Antidiabetic activity of *Warburgia salutaris* (Bertol. f.) Chiov. (Canellaceae)

By

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Declaration

I, Nontokozo Zimbili Msomi state that the dissertation, which I hereby submit entitled "Antidiabetic activity of *Warburgia salutaris* (Bertol. f.) Chiov (Canellaceae)" for Master of Science is my original work. It has not been previously submitted by me for a degree at this or any other tertiary institution.

Student: NZ Msomi ……. Date ……

Supervisor: Dr MBC Simelane ……. Date ……

Co-supervisor: Dr P Murambiwa ……. Date ……

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Abstract

Diabetes mellitus is a chronic metabolic disorder which has become a major risk to the health of mankind, as its prevalence is increasing rapidly, globally. Currently available treatment options in modern medicine have several adverse effects. Thus, an accumulative need to develop alternative, cost effective, safe and active treatment for diabetes is necessary. *Warburgia salutaris* is a traditional medicinal plant which is used in treating numerous ailments. In this study, the stem bark of *W. salutaris* was extracted with dichloromethane from a compound NN-01 was isolated by means of column chromatography technique. Spectroscopic analysis (^{1}H NMR, ^{13}C NMR and MS) was used to identify NN-01 as Mukaadial acetate. The structure was verified by X-ray crystallography technique. Cytotoxicity and glucose utilisation screening assays (*in vitro*) were determined using L6 cells. α- Amylase and α-glucosidase inhibitory activity was executed *in vitro*. 1,1-diphenyl-2- picrylhydrazyl was used to determine antioxidant activity. Antidiabetic study (*in vivo*) was conducted by inducing diabetes mellitus in male Sprague-Dawley rats with a single i.p injection of streptozotocin (60 mg/kg). M. acetate (0.5, 1.5 and 2.5mg/kg) and crude extract (1.5mg/kg) were administered as treatment options, with acarbose as a positive control. Parameters such as blood glucose and insulin concentration, haematology, food intake, water intake, weight change and urine volume were determined. Harvesting of the liver, muscle, kidneys and pancreas was done at the end of experimental study period for further analysis.

The cell viability effect of M. acetate was highest at 3µg/ml with a percentage of 98.4. M. acetate also significantly and dose dependently increased glucose utilisation up to 215.18% (12.5µg/ml). The crude extract and M. acetate dose dependently inhibited the enzymes α-amylase and α-glucosidase. A scavenging activity against DPPH was displayed by both the crude extract and M. acetate in a concentration dependent manner. M. acetate and crude extract did not exhibit blood glucose-lowering activity against STZ, with no significant increase in plasma insulin concentration compared to the control. However, M. acetate and crude extract displayed a protective effect by stabilizing body weight of STZ-induced animals. Furthermore, M. acetate was capable of normalizing some haematological parameters induced by STZ.

The compound M. acetate isolated from *W. salutaris*, revealed considerable cytotoxicity with a significant effect on glucose utilisation *in vitro*. It was further observed that the compound

prevented the delivery and absorption of carbohydrates *in vitro*, by inhibiting the key carbohydrate metabolising enzymes. M. acetate also displayed a promising scavenging effect on free radicals *in vitro.* However, *in vivo* M. acetate exhibited no hypoglycaemic effect, therefore, further studies are required to confirm the promising therapeutic efficacy of M. acetate.

Key words: *Warburgia salutaris*; Mukaadial acetate; Antidiabetic activity; Glucose utilisation

Abbreviations

ATP	Adenosine triphosphate
BA	Basophil
CAD	Coronary artery diasease
CE	Crude extract
DM	Diabetes mellitus
DN	Diabetic nephropathy
DPP-4	Dipeptidyl peptidase
DPPH	1,1-diphenyl-2- picrylhydrazyl
DR	Diabetic retinopathy
EGGG	Epigallocatechin gallate
EO	Eosinophil
ER	Endoplasmic reticulum
GDM	Gestational diabetes mellitus
GLP-1	Glucagon-like peptide
GLUT4	Glucose transporter 4
HCT	Haematocrit
IDDM	Insulin-dependent diabetes mellitus
INS	Insulin
K_{ATP}	Sensitive potassium
LY	Lymphocyte
MA	Mukaadial acetate
MO	Monocyte
NAD	Nicotinamide adenine
NE	Neutrophil
NGB	Haemoglobin
NIDDM	Non-insulin dependent diabetes mellitus
NMR	Nuclear magnetic resonance
RBC	Red blood cell

SGLTZ	Sodium-Glucose co-Transporters
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TLC	Thin layer chromatography
WBC	White blood cell
WHO	World Health Organization

Contribution to Knowledge

See appendix E

A. Publication

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Chapter 1

Introduction

Diabetes is a word denoted from the Greek word siphon, as the affected individuals experience polyuria (pass water like a siphon) [1]. Diabetes is classified into mellitus or less often, insipidus [1]. However, the term is often used without qualification, designating it as diabetes mellitus [1]. Diabetes mellitus (DM) is a metabolic disorder characterised by chronic raised plasma glucose levels as a result of inadequate insulin emission, insulin action or both [2], affecting the body in terms of physical, psychological and social health [3]. The disorder is due to dysfunction of the pancreatic β-cell, whereby insulin production is impaired or lost, or a defective form of insulin is secreted [4].

In 1980, the World Health Organization (WHO) reported that 108 million people were diagnosed with diabetes and it is estimated the number has quadrupled to 422 million thus far [5]. It was estimated between 2000 and 2012 that DM was the eighth prevalent cause of mortality among diseases like Cardio Vascular Disease, Pneumonia, HIV/AIDS and Chronic Obstructive Pulmonary Disease [6]. WHO has projected that by 2030 diabetes will be the 7th main cause of mortality [5], as the number of people with diabetes is likely to double by the year 2030 [2]. This increasing number is due to aging, urbanization, population growth and the escalating prevalence of physical inactivity and obesity [7].

The conventional DM treatment options use blood glucose-lowering agents, however these have limitations [8]. Moreover, in low socio-economic countries conventional drugs are not easily accessible. These restrictions have created a crucial need to find effective and affordable treatments for the progression of DM [9]. Hence, there has been a sudden interest in researchers to evaluate alternative medicine and natural therapies, particularly medicinal plants [2]. These plants have been used in developing countries to treat numerous diseases, including DM [10]. Previous studies have clinically evaluated these plants to possess significant antidiabetic properties [11]. Furthermore, phytochemicals accountable for these antidiabetic properties have been isolated over the years from plants. Thus, WHO has recommended the investigation on medicinal plants as antidiabetic treatment options, as they are deduced to be nontoxic, effective, with minimum adverse effects and affordable [3].

1.1 Structure of the dissertation

Chapter 1: This chapter provides the background on diabetes mellitus, outlining its problematic proclamation.

