stored in sterile bottles in the fridge at 4°C. When the extraction process was complete, three different compounds were formulated with the following medicinal plants; Olea *africana*: *Aspalathus linearis*: *Centella asiatica* at different concentrations per compound (JT2016A, JT2016B, JT2016C) (Table 2.1).

2.4.1 JT2016 Compound Preparation and Ratio Calculations

The Indigenous Knowledge (IK) holders prescribe the dosage of leaves for the infusion/ concoction by measuring these herbs using a handful per day. Based on this information, the medicinal plants used in JT2016 have been previously studied both *in vivo* and *in vitro* for the

treatment of diabetes mellitus (Anoja *et al.,* 2013). Therefore, with both IK and animal equivalent dose (AED) calculations of previous published research papers have been used to build up the ratios used in JT2016. *Aspalathus linearis* (Muller *et al.*, 2012), *Olea africana* (Abunab *et al.*, 2017), and *Centella asiatica* (Kabir *et al.*, 2014; Emran *et al.*, 2015). In table 2.1, JT2016 herbal compound concentrations of the medicinal plants per 100ml are shown.

Table 2.1: JT2016 Compound herb concentrations per 100ml

JT2016 Compound Group	**Olea(g)**	**Rooibos (g)**	**Centella (g)**
JT2016 Compound A	30	30	15
JT2016 Compound B	30	15	15
JT2016 Compound C	15	30	30

Figure: 2.4: A Soxhlet apparatus that was used for solid-liquid extraction of the medicinal plants, Olea, Rooibos and Centella.

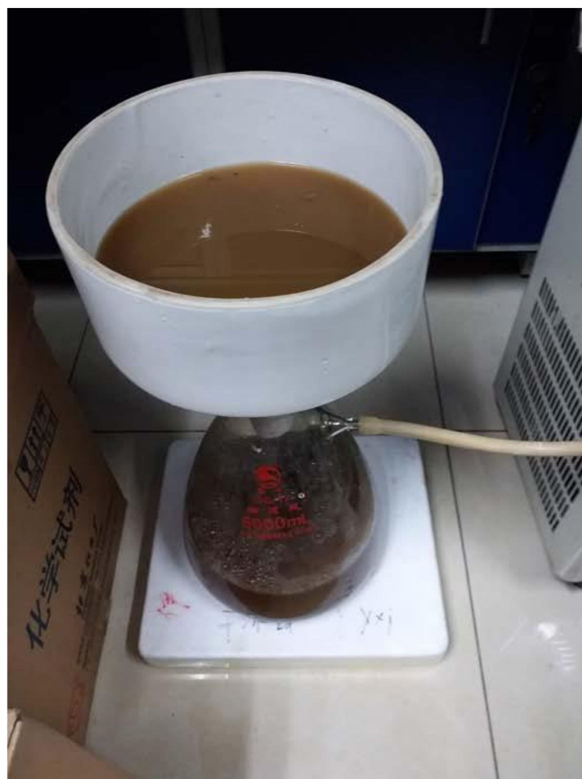

Figure 2.5: Suction filter was used to filter the solutions using the Whatman No.4 filter paper.

Figure 2.6: Vacuum rotatory evaporator, which was used to concentrate the filtered solutions of medicinal plants.

2.5 Animals

A total of 60 male normal Sprague Dawley (SD) rats (210-250g) (seven weeks old) (figure 3.7), were included in the study and were provided by the Model Animal Research Centre of Nanjing University. All animals were housed at the Institute of Basic Theory of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences animal house at an ambient temperature of +/- 23 ℃ and humidity of +/- 55% with a 12-hour light/dark cycle (figure 2.8). The animals were provided with food and water ad libitum, and according to the study design (figure 2.10). The rats were randomly divided into two main groups after two weeks of adaptive feeding: the normal group fed with Normal Pellet Diet (NPD) (n=10), and the experimental group fed with High Fat Diet (HFD) (n=50) (figure 2.9). After two weeks of dietary manipulation, HFD-fed SD rats exhibited significant increase in basal plasma glucose, plasma insulin as compared to NPD-fed rats. HFD-fed rats were then injected intraperitoneally with low dose streptozotocin (STZ) (35mg/kg) suspended in 0.1 mol/L citrate buffer at pH 4.5. Three days later, the animals were fasted, blood taken from tail vein of the rats, and then tested for high blood sugar levels using the commercially bought glucometer; every rat tested positive for hyperglycaemia (+16 mmol/l) was included in the study and randomly assigned into the following five groups: control group, positive drug group (pioglitazone 3 mg/kg), JT2016 compound A (JT2016A), JT2016 compound B (JT2016B), and JT2016 compound C (JT2016C). All medicine was administered intragastrically via gavage: 2ml/kg of all JT2016 compounds were administered daily (Table 2.2; figure 2.10). Body weight and random blood glucose (RBG) levels were measured weekly for the period of six weeks of treatment. After six weeks of treatment, rats were fasted overnight, weight, anaesthetised and blood drawn from the abdominal aorta and tested for the following; fasting blood glucose,

plasma insulin, the levels of inflammatory cytokines (TNF-α, IL-6), the lipid profile (TC, TG, HDLC, LDLC) and free fatty acids (FFA), liver function tests (ALT, AST), and proteins (albumin, globulin, total protein) and executed through euthanasia.

Table 2.2: Administration of drug in the animal groups

GROUP	ANIMAL	DRUG ADMIN/ DAY	DIET	DURATION
1. Normal	Normal SD rats	Saline 2ml/ kg	Normal pellet diet (NPD)	Six (6) weeks
2. Control	STZ induced diabetic SD rats	Saline 2ml/ kg	High fat Diet (HFD)	Six (6) weeks
3. Pioglitazone	STZ induced diabetic SD rats	Pioglitazone 3mg/ kg	High fat Diet (HFD)	Six (6) weeks
4. JT2016A	STZ induced diabetic SD rats	JT2016A 2ml/kg	High fat Diet (HFD)	Six (6) weeks
5. JT2016B	STZ induced diabetic SD rats	JT2016B 2ml/ kg	High fat Diet (HFD)	Six (6) weeks
6. JT2016C	STZ induced diabetic SD rats	JT2016C 2ml/ kg	High fat Diet (HFD)	Six (6) weeks

Figure 2.7: The seven-week old male normal Sprague Dawley (SD) rats, weight 210-250g.

Figure 2.8: The 12-hour light/dark cycle animal house where the rats were kept. **a)** Light room representing day light for the animals, b) dark room representing night for the animals.

Figure 2.9: The animal food, **c)** high fat diet and **d)** Normal pellet diet.

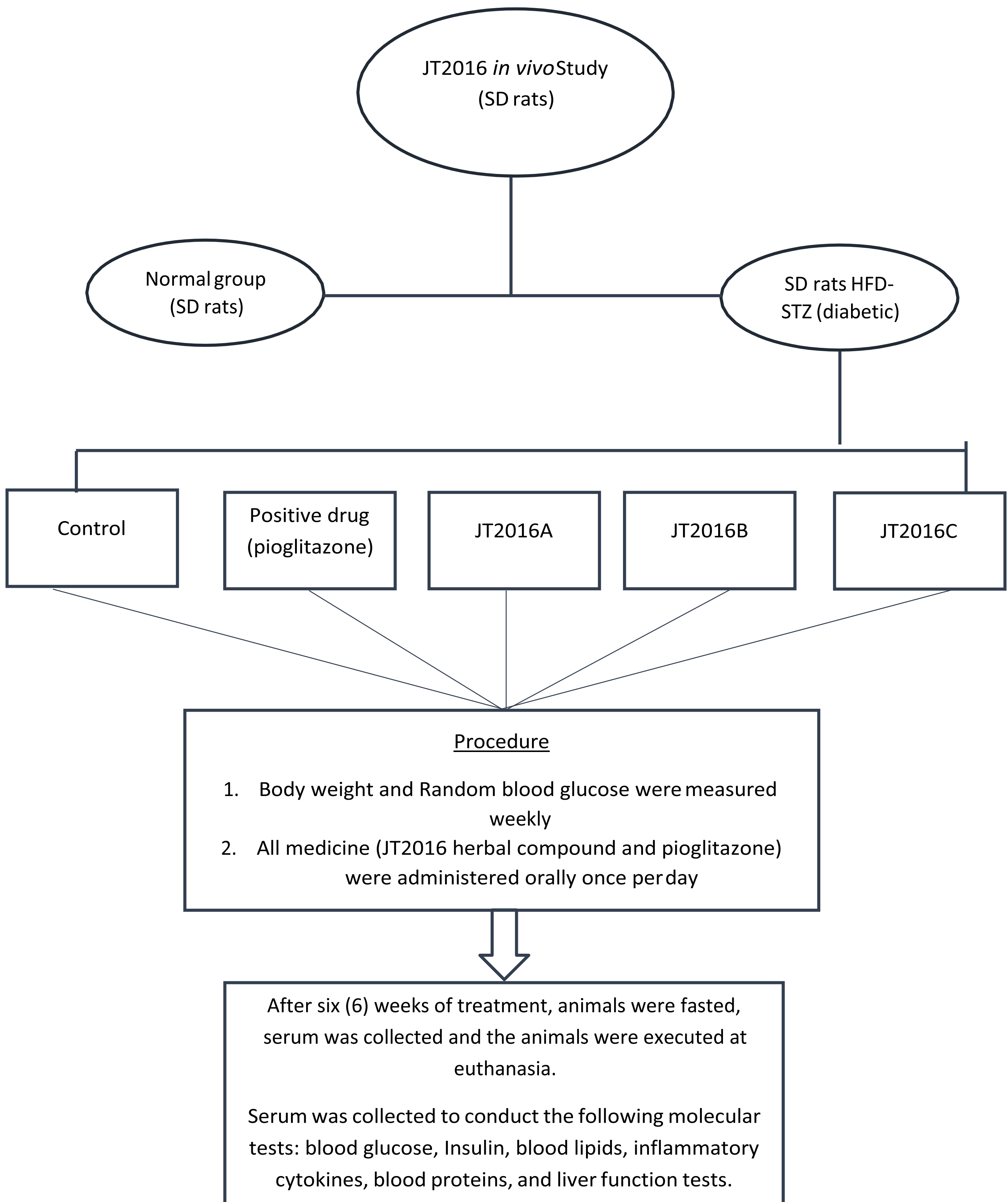

Figure 2.10: Study design for investigation of hypoglycemic effects and safety of JT2016 *in vivo*

2.6 Biochemical Analysis

The animals were fasted overnight and were anaesthetized with sodium pentobarbital (50 mg/kg i.p.). Serum was collected from the abdominal aorta into the specimen collection tubes (serum separator tubes (SST)); Serum samples were prepared by centrifuging the collected blood for 15 minutes at Relative Centrifugal Force (RCF) of 1200 x g.

2.6.1 Blood Glucose

2.6.1.1 Random blood glucose

Weekly, animals were randomly tested for blood glucose levels on day 0, 7, 14, 21, and 28. Random blood glucose (RBG) levels were measured in tail-vein blood ≈0.1 ml each time using a glucose meter kit, and body weight was measured concurrently using an animal weighing scale. Throughout the treatment period, blood glucose levels measured were obtained from blood drawn from the tail-vein using a glucose meter kit bought at Shanghai Dynamic Medical Supplies Co., Ltd. (Shanghai, China).

2.6.1.2 Fasting blood glucose

The animals were fasted for eight hours at the end of the six weeks of treatment. Animals were weight, ≈0.1 ml blood was collected from the tail vein and fasting plasma glucose (FBG) was measured using the commercial glucose meter kit bought at Shanghai Dynamic Medical Supplies Co., Ltd. (Shanghai, China).

2.6.2 Insulin and Insulin resistance

Plasma insulin levels were quantified in duplicate using commercial ELISA kits from Beijing LEYBOLD Cable Technology Co. Ltd. according to the manufacturer's instructions. Standards at a series of concentrations were run in parallel with the samples (Wallace *et al.,* 2004).

2.6.2.1 Determination of insulin resistance (IR)

The insulin resistance was determined using the Homeostatic model assessment (HOMA). The approximating equation for insulin resistance in the model, used a fasting plasma sample and was derived by the use of the insulin-glucose product divided by a constant (Wallace *et al.,* 2004).

Homeostasis model assessment - Estimated insulin resistance (HOMA-IR) was calculated using the following formula (Matthews *et al.,* 1985):

HOMA-IR index = glucose level * serum insulin/ 22.5

2.6.3 Lipid Profile Studies and free fatty acids

The following lipid parameters were measured after six weeks of treatment: total cholesterol, high density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and free fatty acids (FFA). The lipid profile was assessed using a portable Cardiochek™ blood test system. The blood collected was deposited on the lipid panel sensor that was fitted in the Cardiochek ™. Serum cholesterol was estimated according to the COD - PAP method described by Trinder (1969), serum triglycerides were estimated according to the GOP – PAP method described by Lothar (1998). HDL-Cholesterol was estimated by direct

hydrogen peroxide scavenging method and LDL-Cholesterol was estimated according to the direct surfactant removal method described by Jacobs et al. (1990) (Kannan *et al.,* 2014).

Plasma non-esterified fatty acids concentrations were measured in triplicate with a commercial kit, this assay employs fatty acid coated on a 96-well plate with 50 µl standards and 50 µl serum samples pipetted into the wells. Into each well, 2 µl ACR reagents were added and incubated at 37°C for 30 minutes. The 50 µl reaction mix was then added into plates, incubated at 37°C for 30 minutes and the intensity of the color measured at 570 nm. FFA content was determined by the ELISA kit.

2.6.4 Liver function tests

Serum alanine transaminase (ALT) activity was determined according to Reitman and Frankel (1957). Principally, 15µl of standard solution (containing 0.22mg/ml sodium pyruvate, alanine transaminase substrate (0.89g alanine and 0.015g of α-ketoglutarate in 50ml phosphate buffer)) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5µl alanine transaminase substrate for blank and serum were placed in a 96 well plate and incubated at 37^0C for 5 minutes. After which, 2.5µl of serum was added into the appropriate wells and incubated at 37^0C for 30 minutes, followed by 25µl of 2, 4-dinitrophenylhydrazine (0.2%) in 37% HCl and dH_2O added to each well, mixed and incubated at RT for 20 minutes. Into the wells, 250µl NaOH (0.4M) were added, mixed and incubated at RT for 30 minutes. The absorbance readings were taken at 492nm. The activity of ALT in serum was expressed in U/L (figure 2.16).

2.6.4.2 Aspartate aminotransferase (AST) test

Serum aspartate transaminase (AST) activity was determined according to Reitman and Frankel (1957). Principally, 15µl of standard solution (containing 0.22mg/ml sodium pyruvate, aspartate transaminase substrate (0.015g α-ketoglutarate, 1.33g aspartic acid, 12.5ml 1N NaOH, dissolved and adjusted to pH 7.4 with 8.5% phosphoric acid)) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5µl aspartate transaminase substrate for blank and serum were placed in a 96 well plate. After five minutes of incubation at 37^0C 2.5µl of serum was added into the appropriate wells and incubated again at 37^0C for one hour. Thereafter, 25µl of 2, 4dinitrophenylhydrazine (0.2%) in 37% HCl and dH_2O, was added to each well, mixed and incubated at RT for 20 minutes. Into the wells, 250µl NaOH (0.4M) was added, mixed and incubated at RT for 30 minutes and absorbance readings were taken at 492nm. Activity of AST in serum and the results obtained were expressed in U/L (figure 2.17).

2.6.4.3 Total Serum Protein

The total amount of serum protein is measured by the total amount of albumin and globulin in the blood. The total protein test checks the ratio of albumin (A) to globulin (G) in blood, known as the “A/G ratio”. In this study, total serum protein was measured by using commercially available kits (Beijing LEYBOLD Cable Technology Co. Ltd. (Beijing, China)) based on biuret method. The serum albumin was measured by the use of commercially available kit, based on bromocresol green method.

To determine the albumin/globulins (A/G) ratio, total protein and albumin, and globulins are calculated by subtracting the albumin from the total protein.

2.6.5 Anti-inflammatory cytokines test

Anti-inflammatory cytokines are a series of immune-regulatory molecules that regulate the pro-inflammatory cytokine response. Cytokines act in line with specific cytokine inhibitors and soluble cytokine receptors to control and regulate the human immune system response. In this study only tumor necrosis factor-alpha (TNFα) and interleukin 6 (IL-6) were measured.

2.6.5.1 Tumor necrosis factor-alpha (TNFα)

Plasma TNF-α was quantified in duplicate using a commercial ELISA kit (Pierce Endogen, Rockford, IL). To the anti-TNF-α antibody-coated 96-well plate, 50µL of samples or standards were added, followed by one-hour incubation at room temperature. Subsequent to three washes with wash buffer, 50µL of a biotinylated antibody reagent was introduced and the plate was incubated for one hour at room temperature. A second wash cycle was performed followed by addition of 100µL of substrate TMB (3, 3′, 5, 5′- tetramethylbenzidine) and a 30- minutes incubation in a dark room. The reaction was terminated by adding 100µL of stop solution, and the absorbance was read at 450 nm (Kamiya Biomedical Company, Seattle, WA) following the kit instructions.

2.6.5.2 Interleukin 6 (IL-6)

Recombinant IL-6 appropriate for mouse cell culture experiments was obtained from Chengdu PuRuiFa Technology Development Co. Ltd (Chengdu, China) as a lyophilized powder. As per manufacturer instructions, 5µg IL-6 were reconstituted by adding 50µl distilled and sterilized water to achieve a 100 µg/ml solution. This solution was further diluted at 1:10 with culture medium in order to achieve a 10 µg/ml IL-6 stock solution. Aliquots of this stock solution were

frozen at -20ºC until required for experiments. For each experiment, four concentrations of IL- 6 were used: 100 ng/ml, 10 ng/ml, 1 ng/ml, and 0.1 ng/ml. From 10 µg/ml stock solution, a dilution of 1:10 was made with prepared 25mIU/L hCG medium to achieve 1000ng/ml. Further serial dilutions of 1:10 with hCG medium was done to achieve 100, 10, 1 and 0.1ng/ml concentrations for the experiments. After preparation of various IL-6 concentrations in hCG medium, prepared cell culture experimental plates were visually inspected. Medium was removedin sterile conditions, and the four concentrations were added at 300 µl to each well as appropriate, in addition to 300 µl of positive and negative controls. An exposure of 48 hours was allowed before termination of the experiment.

2.7 Statistical Analysis

Statistical significance was calculated using one-way analysis of Variance (ANOVA), Post Hoc tests (Tukey's and LSD) (multiple comparisons) by SPSS Statistics software version 25 (SPSS Inc., Chicago, USA), and GraphPad Prism version 8.4.2 (GraphPad Software Inc., San Diego, CA, USA). All results were expressed as the mean, the standard deviations of mean (SD). Results were considered as significant if $p < 0.05$ and n = 10 for the graphs.

CHAPTER 3

3. RESULTS

3.1 Effects of JT2016 compound on random blood glucose and fasting blood glucose levels

3.1.1 Effects of JT2016 compound on random blood glucose

To study the hypoglycemic effects of JT2016 herbal compound, spontaneous designed HFD- STZ-induced diabetic SD rats were used for six (6) weeks. Figure 3.1 displays the effect of JT2016 on RBG levels over five (5) weeks. When treatment commenced, the RBG levels in the control group (HFD-STZ induced diabetic SD rats) (20.46 ±3.670 mmol/L) were significantly (p
= 0.000) different when compared to the normal group (6.433 ±0.507 mmol/L) (figure 3.1.1). The control group increased in the first week of treatment, the RBG was stable for the next three weeks, and gradually increased in the fifth week (day 28) of treatment, one-way ANOVA
- Post Hoc test, Tukey HSD. Whilst, the RBG remained unchanged and constant in the normal group from day 0 to day 28 (figure 3.1). There was a significant difference (p = 0.000) noted when the control group (29.433 ±6.221 mmol/L) was compared to the normal group (6.633
±0.405 mmol/L) and significantly different (p = 0.018) when compared to JT2016C treatment group (22.53 ±11.06 mmol/L) (figure 3.1.2) after 28 days of treatment. Figure 3.1.2, 3.1.3, and
3.1.4 show random blood glucose levels over three weeks (day 7, 14, 21).

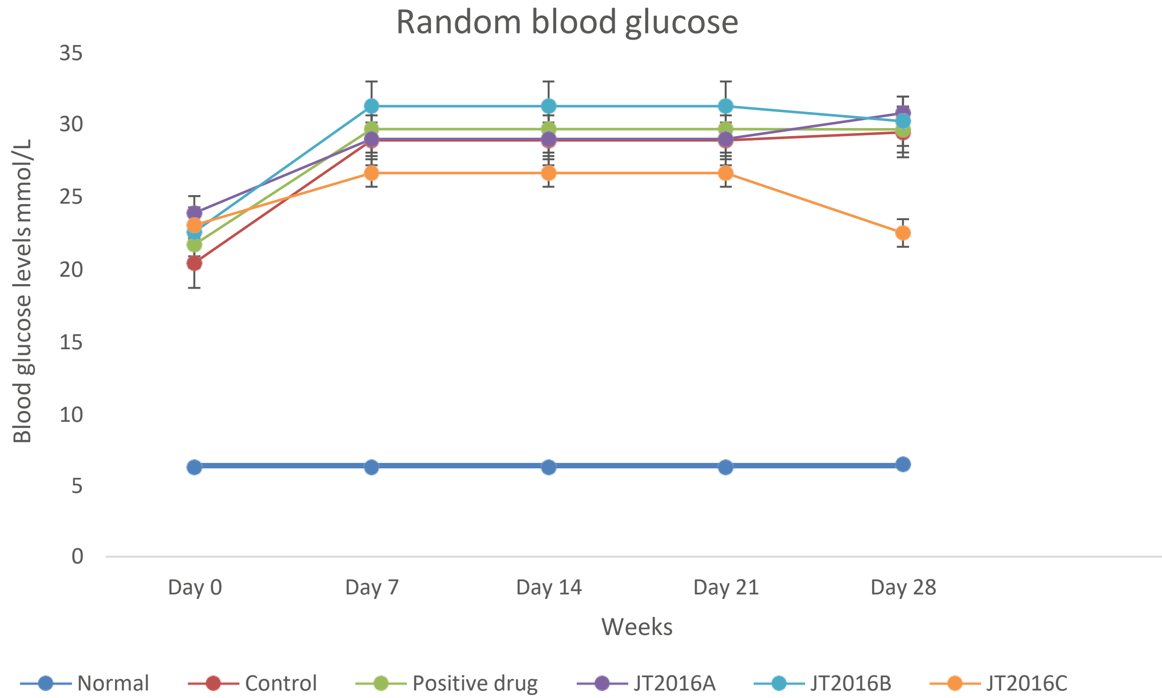

Figure 3.1: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats. The blood was collected from the tail vein in rats, and was measured randomly once every week for five weeks. Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD of animals per group.

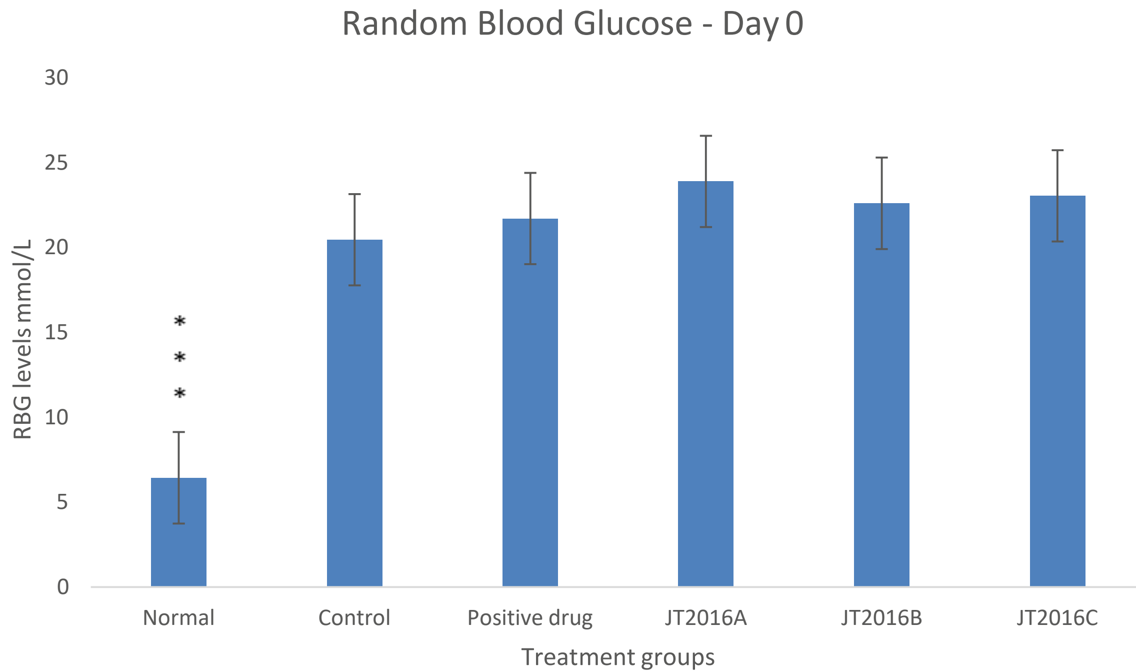

Figure 3.1.1: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats pre-treatment (day 0). The blood glucose levels in the normal group (6.433 ±0.51 mmol/L) were significantly different ($p < 0.000$) to the control group (20.46 ±3.67 mmol/L) (NB: the animals were not fasted). Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD, ***$p < 0.000$.