Chapter 2: This chapter gives detailed information on diabetes mellitus: antiquity, classification, diagnosis, complications and the therapeutic interventions of diabetes. The selected plant for this study has also been discussed briefly in this chapter.

Chapter 3: The chapter provides the methodologic outline of the stated study.

Chapter 4: The chapter gives an overview of the findings of the present study.

Chapter 5: This chapter provides the discussion of the results connecting it to literature.

Chapter 6: This chapter gives a brief conclusion of the findings of the present study. Recommendations are also looked at in this chapter.

Chapter 2

Literature Review

2.1 Prevalence of Diabetes Mellitus in Africa

Diabetes mellitus is a disease that has been rapidly growing. In 1985, an estimated 30 million people suffered from this chronic disease which increased to 230 million in 2006, accounting for 6% of the world population. A percentage of 80 of this number was found to be in developing countries [12], with 5.1% of the population in Africa diagnosed with diabetes and 76% of the mortality rate occurring in diabetic people under the age of 60 [13]. In the next two decades, the number of individuals in Africa to be afflicted with diabetes is estimated to escalate substantially [14]. However, diabetes still remains one of the largest chronic diseases regardless of the alarming statistics [1].

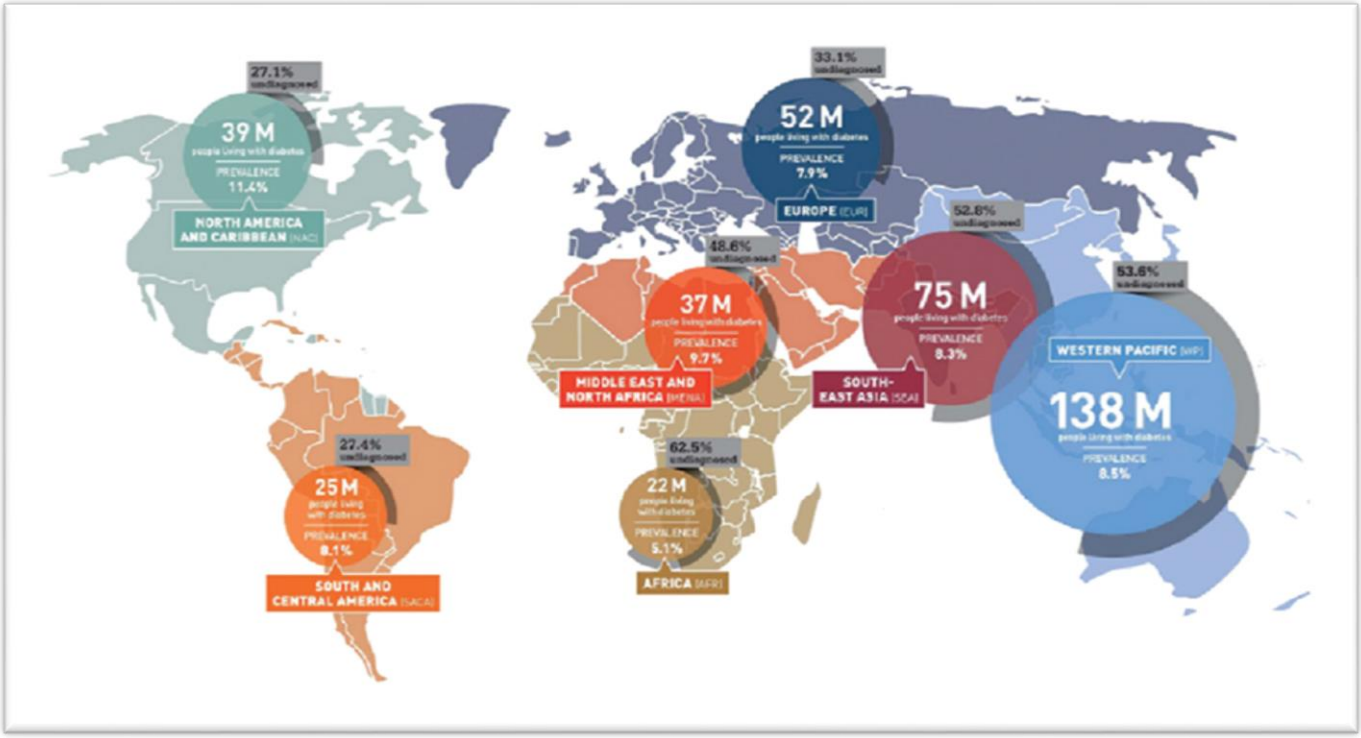


Figure 2.1: Prevalence of Diabetes worldwide with 5.1 % of the African continent diagnosed with diabetes. [15]

2.2 Antiquity of Diabetes Mellitus

Diabetes mellitus (DM) is a disorder which was identified by ancient Egyptians, approximately more than 3000 years ago [16]. Araetus of Cappodocia (81-133AD) invented the word "Diabetes" and in 1675, Thomas Willis (Britain) added the word "Mellitus", as he noted that diabetic urine had a sweet-taste as honey [17]. Mering and Minkowski (1889) discovered that the pancreas played a role in the pathogenesis of DM and this finding later established the basis of insulin isolation by Banting and Best (1921) [17]. Therefore it has been conceptualized that excess glucose production causes DM [2, 18].

2.3 The role of Insulin in Metabolism and Diabetes Mellitus

Insulin, a natural hormone produced by beta cells of the pancreatic islets of Langerhans [19], is a polypeptide with an amino acid sequence, comprising of A and B chains connected by disulphide bridges (Fig 1.2) [19]. It maintains blood glucose levels in the human body by accelerating the uptake of glucose via glucose transporter 4 (GLUT4), into the skeletal muscle fibre and adipocytes [20]. Insulin also regulates the stimulation of lipid, carbohydrate and protein metabolism as well as the transcription of RNA and DNA [21]. Therefore allowing the storage of glucose into glycogen in the liver and skeletal muscle, and deposition of triglycerides in the adipose tissue [20].