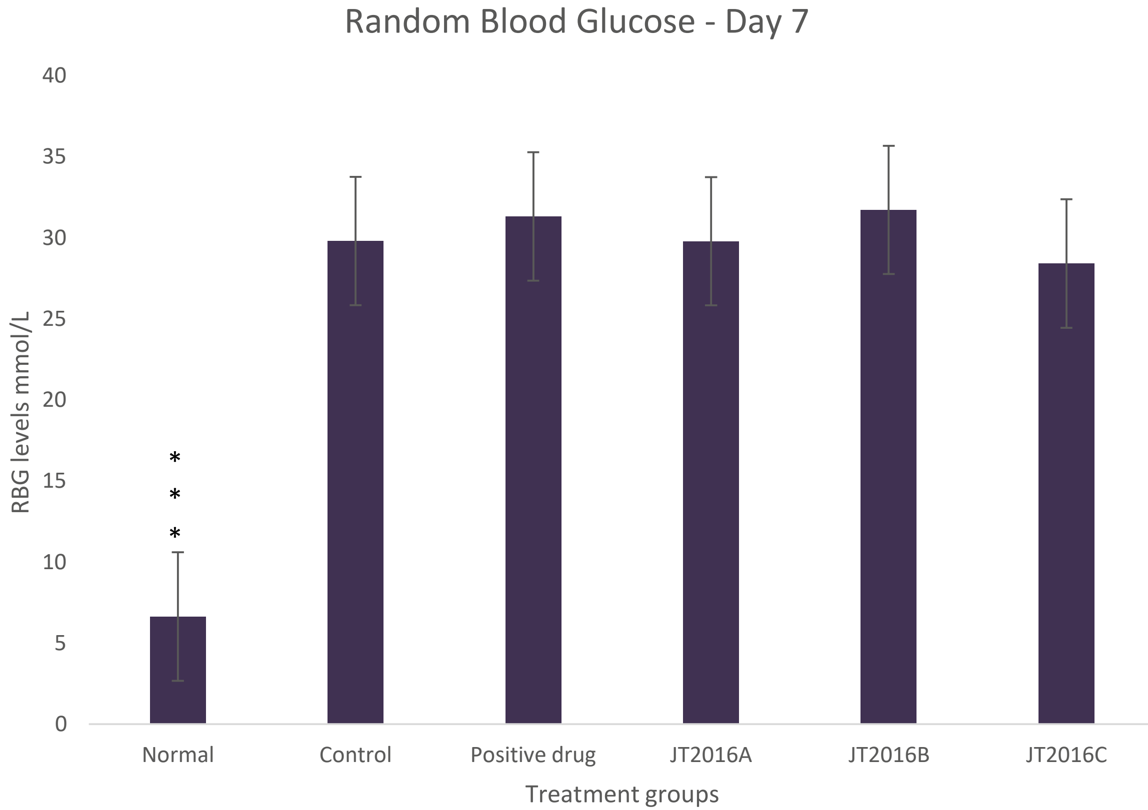

Figure 3.1.2: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats on day 7. The blood glucose levels in the normal group (6.433 ±0.51 mmol/L) were significantly different ($p < 0.000$) to the control group (28.88 ±5.04 mmol/L) and no difference noted when control was compared to other treatment groups (positive drug, JT2016A, JT2016B, and JT2016C) ($p > 0.05$) (NB: the animals were not fasted). Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD, ***$p < 0.000$.

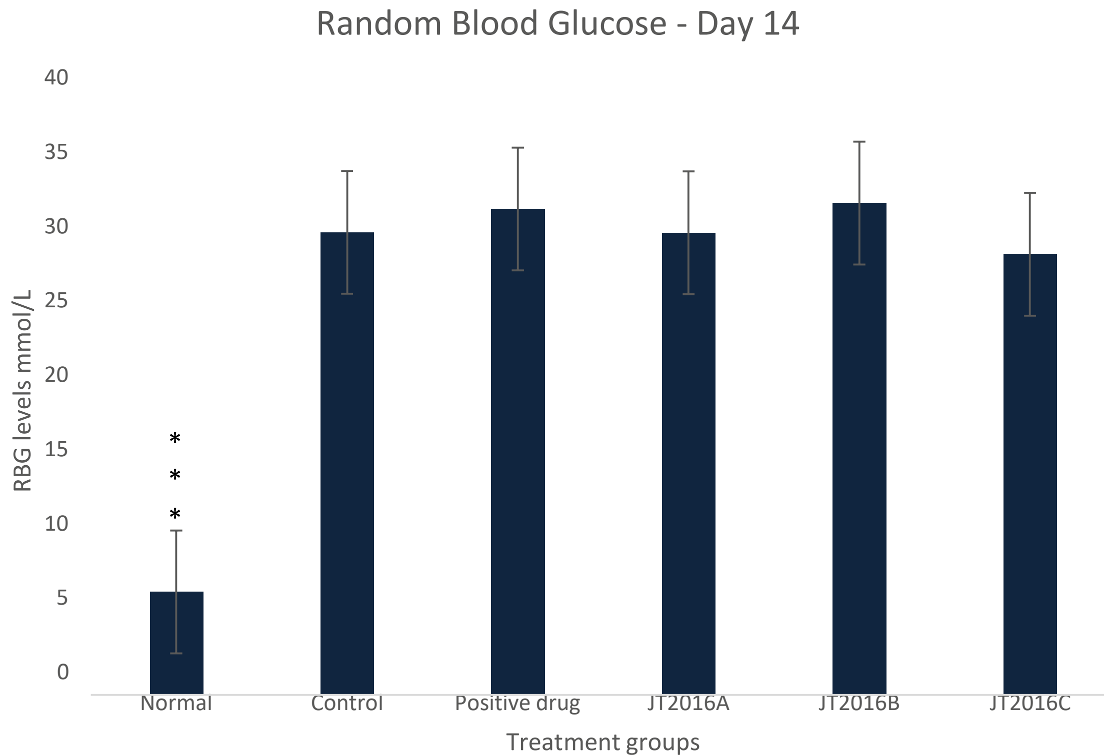

Figure 3.1.3: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats on day 14. The blood glucose levels in the normal group (6.575 ±0.598 mmol/L) were significantly different ($p < 0.000$) to the control group (28.80 ±4.22 mmol/L) and no difference noted when control was compared to other treatment groups (positive drug, JT2016A, JT2016B, and JT2016C) ($p > 0.05$) (NB: the animals were not fasted). Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD, ***$p < 0.000$.

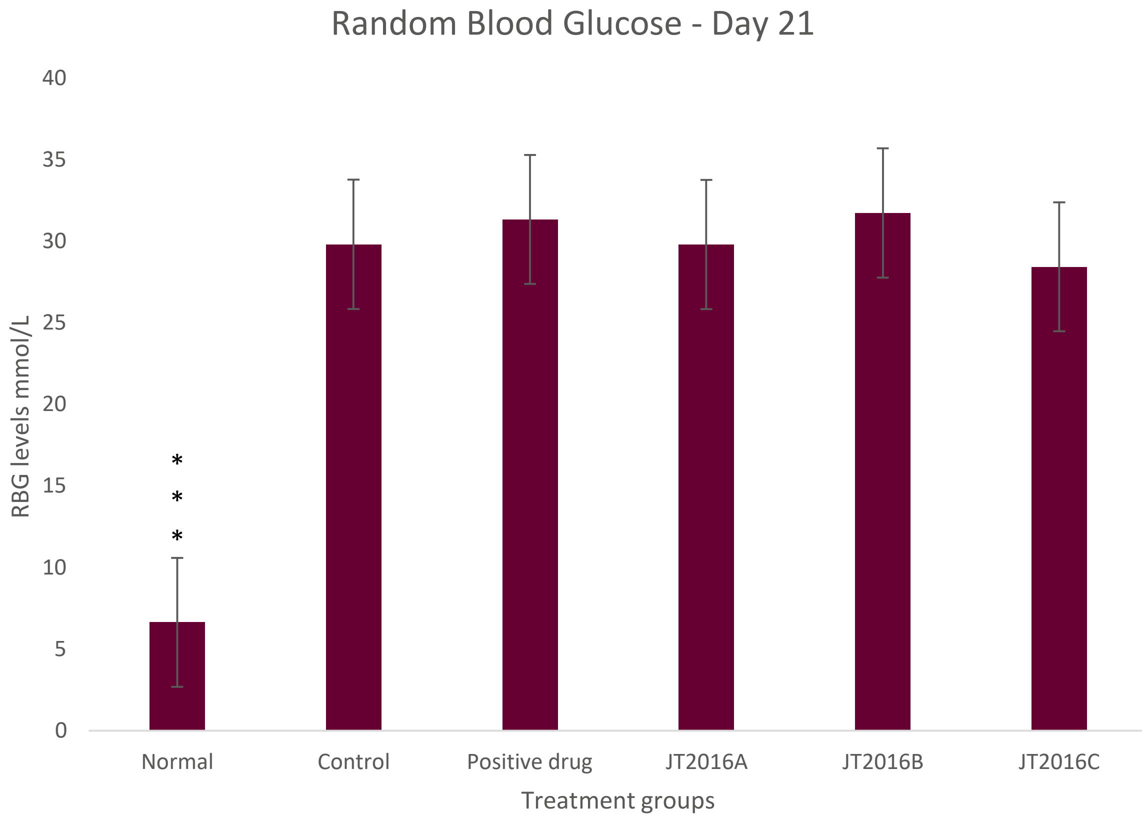

Figure 3.1.4: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats on day 14. The blood glucose levels in the normal (non - diabetic) group (6.633 ±0.41 mmol/L) were significantly different ($p < 0.000$) to the control (diabetic) group (29.81 ±4.65 mmol/L) and no difference noted when control was compared to other treatment groups (positive drug, JT2016A, JT2016B, and JT2016C) ($p > 0.05$) (NB: the animals were not fasted). Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD, $***p < 0.000$.

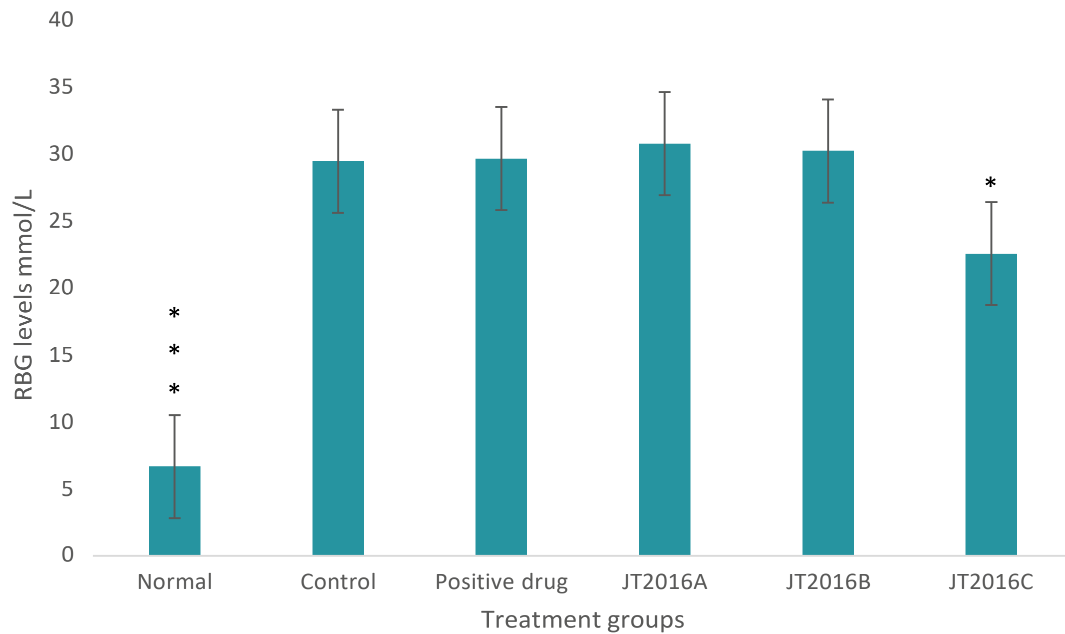

Figure 3.1.5: Effects of JT2016 on random blood glucose level in HFD-STZ diabetic SD rats after 28 days of treatment. The blood glucose levels in the JT2016C treatment group (22.53 ±11.06 mmol/L) were significantly different ($p < 0.05$) compared to the control group (29.43 ±3.49 mmol/L) (NB: the animals were not fasted). Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C. Values are mean +/- SD, $*p < 0.05$, $***p < 0.000$.

At the beginning of the treatment (day 0) in the treatment groups, there was no difference (P = 0.833) in the RBG of the control group (20.46 ±3.67 mmol/L) compared to the JT2016C treatment group (23.05 ±6.61 mmol/L). However, there was a significant difference (P = 0.018) noted between the RBG in the control group (29.43 ±3.49 mmol/L) and the JT2016C (22.53

±11.06 mmol/L) on the 28th day of treatment (figure 3.1).

In JT2016A treatment group, on day 0 was no difference (P = 0.602) of RBG levels in the control

group (20.46 ±3.67 mmol/L) compared to that of JT2016A (23.90 +/- 6.03 mmol/L). There was also no difference (P = 0.998) noted between the RBG levels in the control group (29.43 ±3.49 mmol/L) and the JT2016A (30.75 ±6.21 mmol/L) on the 28th day of treatment (figure 3.1).

In JT2016B treatment group, on day 0 was no difference (P = 0.918) of RBG levels in the control group (20.46 ±3.67 mmol/L) compared to that of JT2016B (22.60 ±5.94 mmol/L). There was also no difference (P = 1.000) noted between the RBG in the control group (29.43 ±3.49 mmol/L) and the JT2016B (30.21 ±4.39 mmol/L) on the 28th day of treatment (figure 3.1).

In positive drug (pioglitazone) treatment group, on day 0 was no difference (P = 0.992) of RBG in the control group (20.46 ±3.67 mmol/L) compared to that of pioglitazone (21.71 ±3.81 mmol/L). There was also no difference (P = 1.000) noted between the RBG in the control group (29.43 ±3.49 mmol/L) and pioglitazone (29.63 ±6.22 mmol/L) on the 28th day of treatment (figure 3.1).

3.1.2 Effects of JT2016 compound on fasting blood glucose levels

Figure 3.2 displays fasting blood glucose levels (eight hours of fast) of normal SD rats and the HFD-STZ induced diabetic rats after six weeks (day 35) of treatment. The illustration shows significant difference between the groups (p = 0.000) one-way ANOVA. FBG was tested at termination of the experiment after 35 days of treatment and the tests results show a significant difference between the control group and the normal group. Further, a significant decrease in FBG levels noted in JT2016C (P = 0.001) (15.34 ±8.96 mmol/L) when compared to the control group (25.18 ±5.74 mmol/L) (figure 3.2). FBG test showed no significant difference (P = 0.916) between JT2016A (28.35 ±6.79 mmol/L) and the control group (25.18 ±5.74

mmol/L).

There was also no significant difference (P = 0.232) noted between JT2016B (18.26 ±5.56 mmol/L) and the control group (25.18 ±5.74 mmol/L). Likewise, FBG tests showed no significant difference (P = 0.771) between pioglitazone (21.24 ±7.95 mmol/L) and the control group (25.18 ±5.74 mmol/L) (figure 3.2).

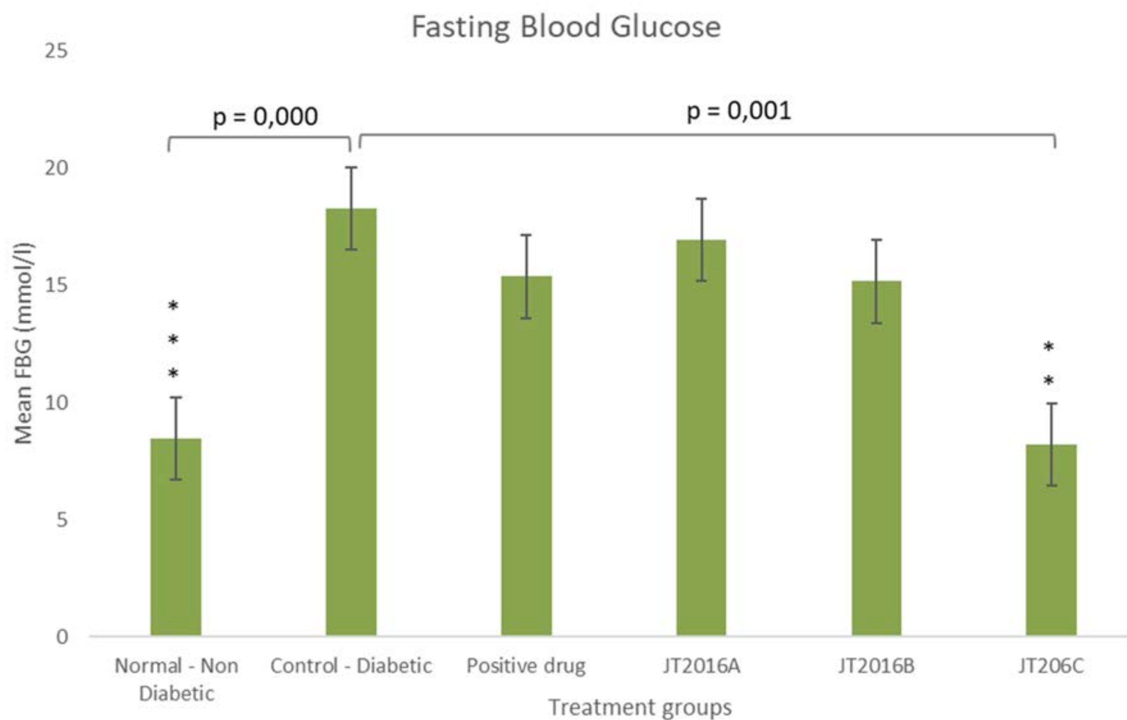

Figure 3.2: Effects of JT2016 in fasting blood glucose level in HFD-STZ diabetic SD rats. The rats were fasted for eight (8) hours before blood collection from the tail vein. Each value represents the mean ± SEM of normal group, control group, positive drug, JT2016A, JT2016B, and JT2016C after six weeks of treatment. JT2016C shows significant decrease in fasting blood glucose compared to the control group (p=0.002), but there is no significant difference between the control group and the other four treatment groups, JT2016A, JT2016B, and positive drug (*p > 0.05*). Values are mean +/- SD, **p <0.05, **p < 0.001,* and *****p< 0.000*.

3.2 Effects of JT2016 compound on Body Weight

The table 3.1 demonstrates body weight of normal SD rats and the HFD-STZ induced diabetic rats in the first week (day 0) of treatment and on the last week (day 35) of treatment. As shown in table 3.1, there was no difference in each treatment group comparing week one (day 0) and

week six (day 35) in all the groups. There was also a significant difference (p=0.000) between the groups noted, the normal group, the control group, positive drug, JT2016A, JT2016B, and JT2016C treatment groups throughout the six weeks of the treatment period. Therefore, JT2016A, JT2016B, and JT2016C, together with the positive drug (pioglitazone) showed no significant effect on the body weight. At the beginning of the treatment there was significant difference (P = 0.000) in the body weight of the control group (259.30 ±17.85 g) and the normal group (311.00 ±25.15 g). However, there was no difference (P = 0.877) noted between the control group and the treatment groups, JT2016A (259.90 ±24.08 g), JT2016B (250.40 ±17.51 g), JT2016C (254.50 ±14.65 g), and the positive drug (251.13 ±13.10 g) (Table 3.1). There was also no difference (P = 0.992) between the body weight of the control group (304.88 ±51.87g) and the treatment groups; JT2016A (306.57 ±59.38 g), JT2016B (209.75 ±29.80 g), JT2016C (300.44 ±59.34 g), and pioglitazone (302.64 ±64.99 g), at the end of the experiment (after 35 days of treatment).

However, the average body weight of the treatment groups decreased/ increased by the following percentages; JT2016A (↑17.96%), JT2016B (↓16.23%), JT2016C (↑18.05%), and positive drug (↑20.34%) after 35 days (week six) of treatment.

Table 3.1 Body weight of Sprague Dawley (SD) rats on day 0 and after day 35 of treatment

MEASUREMENT	NOR	CNT	PD	JT2016A	JT2016B	JT2016C
Week One (DAY 0) Body Weight (g)	311.00 ± 25.15	259.30 ± 17.85	251.13 ± 13.10	259.90 ± 24.08	250.40 ± 17.51	254.50 ± 14.65
Body Weight Increase (+)/ Decrease (-) (%)	+42.98	+17.58	+20.34	+17.96	-16.23	+18.05
Week Six (DAY 35) Body Weight (g)	444.66 ± 65.39	304.88 ± 51.87	302.22 ± 64.99	306.57 ± 59.38	209.75 ± 29.80	300.44 ± 59.34

Table 3.1: Each value represents the mean (SD) of 10 Normal group (NOR), Control group (CNT), Positive Drug (PD), JT2016A, JT2016B, JT2016C Sprague Dawley rats.

3.3 Effects of JT2016 herbal compound on plasma Insulin of HFD-STZ induced diabetic rats

The results displayed in Figure 3.4 shows the serum insulin levels after 35 days of treatment. Figure 3.4 demonstrates the significant difference (p = 0.000) found between the six treatment groups i.e. the normal, control, positive drug, JT2016A, JT2016B, and JT2016C group. Statistically, the serum insulin levels in the normal (non-diabetic) group (p = 0.000) was significantly decreased as compared to the control (diabetic) group. The serum insulin levels in the control group (39.043 ±15.059 mlU/L) compared to the treatment groups, JT2016A (43.90 ±5.515 mlU/L) (p = 0.942), JT201B (38.257 ±13.052 mlU/L) (p = 1.000), and the positive

drug (42.45 ±11.085 mlU/L) (p = 0.984), showed no significant difference (*p > 0.05*), but the JT2016C treatment group (21.01 ±9.627 mlU/L) (p = 0.001) showed a significant difference when compared to the control group.

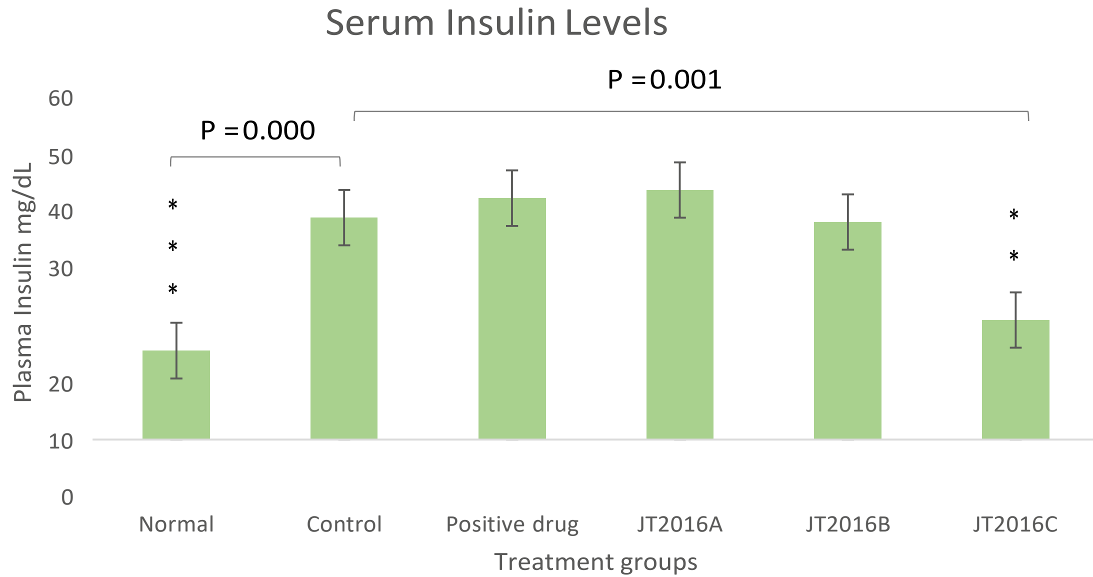

Figure 3.4: The effect of JT2016 on insulin secretion in HFD - STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$, **$p < 0.001$, ***$p < 0.000$. After six weeks of treatment, the treatment groups JT2016A, JT2016B, and positive drug had no significant difference in insulin levels when compared to the control group. Whilst, JT2016C treatment group had insulin levels significantly reduced when compared to control group ($p < 0.05$).

3.4 Effects of JT2016 herbal compound on Insulin resistance

The results displayed in Figure 3.5 show the Insulin resistance (IR) levels after six weeks of treatment. Statistically the IR level in the normal group ($p < 0.001$) and JT2016C ($p < 0.05$) were significantly decreased as compared to the control group. Whilst in other treatment groups JT2016A, JT2016B, and the positive drug, there was no significant difference when compared to the control group ($p > 0.05$).

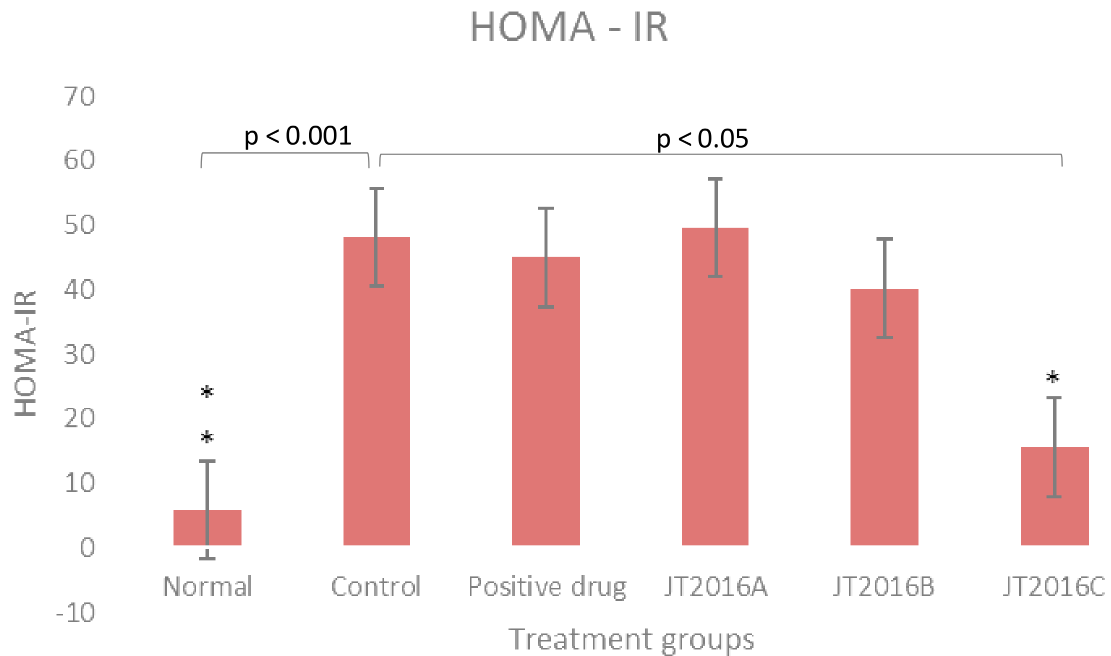

Figure 3.5: The effect of JT2016 on Homeostatic model assessment – Insulin resistance (HOMA–IR) in HFD/ STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$, **$p < 0.001$. JT2016C had the significant effect on HOMA-IR when compared the control group ($p < 0.05$), whilst positive drug, JT2016A, and JT2016B did not have any significant effect in the treatment HFD/STZ diabetic rats as compared to the control group ($p > 0.05$).