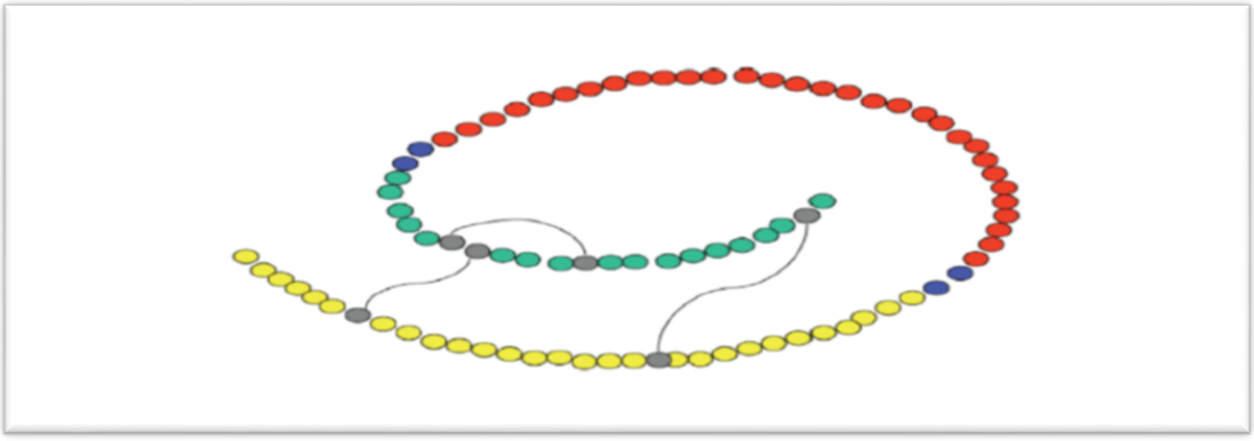


Figure 2.2: Structure of human proinsulin. Insulin's A (green) and B (yellow) chains linked by C-peptide (red) [22]

Insulin synthesis in the beta cells begins with translation of the insulin RNA by ribosomes attached to the endoplasmic reticulum (ER) forming an insulin preprohormone; preproinsulin [23]. This hormone is translocated into the lumen of the ER, cleaving its signal peptide in the ER producing proinsulin. This hormone in turn undergoes scrupulous protein folding, most of this is further cleaved in the Golgi apparatus and packed into secretory vesicles. The translation of proinsulin to insulin occurs in the secretory vesicles and released by exocytosis [24].

Beta cells of the pancreas are responsible for secreting insulin to regulate blood glucose levels. The increase in glucose metabolism elevates adenosine triphosphate (ATP) which affects the plasma membrane by inhibiting the ATP-sensitive K^+ channels. The opening of $Ca2^+$ channels occurs as the cell membrane depolarizes leading to $Ca2^+$ influx. When $Ca2^+$ and ATP levels rise, the plasma membrane fuses with vesicles enclosing insulin, causing insulin release from the cell [25]. Insulin activates translocation of glucose transporters into the adipose and muscle tissue cell membrane, causing glucose to be transported into the cells. To achieve this action insulin binds to plasma receptor proteins situated on insulin responsive tissues. The insulin receptor contains the binding fraction, α_subunit and two β subunits (comprising tyrosine kinase), connected by disulphide bonds. As insulin binds to the receptor it causes autophosphorylation of tyrosine kinase, phosphorylating the insulin receptor substrates (IRS-1, 2, 3 and 4) in turn. The phosphorylated substrates bind to SH2 domains, specifically phosphatidylinositol-3-kinase (PI3-K). The activation of PI3-K promotes phosphatidylinositol 3,4,5-trisphosphate (PIP3) generation. Thus, stimulating the phosphorylation of phosphoinositide-dependent protein kinase 1 (PDK1), activating protein kinase C (PKC) and protein kinase B (PKB) or AKT. The activation of AKT is essential in stimulating GLUT4 translocation towards the plasma membrane, to facilitate the uptake of glucose into the adipose and muscle tissues [8, 26].

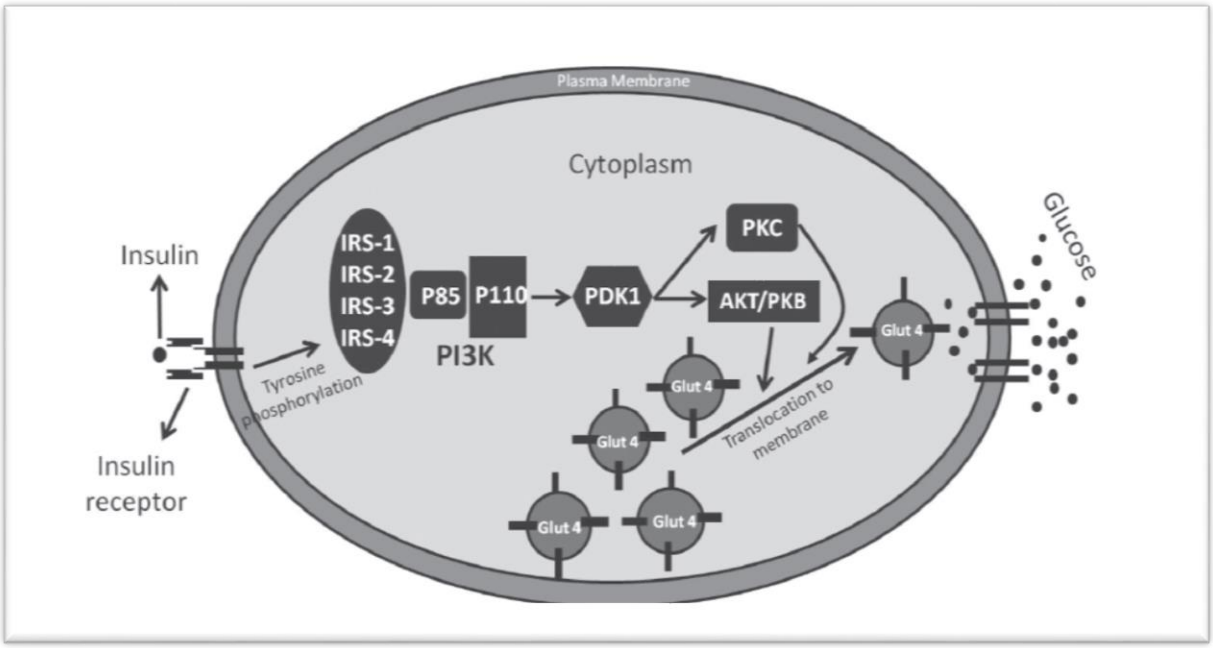


Figure 2.3: The activation of insulin receptors by their ligands. [8] This initiates a number of phosphorylation actions (i.e. phosphorylation of insulin receptor substrate). IRS proteins activates the P13K- pathway by activating P13K, resulting in the production of second messenger PIP3. This activates PDK-1, phosphorylating and activating Akt and PKCs. Therefore, stimulating GLUT4 translocation towards the plasma membrane, enabling the admission of glucose into the adipose and muscle tissues.

Insulin, secreted from the pancreas is released into the portal vein were it is taken up by the liver exerting profound metabolic effects [27]. In the liver, glucose uptake is increased intensely because of the amplified activity of the enzymes glucokinase, phosphofructokinase-I (PFK-l), and pyruvate kinase (PK), the main regulatory enzymes of glycolysis. Glucokinase (GK) acts as a glucose sensor by equipping insulin-secreting cells to connect to changes in glycolytic flux. As the blood glucose concentration is high, insulin secretion is initiated triggering glucose to enter the cell. The phosphorylation of glucose by GK to form glucose-6-phosphate (G6P) leads to a decrease in blood glucose levels. Additionally, due to the incapability of G6P to be transported, it is retained in the hepatocytes acting as a precursor for glycogen synthesis. Following glucose phosphorylation, fructose-6-phosphate (F6P) is generated into fructose-1,6-biphosphate ($F1,6P_2$) by PKF-1. The enzyme PKF-1 is activated by increased levels of adenosine monophosphate (AMP) and fructose 2, 6 bisphosphate ($F2,6P_2$). The generation of $F2,6P_2$ is considered as a regulatory step of glycolysis controlled by the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PFK2/FBPase2). The last

rate limiting step in glycolysis involves the enzyme pyruvate kinase (PK) which is activated by increased levels of $F1,6P_2$. In this step phosphoenolpyruvate is generated into pyruvate. These events lead to diminished blood glucose levels and increased glycogen storage. However, in excessive hepatic glucose production hyperglycaemia may result [28].

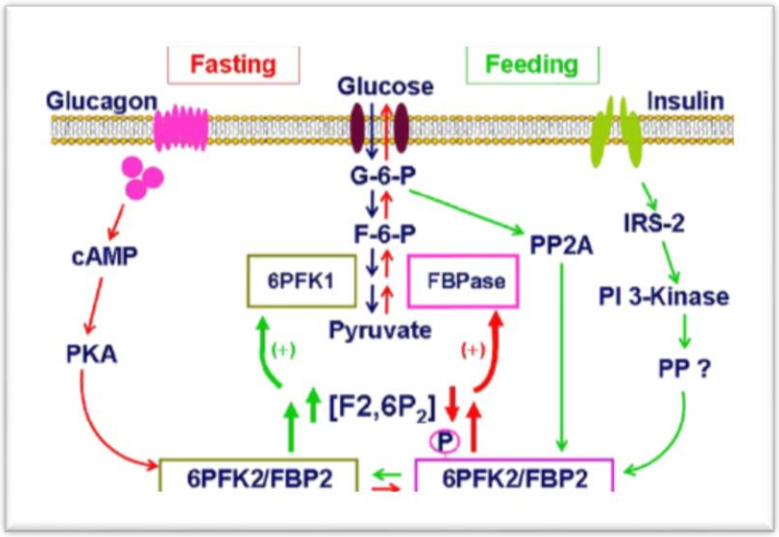


Figure 2.4: The metabolism of glucose in fasted and fed states. **[28]** G-6P: glucose-6-phosphate, F-6-P: Fructose 6-phosphate, 6PFK1: 6-phosphofructo-1-kinase, FBPase: fructose-1,6-bisphosphatase, $F2,6P_2$: fructose 2, 6 bisphosphate, IRS-2: Insulin receptor substrate 2, PI: Phosphoinositide, cAMP: Cyclic adenosine monophosphate, PKA: Phosphofructokinase.

2.4 Pathophysiology of Hyperglycaemia

Hyperglycaemia is a condition due to inadequate insulin for the regulation of blood glucose levels, maintaining the balance between insulin secretion and insulin action [29]. At normal, the pancreatic β-cell adapts to insulin action variations, as insulin sensitivity increases its secretion increases (vice versa) [30]. Figure 1.5 elucidates the relation between β-cell function and insulin sensitivity at normal. As insulin action decreases, the body attempts to compensate by inducing β-cell function [29]. However, β-cell function becomes inadequate resulting in a dysfunction which causes elevated blood glucose levels, thus negatively affecting insulin sensitivity resulting in hyperglycaemia [29, 30]. Prolonged hyperglycaemia produces toxic glucose levels which affects the polyol pathway, PKC activation, hexosamine pathway, glucose autoxidation, methylglyoxal pathway, methylglyoxal formation and glycation, and

oxidative phosphorylation [31]. These pathways lead to oxygen free radical production upsurge resulting in the formation of chronic oxidative stress [32, 33]. Which subsequently damages enzymes and cellular organelles, induces lipid peroxidation, and the development of insulin resistance [34]. These consequences stimulate the development of the disease DM [35, 36].

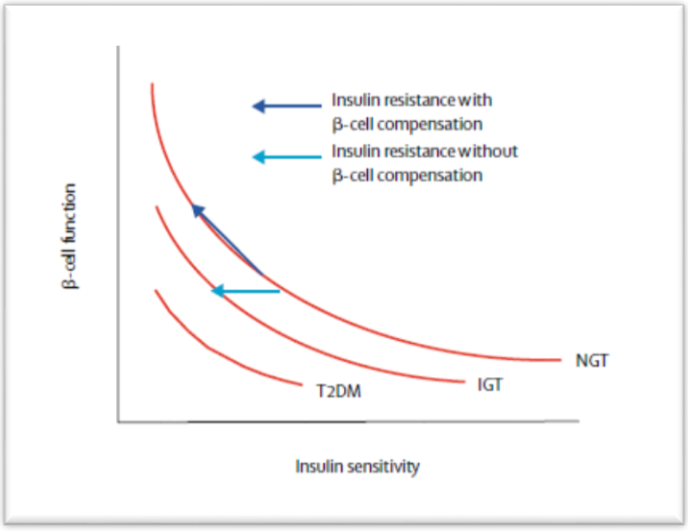


Figure 2.5: The relation between insulin sensitivity and β-cell function. [29] Normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and type 2 diabetes (T2DM)

2.5 Diagnosis of Diabetes Mellitus

According to the Society for Endocrinology, Metabolism and Diabetes of South Africa (SEMDSA), the standard criteria for the diagnosis of DM, to eliminate diabetes a fasting blood glucose level of <5.6mmol/L , for at risk individuals 6.0 to 6.9 mmol/L and for a diabetic individual >7.0mmol/L [37]. The associated symptoms include polyphagia, polydipsia, polyuria and weight loss [16]. Furthermore, to determine glucose tolerance, an oral glucose tolerance test is used (140-199 mg/dL of glucose after a 75g glucose intake) or diabetes (glucose >200mg after 2 hours) [38].

Table 2.1: Diagnostic criteria of Diabetes Mellitus [29]

	Glucose concentration in venous plasma (mmol/L)
Diabetes mellitus	Fasting ≥7 or 2-h post glucose load ≥11.1
Impaired glucose tolerance	Fasting (if measured) <7.0 and 2-h post glucose load ≥ 7.8 and <11.1
Impaired fasting glucose	Fasting ≥6.1 and <7.0 and 2h post glucose load (if measured) <7.8
Glucose load=75g glucose orally	

2.6 Classification of Diabetes Mellitus

In 1997, DM classification was established by the Expert Committee. It is a disorder with multifactorial aetiologies categorized into two main groups Type 1 and Type 2, and gestational diabetes as a third type [30].