3.5 Effects of JT2016 herbal compound on lipids in HFD-STZ induced Diabetic SD rats

In the presented results, preceding the six weeks of treatment, there was significant difference between the groups identified in the following tests taken: Plasma Total Cholesterol (TC)was significantly increased in the control group when compared to the normal group (p = 0.025) (figure 3.6); High Density Lipoprotein Cholesterol (HDLC) was significantly increased in the control group when compared to the normal group (p = 0.009) (figure 3.9); Low Density Lipoprotein Cholesterol (LDLC) was significantly increased in the control group as compared to

the normal group (p = 0.001) (figure 3.10). Triglycerides (TG) and non-esterified free fatty acids (FFA) had no significant changes identified between the control group and the normal group. Triglycerides had the p = 0.467 normal group compared to the control groups (figure 3.11), and the non-esterified free fatty acids had the normal group (p = 0.467) when compared to the treatment groups (figure 3.12).

The results in Figure 3.6 shows the TC levels in six weeks of treatment with significant difference between groups (p = 0.025) one-way ANOVA - Post Hoc test, Tukey HSD. Statistically, the TC levels in the control group were significantly raised compared to the normal group (p = 0.026). In other treatment groups there was no significant difference found: the positive drug treatment group (12.00 ±12.22 mmol/L) (p = 0.966), JT2016A (15.00 ±15.523 mmol/L) (p = 1.000), JT2016B (14.280 ±12.217 mmol/L) (p = 1.000), and JT2016C (9.302 ±9.323 mmol/L) (p = 0.730) compared to the control group.

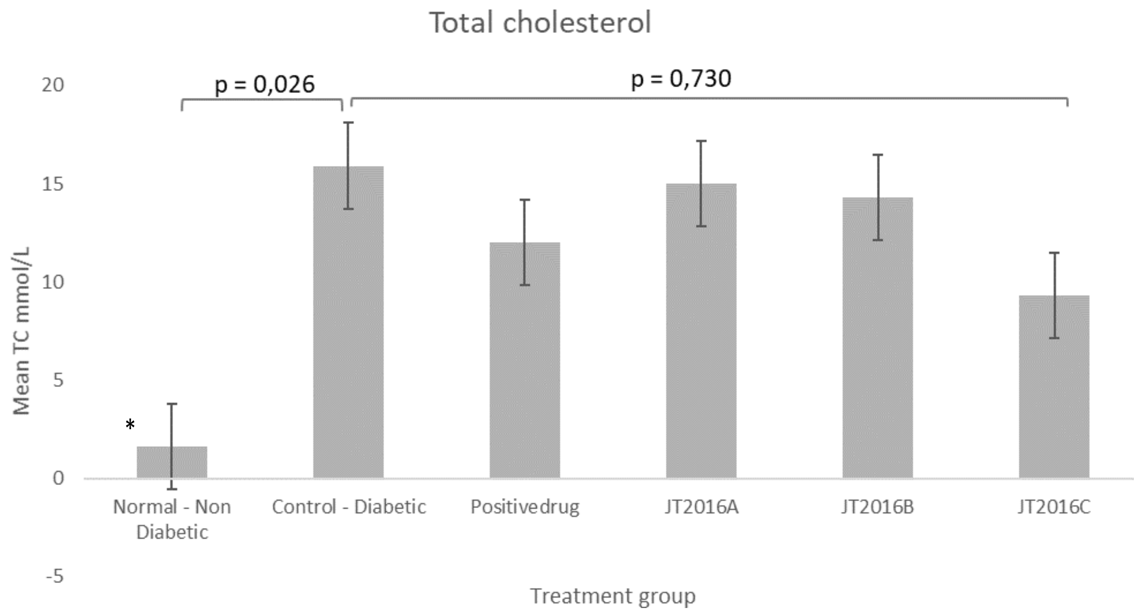

Figure 3.6: The effect of JT2016 on TC levels in HFD-STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$. When six weeks of treatment had elapsed, the control group had a significant increase in TC when compared to the normal group ($p < 0.05$). Although, there was no significant change in treatment groups JT2016A, JT2016B, JT2016C, and positive drug when compared to the control group ($p > 0.05$).

Figure 3.7 shows the HDLC levels after six weeks of treatment with significant difference between groups (p = 0.009) one-way ANOVA, Post Hoc test, Tukey HSD. The HDLC levels in the control group were significantly raised compared to the normal group (p = 0.005). The treatment groups had no significant difference when compared to the control group: the positive drug treatment group (1.600 ±0.634 mmol/L) (p = 0.869), JT2016A (1.634 ±0.747

mmol/L) (p = 0.929), JT2016B (1.811 ±0.735 mmol/L) (p = 0.999), and JT2016C (1.533 ±0.615

mmol/L) (p =0.730).

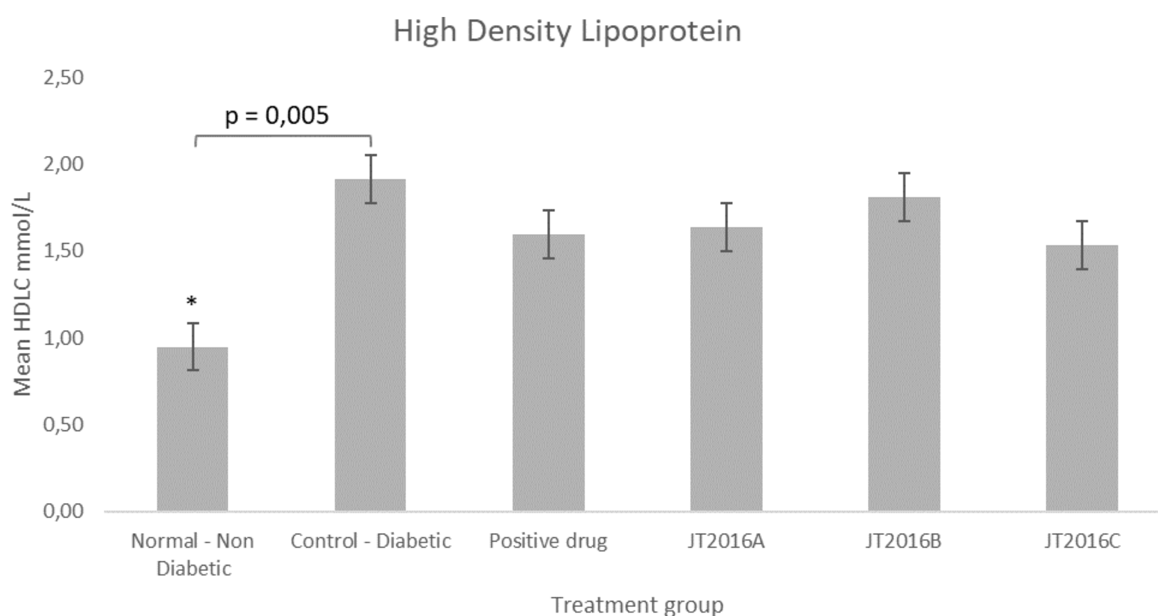

Figure 3.7: The effect of JT2016 treatment on HDLC levels in HFD-STZ induced diabetic SD rats. Values are mean +/- SD, ***p < 0.001*. When six weeks of treatment had elapsed, the control group had a significant increase in HDLC when compared to the normal group (*p < 0.001*), and no significant difference in the treatment groups JT2016A, JT2016B, JT2016C, and positive drug when compared to the control group (*p >0.05*).

The results in figure 3.8 show the LDLC levels after six weeks of treatment with significant difference between groups (p = 0.001) one-way ANOVA, Post Hoc test, Tukey HSD. Statistically, the LDLC levels in the control group were significantly raised compared to the normal group (p = 0.001). JT2016A (2.903 ±3.044 mmol/L) (p = 0.021), showed a significant difference when compared to the control group. Whilst other treatment groups presented non-significant difference when compared to the control group: the positive drug treatment group (3.488 ±3.809 mmol/L) (p = 0.276), JT2016B (6.843 ±5.076 mmol/L) (p = 1.000), and JT2016C (4.411 ±4.286 mmol/L) (p = 0.544).

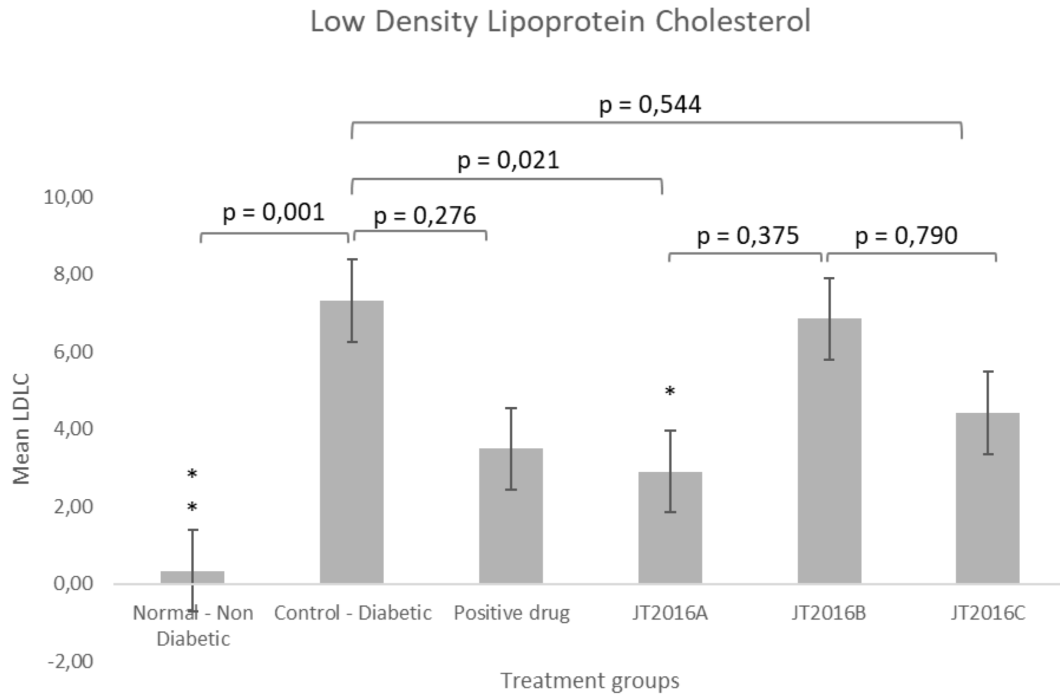

Figure 3.8: The effect of JT2016 treatment on LDLC levels in HFD-STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$, **$p < 0.001$. After six weeks period of treatment, the control group had a significant increase in LDLC when compared to the normal group ($p < 0.001$), and no significant difference noted in treatment groups JT2016A, JT2016B, JT2016C, and positive drug when compared to the control group.

The results in figure 3.9 show the triglycerides levels after six weeks of treatment with no significant difference between groups (p = 0.467) one-way ANOVA, Post Hoc test, Tukey HSD. Statistically, triglyceride levels in the control group were found to have no significant difference when compared to the normal group (p = 0.952). The treatment groups had no significant difference when compared to the control group ($p > 0.05$); the positive drug treatment group (3.678 ±5.354 mmol/L) (p = 0.986), JT2016A (5.404 ±6.107 mmol/L) (p = 1.000), and JT2016B (1.85 ±1.058 mmol/L) (p = 0.915), and JT2016C (1.191 ±1.053 mmol/L) (p = 0.855) at the end of treatment.

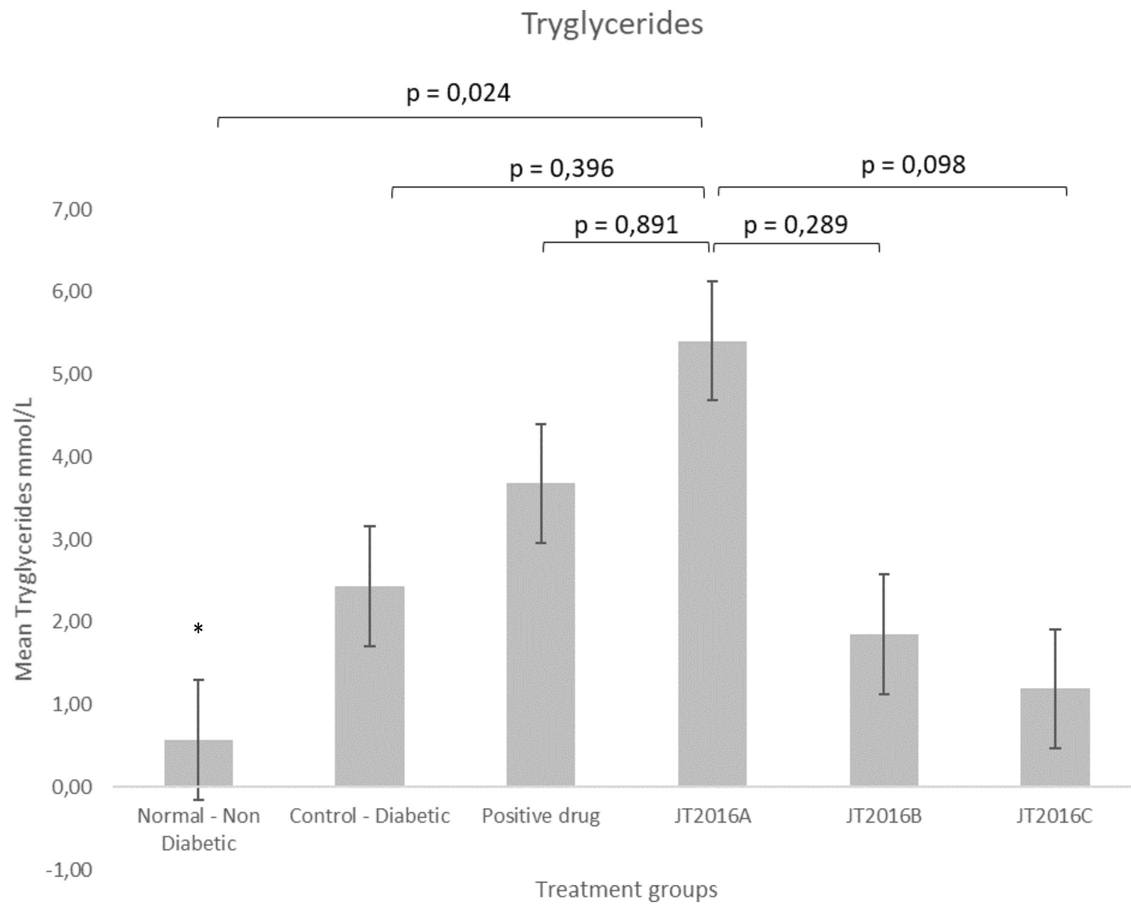

Figure 3.9: The effect of JT2016 treatment on triglycerides (TG) levels in HFD-STZ diabetic SD rats. Values are mean +/- SD. After six weeks of treatment, the control group had a no significant difference in triglycerides when compared to the normal group ($p > 0.05$), and no significant difference in treatment groups JT2016A, JT2016B, JT2016C, and positive drug when compared to the control group ($p > 0.05$).

The results in figure 3.10 show the FFA levels after six weeks of treatment with no significant difference between groups (p = 0.467) one-way ANOVA, Post Hoc test, Tukey HSD. Statistically, FFA levels in the control group had no significant difference when compared to the normal group (p = 0.952). The treatment groups were also found to have no significant differences when compared to the control group: the positive drug (0.776 ±0.790 mmol/L) (p = 0.986), JT2016A (0.654 ±0.390 mmol/L) (p = 1.000), and JT2016B (0.481 ±0.122 mmol/L) (p = 0.915),
and JT2016C (0.468 ±0.076 mmol/L) (p = 0.855).

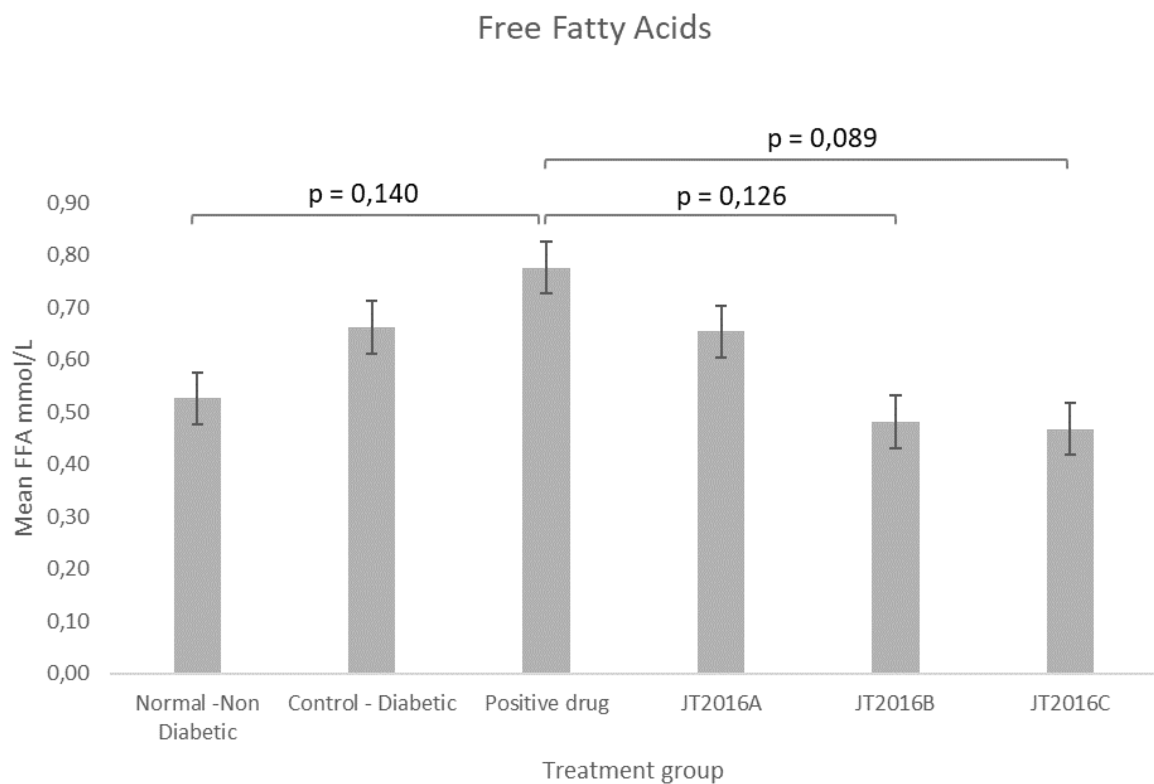

Figure 3.10: The effect of JT2016 treatment on FFA levels in HFD-STZ induced diabetic SD rats. Values are mean +/- SD. After six weeks of treatment, the control group had no significant change in FFA compared to the normal group ($p > 0.05$), and no significant change in treatment groups JT2016A, JT2016B, JT2016C, and positive drug were noted when compared to the control group ($p > 0.05$).

3.6 Effects of JT2016 herbal compound on inflammatory cytokines in HFD-STZ induced Diabetic rats

The results presented in figure 3.11 show the inflammatory cytokine levels of interleukin-6 (IL- 6) after six weeks of treatment. Statistically, there is a significant difference noted between the treatment groups ($p = 0.000$) one-way ANOVA, Post Hoc test, Tukey HSD. The IL-6 levels in the control group were significantly increased when compared to the normal group ($p = 0.000$). There was a significant difference/ reduction of IL-6 in JT2016C treatment group when compared to the control group ($p = 0.000$). But there was no significant difference in the treatment groups JT2016A, JT2016B, and positive drug when compared to the control group ($p > 0.05$), i.e. the positive drug treatment group (164 ±15.316 pg/ml) ($p = 0.979$), JT2016A (155 ±10.451 pg/ml) ($p = 0.358$), and JT2016B (138.236 ±15.86 mmol/L) ($p = 0.128$).

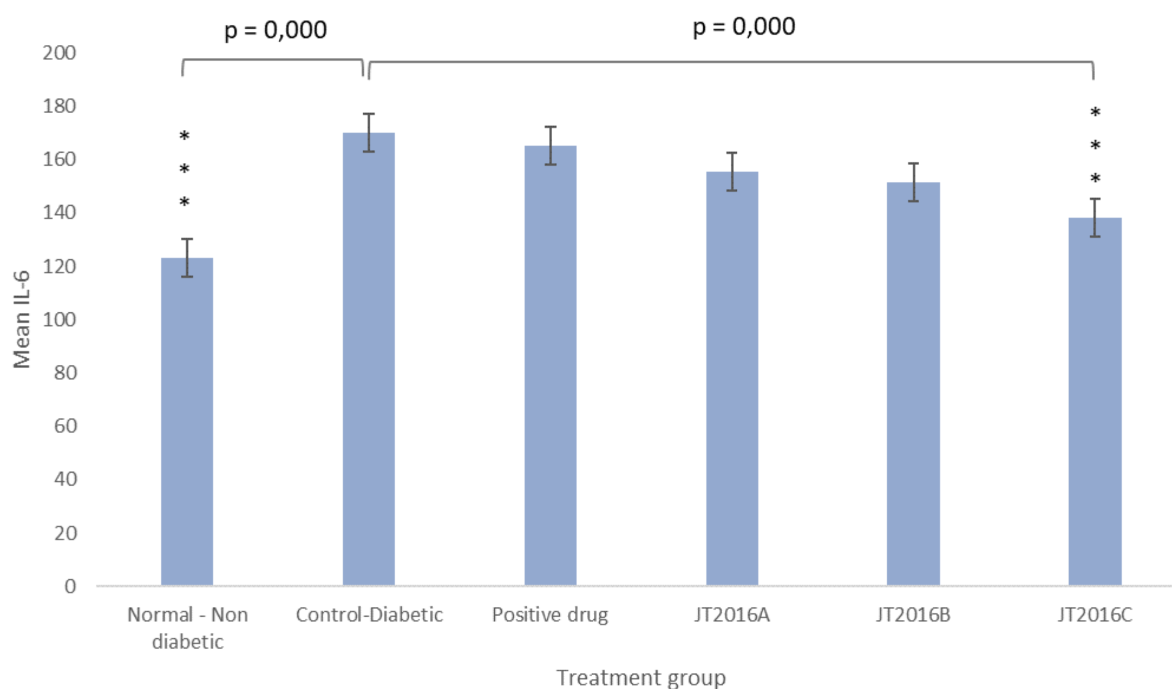

Figure 3.11: The effect of JT2016 treatment on inflammatory cytokine IL-6 levels in HFD -STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$. After six weeks of treatment, the control group had a significant increase in inflammatory cytokine IL-6 when compared to the normal group ($p < 0.000$). JT2016C significantly reduced IL-6 when compared to control group ($p < 0.0001$), but there was no significant difference noted in the treatment groups, positive drug, JT2016A, and JT2016B when compared to the control group ($p > 0.05$).

The results in figure 3.12 shows the inflammatory cytokine levels of Tumor necrosis factor (TNF-α) after six weeks of treatment with significant difference noted between groups (p = 0.000) one-way ANOVA, Post Hoc test, Tukey HSD. Statistically, TNF-α levels in the control group were significantly increased when compared to the normal group (p = 0.000). JT2016C treatment had a significant difference in TNF-α levels when compared to the control group

(61.569 ±5.461 pg/ml) (p = 0.001). On the other hand the treatment groups, positive drug, JT2016A, and JT2016B had no significant difference noted when compared to the control group ($p > 0.05$): positive drug (67.425 ±8.996 pg/ml) (p = 0.142), JT2016A (66.279 ±7.090 pg/ml) (p = 0.087), and JT2016B (68.829 ±4.806 pg/ml) (p = 0.342).

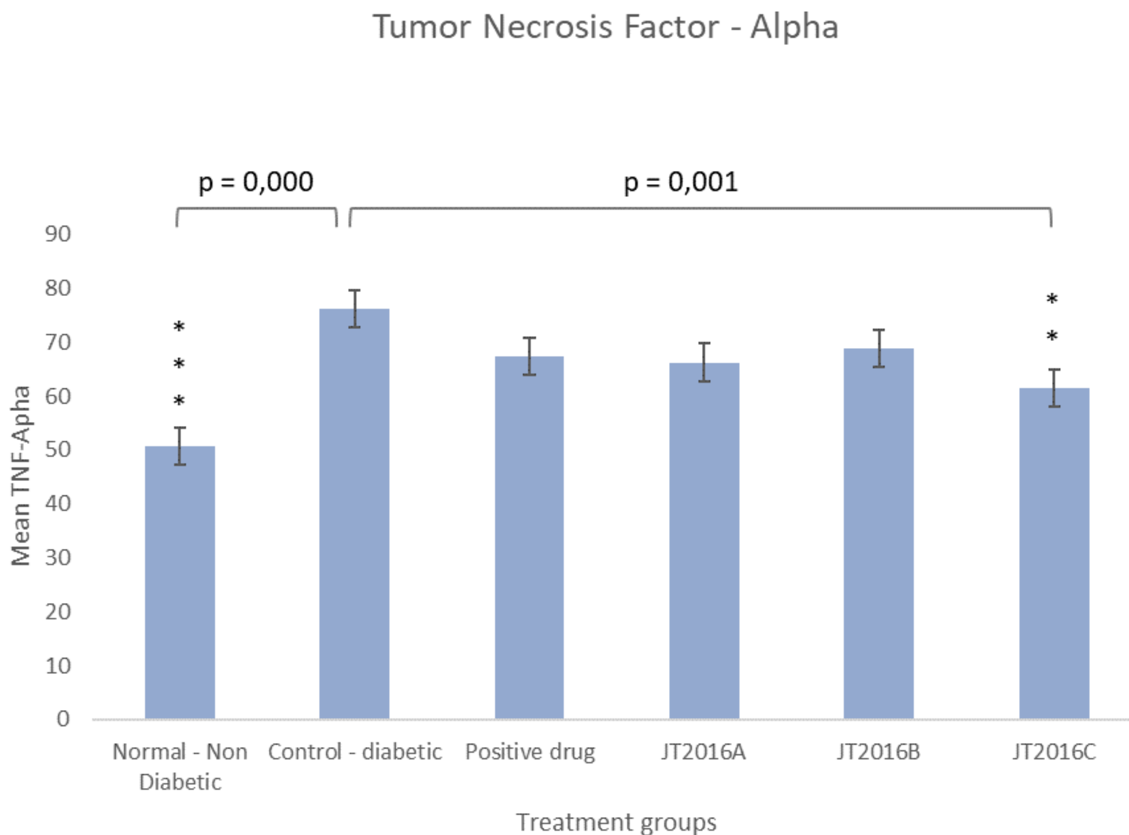

Figure 3.12: The effect of JT2016 on inflammatory cytokine TNF – α levels in HFD STZ induced diabetic SD rats. Values are mean +/- SD, *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. After six weeks of treatment, the control group had a significant increase in inflammatory cytokine TNF-α when compared to the normal group ($p < 0.001$). JT2016C significantly reduced TNF-α when compared to control group (p < 0.0001), whilst the other treatment groups, positive drug, JT2016A, and JT2016B had no significant difference in TNF-α levels when compared to the control group ($p > 0.05$).