2.6.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is identified as insulin-dependent diabetes mellitus (IDDM), also known as Juvenile type as it occurs in children and young adults. IDDM is characterized by the immune mediated pancreatic destruction of beta cells leading to the loss of insulin production. This condition accounts for approximately 5-10% of diabetes [18]. The deficiency of insulin leads to ketoacidosis and death if untreated [30]. Autoimmune disease is the most common form of IDDM whereby beta cells are selectively destroyed [30].

2.6.1.1 Pathogenic factors of Type 1 Diabetes Mellitus

2.6.1.1.1 Genetic Predisposition

Type 1 diabetes is a disorder that involves countless genes. It has been established that there are two approaches to track record of the natural history of T1DM. Firstly, the risk of children developing T1DM is whether the sibling (8%), father (5%) or mother (3%) has the disorder [39]. Secondly, T1DM is associated with alleles including HLA-D antigens, HLA class II DR3 and DR4. The combination of DR3 and DR4 alleles is associated with an increased risk for this condition [40].

2.6.1.1.2 Environmental Factors

2.6.1.1.2.1 Infections

Viral infections have been reported to be a potential cause of T1DM in case reports, seroepidemiological studies and early ecological reports. A number of viruses have been implicated, particularly enteroviruses [41]. In the presence of viruses, the injured pancreas experiences intense inflammation, destroying the β-cells and the surrounding exocrine pancreatic tissue. Recent studies have associated the occurrence of T1DM following enterovirus epidemics by the recognition of enteroviral RNA in the blood and the expression of viral capsid protein VP1 in the islets of T1DM patients [42].

2.6.1.1.2.2 Nutrition

2.6.1.1.2.2.1 Cow's milk proteins

The hypothesis of "cow's milk and T1DM" has been questioned for more than 15 years. *In vivo* studies have verified deleterious effects of proteins from cow's milk in the progression of T1DM. These proteins include bovine serum albumin and b-lactoglobulin which have been observed in diagnosed patients with enhanced humoral and cellular responses to cow's milk [43].

2.6.1.1.2.2.2 Vitamin D

The deficiency of vitamin D in infancy increases the possibility of T1DM, especially in countries located far away from the equator. Therefore, vitamin D supplementation is required daily in the infant, to reduce the possibility of developing T1DM [44]. The protective factor of vitamin D is due to its active role in regulating the immune system and the metabolic pathways related to diabetes [41].

2.6.1.1.2.3 Autoimmunity

Autoimmunity in T1DM is a disease initiated by genetic susceptibility when interacting with environmental factors such as viruses and exposure to parasites/bacteria to cause the immune-mediated process. This succeeds to autoimmunity leading to pancreatic β- cell destruction [45]. Autoantibodies often expressed first are insulin autoantibodies which occur particularly in young children. These autoantibodies may exist for years before the onset of diabetes [46]. Throughout this phase, the secretion of insulin is reduced, developing at quite an adaptable rate towards diabetes [45].

2.6.1.1.2.4 Cytokines

Cytokines are proteins secreted and released by certain immune system cells which have an effect on the communication and interaction between cells. Cytokines include interferons, lymphokines, chemokines, interleukins and tumor necrosis factors. These respond to immune responses, infections, trauma and inflammation [47]. In the instigation of T1DM, infiltrated macrophages during islet injury discharge proinflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-1β (IL-1β) which attract immune cells such as T lymphocytes. β –cells specific antigens in the T cells are activated, infiltrating the exacerbated islets and attack the β-cells. Thus leading to the inability to release insulin with increased blood glucose levels, resulting in the pathogenesis of T1DM [48].

2.6.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is identified as non-insulin dependent diabetes mellitus (NIDDM), accounting for 90-95% of DM [18, 49]. This disorder occurs among the elderly known as "Adult Type" [3], however over the years it has been exhibited in younger individuals referenced as maturity-onset diabetes of the young (MODY) [50]. This form of diabetes signifies disordered metabolism of proteins, carbohydrates and fat [30] which is triggered by insulin deficiency and reduced sensitivity of insulin to the target tissues [9, 30]. Hyperinsulinism is a condition that prevails due to these defects [51]. There are probably many different causes of T2DM, however obesity and lifestyle appear to be the triggering factors in its pathogenesis, in other cases genetic predisposition is also intricate [30].

2.6.2.1 Pathogenic factors of Type 2 Diabetes Mellitus

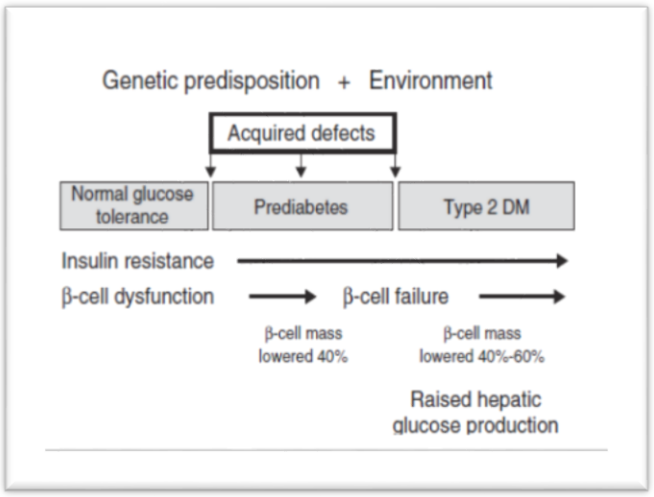


Figure 2.6: Illustrates the pathological features of type 2 diabetes [51]

2.6.2.1.1 Genetic Predisposition Factors

A family history with a positive T2DM increases the risk of developing this disease [29]. A study on identical twins in the United Kingdom revealed a genetic link of 100% concordance rate for T2DM [51]. The genetic basis of diabetes discovered thus far includes Wolframs syndrome, mitochondrial genome defects, MODY syndromes, unusual disorders of extreme

insulin resistance and obesity [51]. Genetic insight has been elusive, genes that have been identified to underlie T2DM include calpain-10 [51], ABCC8, KCNJ11 [52], SLC30A8, JAZF1, TCF7L2 ,PPARG, NOTCH2, WFSI and LGF2BP2 [16].

2.6.2.1.2 Environmental Factors

The environmental factors associated with T2DM are nutrition and physical activity [30, 51]. Additional calorie intake, high fat consumption and lack of physical activity account for these factors leading to obesity and insulin resistance [30]. Deteriorating insulin resistance and the impairment of β-cell function negatively affect the glucose homeostasis system [51]. Therefore increasing the risk of the progression of hyperglycaemia [30, 51]. Furthermore, pharmalogical drugs including diuretics and phenytoin negatively affect glucose metabolism adding to the development of hyperglycaemia [38].