3.7 Effects of JT2016 herbal compound on Proteins in HFD-STZ induced Diabetic rats

The results in figure 3.13 shows the serum albumin levels after six weeks of treatment with no significant difference between groups (p = 0.102) one-way ANOVA, Post Hoc test, Tukey HSD. Statistically, serum albumin levels in the control group compared to the normal group had no significant change (p = 0.868). The treatment groups were found to have no significant difference when they were compared to the control group: Pioglitazone, the positive drug treatment group (30.142 ±5.108 g/l) (p = 0.483), JT2016A (30.653 ±5.132 g/l) (p = 0.338),
JT2016B (26.808 ±2.832 g/l) (p = 0.999), and JT2016C (27.200 ±3.259 g/l) (p = 1.000).

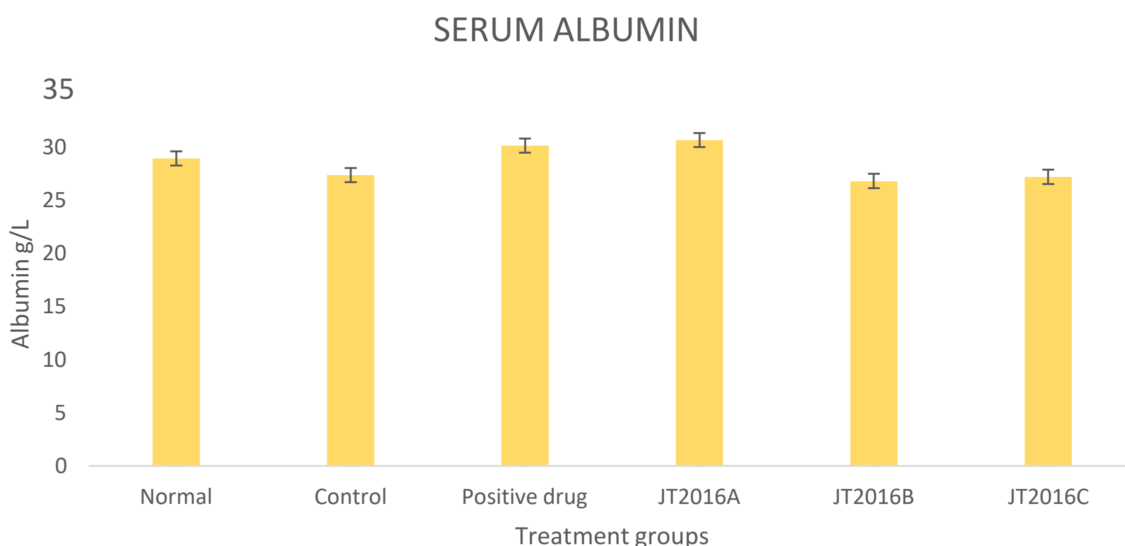

Figure 3.13: The effect of JT2016 treatment on serum albumin in HFD-STZ induced diabetic SD rats. Values are mean +/- SD. After six weeks of treatment, control group had no significant effect in serum albumin compared to the normal group ($p > 0.05$). The treatment groups JT2016A, JT2016B, JT2016C, and positive drug had no significant effect on serum albumin levels when compared to the control group ($p > 0.05$).

The results in figure 3.14 show the serum globulin levels after six weeks of treatment with no significant difference between groups (p = 0.248) as determined by one-way ANOVA, Post Hoc test, Tukey HSD. Serum globulin levels in the control group, when compared to the normal group, had no significant change (p = 0.420). The treatment groups had no significant effect on globulin when compared to the control group: the positive drug treatment group (35.371 ±2.725 g/l) (p = 0.969), JT2016A (33.761 ±2.545 g/l) (p = 0.552), JT2016B (33.594 ±3.035 g/l) (p = 0.491), and JT2016C (36.314 ±3.778 g/l) (p = 1.000).

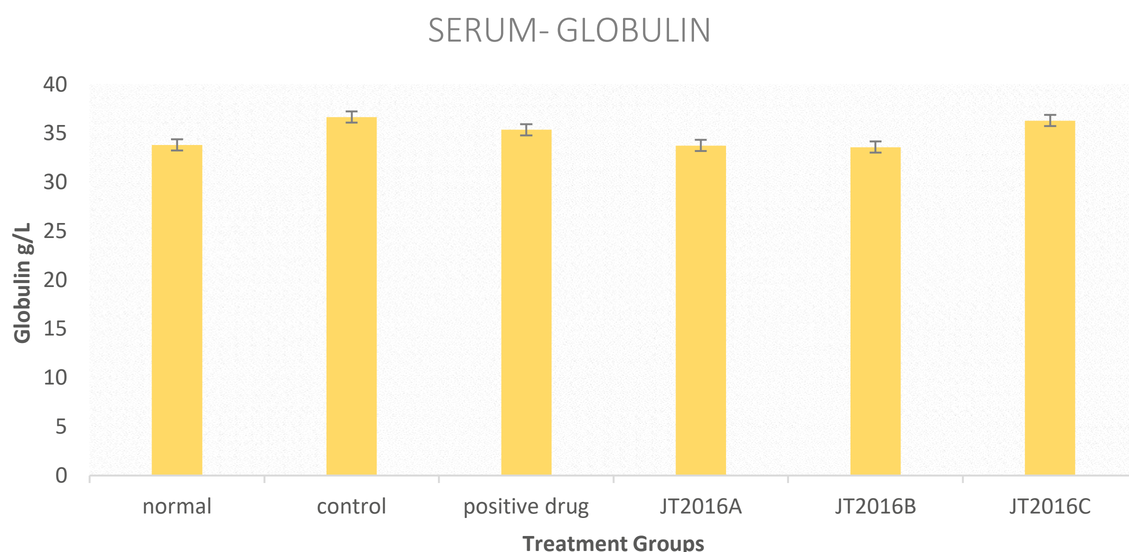

Figure 3.14: The effect of JT2016 treatment on serum globulin in HFD-STZ induced diabetic SD rats. Values are mean +/- SD. After six weeks of treatment, the control group had no significant effect in serum globulin levels when compared to the normal group (*$p > 0.05$*). The treatment groups, JT2016A, JT2016B, JT2016C, and positive drug had no significant effect on serum globulin levels when compared to the control group (*$p > 0.05$*).

The results in figure 3.15 show the total protein levels after six weeks of treatment with no

significant difference between groups (p = 0.595) one-way ANOVA, Post Hoc test, Tukey HSD.

Statistically, total protein levels in the control group when compared to the normal group had no significant effect (p = 0.994). The treatment groups had no significant effect in the total protein levels when compared to the control group: the positive drug treatment group (65.514 ±7.166 g/l) (p = 0.993), JT2016A (64.414 ±7.267 g/l) (p = 1.000), and JT2016B (60.402 ±3.702 g/l) (p = 0.768), and JT2016C (63.514 ±5.569 g/l) (p = 1.000).

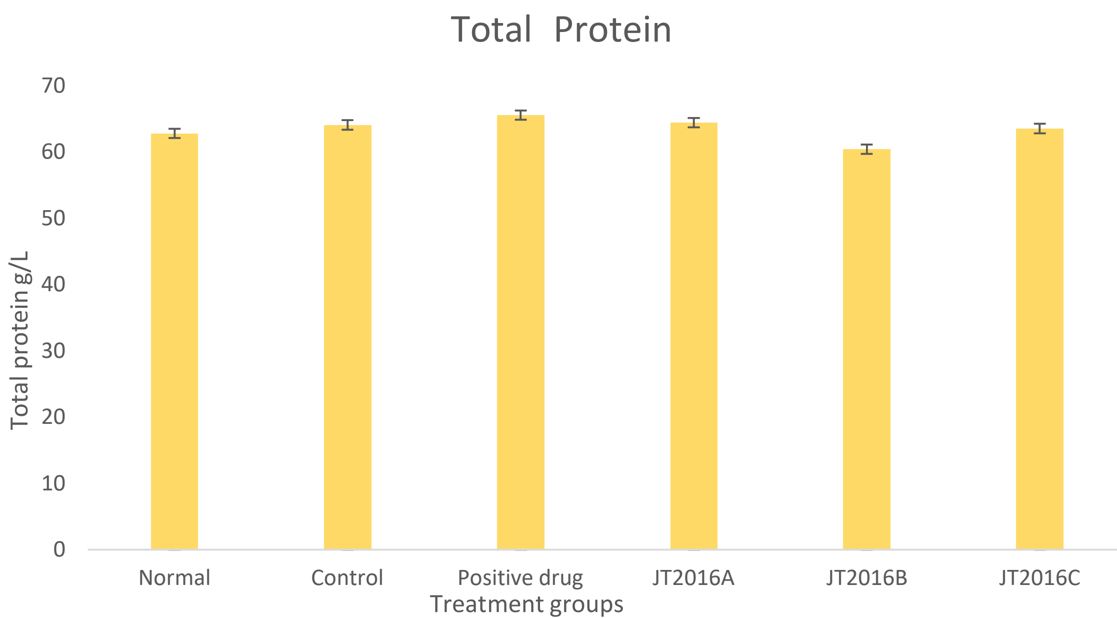

Figure 3.15: The effect of JT2016 treatment on Total Protein in HFD-STZ induced diabetic SD rats. Values are mean +/- SD. After six weeks of treatment, the control group had no significant effect in total protein levels when compared to the normal group ($p > 0.05$). The treatment groups, JT2016A, JT2016B, JT2016C, and positive drug had no significant effect in total protein levels when compared to the control group ($p > 0.05$).

3.8 Effects of JT2016 herbal compound on Hepatic Function in HFD-STZ induced Diabetic rats

The results in figure 3.16 show the serum Alanine Transaminase (ALT) levels after six weeks of treatment with significant difference between groups (p = 0.034). A significant difference in ALT levels noted between the control group and the normal group (p = 0.008). The treatment groups, JT2016A (73.33 ±39.86 IU/l) (p = 0.052) illustrated a significant difference in ALT levels when compared to the control group ($p < 0.05$), but no significant difference noted in JT2016B (254.64 ±284.91 IU/l) (p = 1.000), JT2016C (100.63 ±70.77 IU/l)(p = 0.071), and positive drug

(90.94 ±39.69 IU/l) (p = 0.070) treatment groups when compared to the control group ($p > 0.05$).

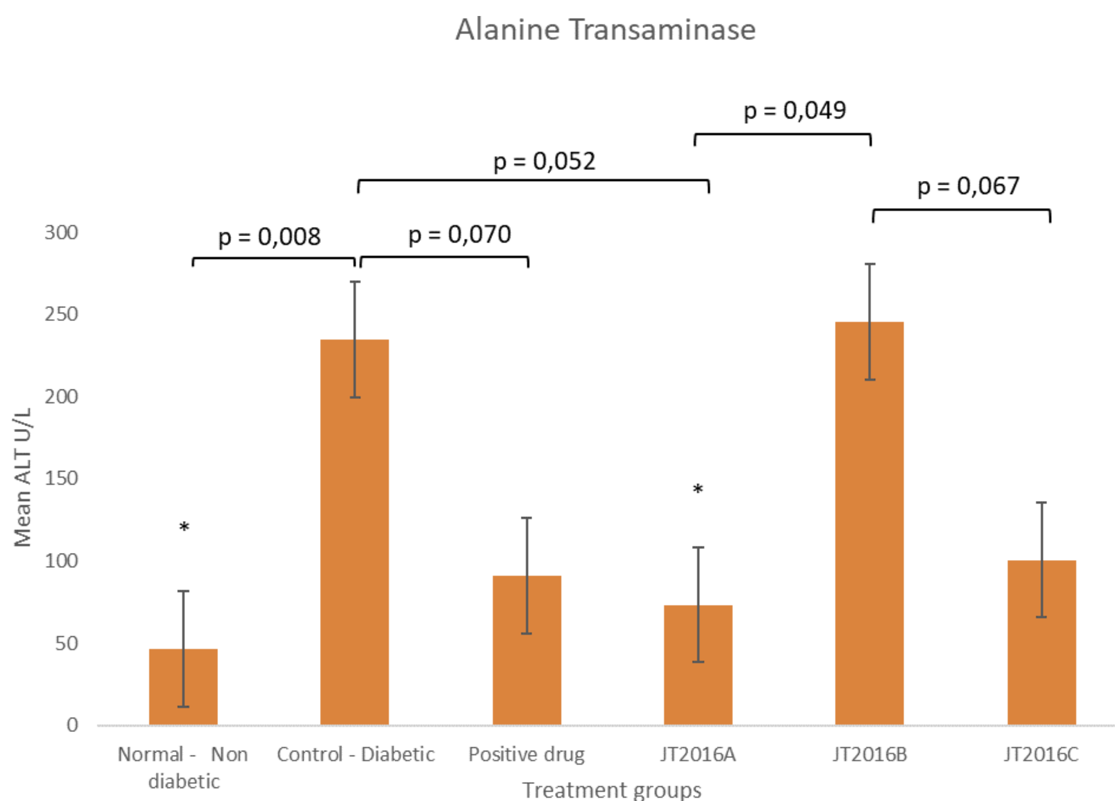

Figure 3.16: The effect of JT2016 treatment on Alanine amino transaminase (ALT) in HFD-STZ diabetic SD rats. Values are mean +/- SD, *$p < 0.05$. After six weeks treatment, the control group had a significant increase in ALT when compared to the normal group ($p < 0.05$) and a significant decrease in

JT2016A treatment group when compared to the control group (*$p < 0.05$*). The treatment groups, JT2016B, JT2016C, and positive drug had no significant effect in ALT levels when compared to the control group (*$p > 0.05$*).

Figure 3.17 shows the Aspartate amino transaminase (AST) levels after six weeks of treatment with no significant difference between groups ($p = 0.142$) one-way ANOVA, Post Hoc test, LSD. Statistically, the AST levels in the control group were significantly raised compared to the normal group ($p = 0.017$), whilst in the treatment groups, JT2016A, JT2016B, JT2016C and the positive drug had no significant difference when compared to the control group: the positive drug treatment group (172.98 ±19.54 IU/l) ($p = 0.073$), JT2016A (185.58 ±36.81 IU/l) ($p = 0.147$), JT2016B (241.45 ±131.70 IU/l) ($p = 0.741$), and JT2016C (186.13 ±40.22 IU/l) ($p = 0.091$).

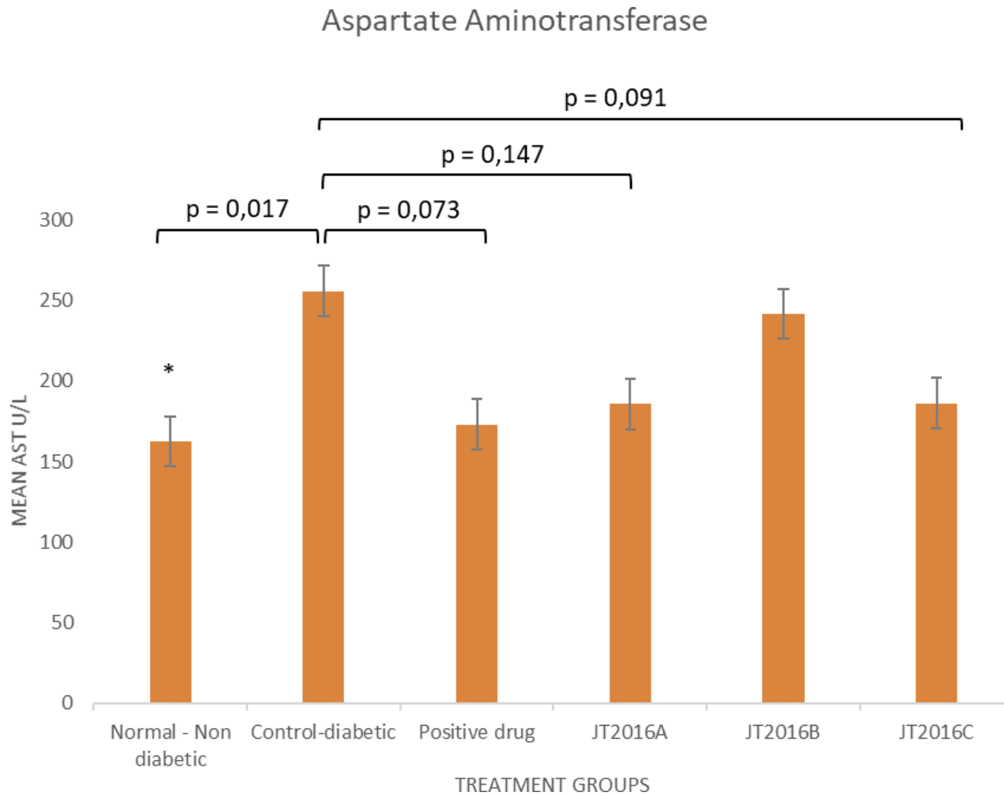

Figure 3.17: The Aspartate amino transaminase (AST) levels in HFD-STZ diabetic SD rats in different treatment groups. Values are mean +/- SD. After six weeks of treatment, the control group had no significant effect in AST when compared to the normal group ($p > 0.05$), and no significant difference noted in treatment groups, JT2016A, JT2016B, JT2016C, and the positive drug compared to the control group ($p > 0.05$).

CHAPTER 4

4. DISCUSSION

4.1 HFD/STZ Induced T2DM animal model

The Type 2 Diabetes Mellitus (T2DM) pathology is multifaceted, with several stages culminating in a functional β-cell mass which is not sufficient to meet needs of the body. To elucidate the pathobiology of this disease, animal models mimicking the pathology of human T2DM characterized by impaired regulation of hepatic glucose production, peripheral insulin resistance and declining beta-cell function, were to be used. The Sprague Dawley (SD) rat models treated with high fat diet (HFD) and low dose Streptozotocin (STZ) were used in this study for the T2DM investigation and pharmacological screening. The model was easily accessible, and took a relatively short period of time for development and yet adequate enough to allow for invasive procedures (Guo *et al.,* 2018; Srinivasan *et. al.,* 2015). This animal model closely reflected the natural history and metabolic characteristics of human T2DM. It is therefore, normally and widely used to study T2DM by numerous investigators especially for new drug development research studies (Skovsø, 2014).

In this study, the T2DM animal model was induced with low dose STZ (35mg/kg) treatment following a period of two weeks of high fat diet (HFD) dietary manipulation. Khan et al. (2014) studies confirm that HFD manipulation of two weeks and low dose STZ (35mg/kg) treated male SD rats can induce T2DM. In addition, a study done by Srinivasan et al. (2005) using different doses of STZ (25, 35, 45, 55 mg/kg) confirmed that 35mg/kg is the right dose to induce T2DM in male SD rats. The same study shows that 25 mg/kg STZ did not produce any significant hyperglycaemia in both the Normal Pellet Diet (NPD) as well as HFD-fed SD rats,

and therefore, was regarded insignificant for the research study (Srinivasan *et al.,* 2015). On another note, the amount of 45mg/kg and 55mg/kg STZ treatment induced T1DM and was considered a high dose to induce T2DM. According to Dekel et al. (2009), a high single dose of STZ (± 65mg/kg) in rats is used to induce T1DM which leads to the destruction of the pancreatic beta cells and absence of insulin production. Whilst low dose STZ and HFD treatment in rodents is able to produce insulin resistance and hyperglycaemia (Dekel *et al.,* 2009). Ming Jang et al. (2008) study supports the combination of HFD with low dose of STZ for at least two weeks as a better way for inducing a stable animal model for T2DM, and good for pharmacological screenings (Ming Jang *et al.,* 2008).

Moreover, SD rats in this current study developed T2DM following injection of STZ (35mg/kg) and of two weeks pre-dietary manipulation of HFD. Following the three days of 35mg/kg STZ injection, the HFD/STZ SD rats developed hyperglycaemia with FBG levels increased (20.46 ±3.670 mmol/l) in the control group as compared to those in the normal group (6.4333 ±0.507 mmol/l), the body weight of 259.3 ±17.857g in the control group as compared to 311.00 ±25.15g of the normal group. These results suggest that the rodents were successfully induced with hyperglycaemia.

Subsequent six weeks, animals were re-tested for FBG, tested for plasma insulin (PI), HOMA- IR, low density lipid cholesterol (LDL-C), high density lipid cholesterol (HDL-C), triglycerides, total cholesterol (TC), free fatty acids (FFA) levels etc. and the control group of the latter was compared to the normal group with the following results:

The animals tested for Insulin resistance and hyperinsulinemia, to ensure that the HFD/STZ treated rats were induced with T2DM (Matthews *et al.,* 1985). The following results were discovered when HOMA-IR was calculated: a significant difference between the normal group

and the control group ($p < 0.001$), which verifies that the animals in the treatment groups were Insulin resistant. The fasting blood insulin levels (FBI) also in the control group (39.0427 ±15.059 mIU/l) were significantly higher ($p < 0.05$) when compared those of the normal group (5.898 ±3.69 mIU/l), indicating hyperinsulinemia in the HFD/STZ treated SD rats. These results therefore indicate that HFD/STZ treated SD rats were successfully induced with T2DM.

Srinivasan et al., (2005) substantiates the latter results with their research with which the combination of HFD-fed and low-dose STZ-treated (35mg/kg) male Sprague–Dawley rats (160–180g) were successfully induced with Insulin resistance according to the HOMA-IR results simulating the human syndrome that is suitable for testing anti-diabetic agents (Srinivasan *et al.,* 2005). Qian et al. (2015) indicates that HFD combined with a single or multiple low dose of STZ at weekly intervals for two weeks has evidenced to be a better way for inducing a stable animal model of T2DM (Qian *et al.,* 2015).

The results on lipids indicate that LDL-C in the control group (7.32 ±4.72 mmol/l) were significantly higher ($p < 0.001$) when compared to those in the normal group (0.33 ±0.08 mmol/l) (Figure 3.12) indicating high levels of bad cholesterol in the blood in all the treatment groups. These could mean build-up of cholesterol in the arterial walls, and possible hardening and narrowing of the arterial walls increasing the risk of cardiovascular disease (CVD) and stroke. The serum TG levels in the control group (2.44 ±1.35 mmol/l) compared to those in the normal group (0.57 ±0.20 mmol/l) were not different ($p > 0.05$) (Figure 3.13) which means the serum TG were not affected. However, the TC levels in the control group (15.91 ±10.06 mmol/l) were significantly higher ($p < 0.05$) when compared to those in the normal group (1.63 ±0.25 mmol/l) (Figure 3.14), meaning high risk of CVD and stroke. When cholesterol levels are too high, cholesterol can be harmful by contributing to narrowed or blocked arteries resulting to CVD.

Unfortunately, diabetics are prone to bad high cholesterol levels. The FFA levels in the control group (0.66 ±0.27 mmol/l) compared to those in the normal group (0.52 ±0.14 mmol/l) were not different ($p > 0.05$) (Figure 3.15). These results therefore, confirmed a significant dyslipidaemia in the HFD/STZ induced diabetic SD rats.

Overall, the rats showed significant hyperglycaemia, insulin resistance, and dyslipidaemia, therefore suggesting that the HFD/STZ treated SD rats were successfully induced with T2DM.

4.2 The effect of JT2016 on blood glucose in HFD/STZ Induced diabetic SD rats

Jiang Tang 2016 (JT2016) is the herbal polyherbal product made up of three indigenous medicinal plants that have remained in use for decades as traditional medicine for the treatment of diabetes. The anti-diabetic polyherbal products are made to target different pathological events throughout instigation and development of diabetes from different mechanistic approaches, to annul the symptoms, treat insulin resistance and improve hyperglycaemia and the overall quality of life in patients. The JT2016 herbal compound is made of the following medicinal plants: *Aspalathus linearis, Olea africana* and *Centella asiatica* formulated to effectively treat and manage diabetes via multiple mechanisms of the medicinal plants within the compound. Thus, purposeful mixtures of the medicinal plants mentioned above have been comprehensively evaluated for their effective uses in diabetic patients. Therefore, JT2016 polyherbal products are as follows: JT2016A, JT2016B, and JT2016C made of the same three medicinal plants using different ratios. The different ratios of JT2016 compound were prepared according to Indigenous knowledge.

CHAPTER 5

5. CONCLUSION

Jiang Tang 2016 is an anti-diabetic herbal compound made of South African medicinal plants, developed for the management and treatment of T2DM. JT2016 was tested for its safety and hypoglycaemic effects in HFD/STZ diabetic SD rats in this study. Based on the results obtained, the anti-diabetic effects of JT2016 in HFD/STZ diabetic SD rats were positive. JT2016 significantly lowered the blood glucose levels showing positive hypoglycaemic effects and was shown to significantly alleviate the insulin resistance in HFD/STZ diabetic SD rats. Moreover, JT2016 significantly lowered inflammatory levels in experimental rats (HFD/STZ diabetic SD rats) confirmed by significantly lowered plasma levels of the anti-inflammatory cytokines, TNF-alpha and IL-6.

Furthermore, according to this study JT2016 is found to be safe to use, the AST and the proteins (albumin, globulin, and total proteins) are shown to be unaffected. The overall results of these study thus confirm that JT2016 is a potential hypoglycaemic compound that can be developed as an effective and safe anti-diabetes herbal compound for the treatment and management of T2DM.

Therefore, further *in vivo* and *in vitro* investigations are required to persuasively elucidate JT2016's anti-diabetic effects with the use of different cell lines e.g. fat cells (3T3-L1), muscles cells (C2C12), liver cells (CT-1) etc. and different diabetic animal models with genetic mutation e.g. db/db mice for a longer than 6 weeks treatment period with JT2016. This is to investigate more details on how JT2016 impacts blood glucose, IR, body weight, blood lipids, as well as other underlying molecular mechanisms.

CHAPTER 6

6. REFERENCES

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