2.6.3 Gestational Diabetes

Gestational diabetes mellitus (GDM) is distinguished as impaired glucose intolerance identified during pregnancy. It develops during the third trimester of pregnancy [30] . This condition affects approximately 7% of pregnancies causing complications which result in more than 200 000 cases annually. The risk of developing T2DM is more frequent in women with prior GDM [53]. The incidence of fasting hyperglycaemia >105mg/dl in GDM is associated with foetal death. The management of GDM is usually by diet, in some cases insulin therapy is recommended [54].

2.6.4 Complications of Diabetes Mellitus

Diabetes mellitus has various forms of complications which are either macrovascular or microvascular [16]. These often result in kidney failure, amputations, fractures, frailty, depression [55] and premature death due to the late recognition and insidious progression, particularly in low socio-economic countries [16]. Macrovascular diseases include diabetic ketoacidosis [56], coronary artery disease (CAD), cerebrovascular disease (CVD) or peripheral arterial disease (PAD) [57]. Whereas, microvascular include retinopathy, neuropathy and nephropathy [56], macrovascular diseases occur due to damage of large blood vessels (veins

and blood vessels arteries) and microvascular diseases are caused by the damage of small blood vessels [31].

2.6.4.1 Macrovascular Diseases

2.6.4.1.1 Diabetic Ketoacidosis

Diabetic ketoacidosis is a metabolic disorder as a resultant of insulin deficiency from new onset diabetes. Deficiency of insulin elevates counter regulatory hormones (cortisol, catecholamines and glucagon). The capability of the cells to use glucose is deprived causing the body to rely on burning fat for energy and producing ketones which accumulate rapidly. Proteins and glycogen are broken-down to form glucose. Thus, hyperglycaemia develops from these factors, causing a hyperosmolar state, metabolic acidosis and osmotic diuresis [58].

2.6.4.1.2 Coronary Artery Disease

Type 2 diabetes mellitus has a strong distinctive association with CAD. The risk factors in diabetic patients related with the development of CAD include insulin resistance, dyslipdemia and hyperglycaemia, which result in endothelial cell, impaired platelet function, vascular smooth muscle dysfunction and abnormal coagulation [59].

2.6.4.2 Microvascular Diseases

2.6.4.2.1 Retinopathy

Diabetic retinopathy (DR), a microvascular complication affecting the retinal vasculature which eventually leads to visual impairment and blindness [60]. In South Africa the prevalence of DR is projected between 5% and 10% [61]. The risk factors for the progression of DR involve prolonged period of diabetes and reduced glycaemic and blood pressure management [62]. The phenomena contributing to pathophysiology of the retinal vasculature destruction include PKC activation, oxidative stress, accumulation of glycation end products and polyols [63].

2.6.4.2.2 Nephropathy

Diabetic nephropathy (DN) is a microvascular complication which causes destruction to capillaries of the kidneys [31]. It is distinguished by the accumulation of proteinuria > 500mg in 24 hours [36]. In Africa the prevalence of DN ranges from 32-57% [64], affecting one patient out of three with T2DM, developing from end-stage renal disease [9]. Studies have identified numerous risk factors related to the progression of DN including high levels of insulin resistance, elevated levels of blood pressure and glycosylated haemoglobin [65].

2.6.4.2.2 Neuropathy

Diabetic neuropathy is long-term complication affecting almost half of diabetic patients [66-68]. The pathogenesis of this disorder is unknown therefore various studies have tried to elucidate its mechanism [67]. These numerous hypotheses include advanced glycation end products, protein kinase C, cytokines, polyol, aldose reductase and reactive oxygen species [67]. This disorder has various symptoms such as burning, "pins and needles", crawling, itching [66], stabbing and unpleasant electric shock [68].Uncontrolled diabetes can result in severe macroangiopathies as well as outcomes such as ictus and stroke [66].

2.7 The management of Diabetes Mellitus

2.7.1 Exercise

Glucose is an essential fuel for endurance exercise in humans. Muscle contraction is an effective stimulus of glucose uptake in skeletal muscle during exercise [69]. The contraction occurs by facilitated diffusion which is reliant on GLUT4 in an insulin independent manner, resulting in glucose uptake [70]. The activation of AMPK has been suggested to promote glucose uptake during exercise [71]. Recent studies *in vivo* have shown that contraction stimulated glucose disposal is not only mediated by AMPK, other signal intermediates suggested include calcium, nitric oxide, bradykinin, and the Akt substrate, AS160 [72]. In type 2 diabetic individuals, exercise reduces circulating blood glucose concentrations and decreases the occurrence of hyperglycaemic episodes throughout the day [73]. This therefore increases insulin sensitivity in the body which lasts up to 48 hours [73]. Prolonged exercise is required, as inactivity increases the risk of complications [74]. Exercise affects fuel metabolism by

shifting from the reliance of free fatty acids to a combination of glucose, fat and muscle glycogen [74].

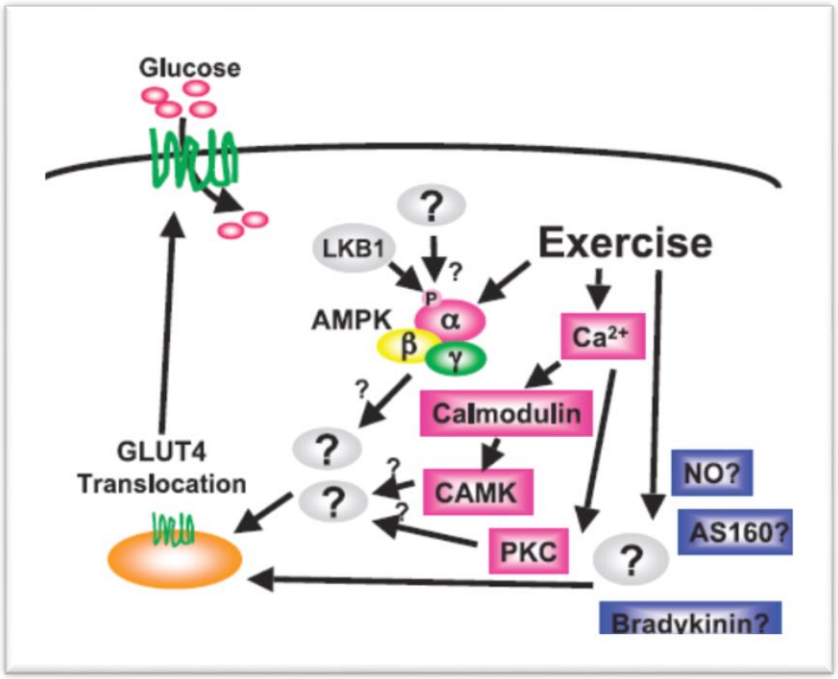


Figure 2.7: The translocation of glucose transporter proteins (GLUT4). **[72]** GLUT4 can be translocated to the cell membrane by muscle contraction. Contractions activate a variety of proteins that may be associated in the signalling mechanism of exercise (i.e. AMPK, nitric oxide, calcium, bradykinin, AS160 and ROS).

2.7.2 Diet

Diabetes is a dietary related disorder, calorie-dense, nutrient depleted and highly processed diet leads to exaggerated blood glucose levels. It has been revealed that diabetic patients fail to control their dietary behaviour which is problematic [75]. The use of dietary fibres delays glucose diffusion, efficiently absorbs glucose and obstructs the action of α- amylase, resulting in depressed blood glucose levels [3]. Obesity is a condition arising from chronic ingestion of calories in surplus of the required needs. It results in excessive growth of adipose tissue. This condition has been linked to T2DM, with regards to epidemiological data [76]. Weight loss in obese diabetic patients is a significant component in the management of diabetes; it positively affects blood pressure, cholesterol and blood glucose levels. A well balanced diet can

accomplish weight loss. However, probable side effects may occur in patients adhering to a diet with low carbohydrates, such as headaches, constipation and hypoglycaemia [77]. The ability of AMPK to switch on catabolic pathways suggests that kinase activators can be effective agents in treating obesity. Thus, reducing the risk of the development of T2DM. The reduction of plasma glucose can occur via the activation of AMPK by inhibiting gluconeogenesis enzymes expression in the liver or by increasing uptake of glucose by muscle and other tissues [78].

2.7.3 Insulin Therapy

Insulin has become the basis for the treatment of diabetes since its discovery in 1922. Insulin therapy suppresses ketogenesis, restores normoglycemia, delays or arrests diabetic complications [79]. Insulin delivery methods include using syringes, insulin pens and subcutaneous insulin infusion [80]. The use of insulin injections may cause weight loss or gain. Insulin can cause hypokalemia by driving potassium into the cell. The combination of insulin injection and other drugs such as thiazolidinedione's can result in cardiac failure. Constituents of insulin preparation can cause allergy [77].

2.7.4 Pharmacological treatments and their limitations

Diabetes has a long progression and grave complications often resulting especially in developing countries due to reconstruction of lifestyle. If exercise and diet are not effective, pharmacological agents are prescribed to the patients [3]. These medications are mostly biochemical or chemical agents [18], which increases secretion of insulin by either acting on pancreatic β-cells (metaglinides and sulphonylureas) or enhancing the peripheral sensitivity to insulin (thiazolidinediones and biguanides). Furthermore, agents with inhibitory effect on intestinal α-glucosidase are recommended, these are able to decrease postprandial hyperglycaemia [8].

2.7.4.1 Biguanides

Biguanides are drugs reducing the hepatic glucose production [38]. Metformin is a biguanide

drug derived from the plant *Galega officinalis*, used as the first line of treatment for T2DM [81]. Studies have reported that treatment with metformin in controlling glucose has reduced the risk of diabetes related complications and mortality in diabetic obese patients, and also related to less hypoglycaemic attacks when compared to other antidiabetic drugs [82]. The key function of metformin is to reduce hepatic glucose production by inhibiting the hepatic glucose output [29, 81]. Metformin also improves cardiovascular safety and peripheral insulin sensitivity [81]. The mechanism of action of metformin includes AMPK activation in the hepatocytes [83], stimulated by the inhibitory effect of the drug on the respiratory chain complex I [82] and by blocking adenylyl cyclase via the inhibitory effect on glucagon-induced cAMP production [84]. However metformin has side effects including nausea, weight gain, diarrhoea, abdominal discomfort [38] and lactic acidosis [81].

2.7.4.2 Sulfonylureas

Sulfonylureas increases the production of insulin by stimulating pancreatic beta cells [3]. In the ATP-sensitive potassium (K_{ATP}) channel these bind to SUR subunit therefore closing the channel [85], resulting in enhanced insulin secretion[29]. However, the closure of the channels is not absolute, consequently the inhibition of the high-affinity sulfonylureas is not complete [85]. Sulfonylureas in the liver limits gluconeogenesis by decreasing the metabolism of lipids and reducing the clearance of insulin. These agents are grouped in to two categories: first-generation agents (tolbutamide, chlorpropamide and tolazamide) and second-generation agents (glyburide, glipizide and glimepiride) [77]. These drugs proliferate the risk of weight gain [81], cardiovascular disease and hypoglycaemia [86].

2.7.4.3 Non-sulfonylurea secretagogues

Non-sulfonylurea secretagogues are agents acting on the K_{ATP} channel in the pancreas, stimulating the beta cells to release insulin. These are similar to sulfonylureas however act at different binding sites [16]. These include nateglinide and repaglinide [81]. The action of meglitinide has a short duration and rapid onset hence it has a lower risk of hyperglycaemia [16].

2.7.4.4 Sodium-Glucose co-Transporters (SGLT2) Inhibitors

SGLT2 inhibitors are a class of antidiabetics which act by inhibiting renal glucose reabsorption in the kidneys. These are the main cotransporters for tubular glucose reuptake, in abundance of SGLT2 cotransporter's glycosuria arises. Therefore, inhibitors of this activity reduce blood glucose levels by blocking glucose reabsorption from the renal tubule. Several trails have investigated the safety and efficacy of these inhibitors, displaying body weight reduction, amended glucose control and blood pressure with a low risk of hyperglycaemia [87].

2.7.4.5 Incretin Mimetics

Glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic peptide are the main incretin hormones. These are secreted from the small intestine endocrine cells in the epithelium, stimulated by induced concentration of glucose in the intestinal tract. Incretins promotes insulin secretion by stimulating the pancreatic β-cells but inhibit α-cells from discharging glucagon. Both these hormones are rapidly degraded by dipeptidyl peptidase (DPP)-4 which is a serine protease. *In vivo* studies have recognised the importance of DPP-4 as a determining factor of incretin concentrations because DPP-4 inhibitors avert the breakdown of glucose-dependent insulinotropic peptide and GLP-1. Thus, there are 2 classes of incretin drugs which have been developed: DPP-4 inhibitor and GLP-1 analogue [88].

2.7.4.6 Glucagon-like peptide-1 (GLP-1) Receptor Agonists

GLP-1 is a hormone that increases insulin secretion, decreases glucagon secretion and maintains blood glucose levels. The excretion of GLP-1 is often reduced in diabetic patients. GLP-1 receptor agonists currently available include liraglutide and exenatide. These agents exhibit augmented resistance to enzymatic degradation by DPP4 [89].

2.7.4.7 Dipeptidyl peptidase 4 (DPP-4) inhibitors

In managing T2DM by inhibiting DDP-4, it prevents the inactivation of GLP-1. Therefore, increasing the secretion of insulin and reducing glucagon secretion, in turn lowering blood glucose levels [90]. DDP-4 inhibitors include vidagliptin, linagliptin, sitagliptin, saxagliptin and alogliptin [77].

2.7.4.8 Thiazolidinedione's

Thiazolidinedione's improve insulin sensitivity to the muscles and other tissues [3, 81]. These agents act on peroxisome proliferator-activated receptor alpha (PPARY), causing it to bind to 9-*cis* retinoic acid receptor. The binding regulates genetic transcription and translation of proteins involved in glucose and lipid metabolism[91]. Thiazolidinedione's include pioglitazone and rosiglitazone [81]. The use of rosiglitazone is restricted due to the increased risk of cardiovascular complications [16]. Pioglitazone can be used in renal impairment cases, as it is not associated with hypoglycaemia however it raises concerns regarding fluid retention, peripheral edema and risk fracture in women [16]. Side effects of thiazolidinedione include weight gain and edema [81].

2.7.4.9 α- Glucosidase inhibitors

α-Glucosidase inhibitors interrupt the carbohydrate absorption from the gastrointestinal tract [3]. These include acarbose, voglibose and miglitol. α-Glucosidase inhibitors are mostly effective for postprandial hyperglycaemia [16] and also modulating the incretin hormones i.e. increasing endogenous glucagon-like peptide-1 or replicate the action of GLP-1. Side effects include abdominal pain, diarrhoea and flatulence [81].

2.7.5 Supplements

2.7.5.1 Vanadium

Vanadium has not been recognized as an essential nutrient; it exists in vanadyl and vanadate forms. It has been shown in animal models to facilitate amino acid, lipid and glucose metabolism, the uptake and improvement of insulin sensitivity. In humans, it improves glucose oxidation, hepatic glucose output and glycogen synthesis. Vanadium affects numerous points

in the pathway of insulin signalling and may result in the upregulation of the insulin receptor, leading to increased tyrosine serine threonine kinase activity, inhibition of hepatic gluconeogenesis and improved synthesis of glycogen. Oral supplementation of vanadium may cause vomiting, nausea, flatulence, cramping and diarrhoea [92].

2.7.5.2 Chromium

Chromium is a trace element that is necessary for the regulation of normal glucose metabolism. A deficiency of chromium results in impaired glucose tolerance which can be improved by adding chromium to the diet. Chromium has been reported to decrease fasting glucose levels, decrease insulin levels, and reducing total triglycerides and cholesterol whereas HDL cholesterol being increased. This element performs by enhancing insulin actions through an increase in the sum of insulin receptors and increased binding of insulin to the insulin receptors. Studies in vitro have suggested that chromium picolinate at high doses may result in chromosomal damage at increased rates. Chromium at high doses has been shown to cause hepatic and renal toxicity, psychiatric disorders, hypoglycaemia, rhabdomylosis, competes with iron for transport on transferrin and decreases zinc absorption [92].

2.7.5.3 Nicotinamine

Vitamin B_3 occurs in two forms, nicotinamide and nicotinic acide. Nicotinamide adenine dinucleotide NAD and NAD phosphate are the active coenzyme forms responsible for the functions of numerous enzymes and standard lipid, protein and carbohydrate metabolism. Studies suggest that nicotinamide acts as a defensive mechanism for the pancreatic β-cell against autoimmune destruction. However, side effects associated with nicotinamine include dizziness, nausea, heartburn, skin reactions, vomitting, sore mouth, fatigue and hepatotoxicity [92].

2.7.5.4 Magnesium

Magnesium is a mineral that acts as a cofactor for more than 300 enzymes. A deficiency of magnesium is connected to insulin resistance, complications of diabetes, dyslipidemia and glucose intolerance. However, the mechanism by which magnesium affects these

phenomenons is unknown. High doses of magnesium may cause nausea, headaches, central nervous system disorders, altered cardiac function and death [92].

2.7.5.5 Vitamin E

Vitamin E is a vital fat-soluble vitamin which acts as an antioxidant. Low levels of Vitamin E are linked to increased occurrences of diabetes, diabetic people are suggested to have reduced levels of antioxidants. Vitamin E functions to counteract free radical species developed during cellular metabolism, shielding cellular membranes and lipoproteins. It may also treat and prevent diabetes complications such as neuropathy and nephropathy. Vitamin E has been shown to cause increased risk of haemorrhage stroke [92].

2.8 Medicinal Plants in managing Diabetes Mellitus

Diabetes treatment requires immense quantities of resources including physical training, diets and medicines in all countries [18]. Conventional drugs also have adverse effects such as lactic acid intoxication, gastrointestinal upset and hypoglycaemia [18]. However, traditional medicine has been perceived to have minimal side effects in scientific research and moderately low costs [2]. Plants have been an effective source of medication against numerous diseases since ancient times [93]. Asia and Africa have a profound culture on the use of traditional remedies [94]. It is approximated that 80 percent of South Africans rely on medicinal plants for their health care needs [95]. These medications have been recognized as an alternative measure in managing diabetes [18]. Hypoglycaemic action of medicinal plants has been established in *in vivo* and *in vitro* studies [3]. Numerous compounds have been identified and isolated from plants used as antidiabetics [96].

2.8.1 Plants used in the management of Diabetes Mellitus

2.8.1.1 *Aloe vera*

Aloe vera is a plant that has been used in herbal medicine for centuries in numerous cultures. [97]. It is botanically called Aloe barbadenis miller, belonging to the family Asphodelaceae (Liliaceae) [98]. *Aloe vera* has been used intensively as a folk remedy in treating burns, eczema, wounds and psoriasis [99]. The active components of this plant are found in the rind of the leaves and gel [100]. Pharmacological study in streptozotocin-induced diabetic rats revealed

the significant reduction in blood glucose after ethanolic extract administration of *Aloe vera* gel [97, 100]. Hypoglycaemic activity has also been investigated in alloxan-diabetic rats [99].

2.8.1.2 *Panax ginseng*

Panax ginseng is a herbal plant from the family *Araliaceae* [101], native to Korea and China with active agents known as ginsenosides which are triterpene saponins [101, 102]. It is used traditionally for hypotonia, insomnia, neuralgia, neurasthenia, depression, as a stimulant, stomachic and diuretic [99]. The root has been used as a tonic without any side effects for years [103]. A pharmacological study by Chung et al (2016), revealed a reduction in hyperlipidaemia, body weight gain and hyperglycaemia in diabetic animals receiving ginseng in their diet [102]. In addition, *P. ginseng* extract has been reported to increase the number of insulin receptors in the bone marrow and decrease the number of glucocorticoid receptors in the rat brain. These parameters contribute to the antidiabetic action of *P. ginseng* [104].

2.8.1.3 Green Tea

Green tea is formed by enzymatic inactivation of *Camellia sinensis* leaves [105], it is the most consumed beverage [106]. Botanical evidence suggests that the cultivation of green tea occurred in China and India originally [107]. It is abundant in catechins, mainly epigallocatechin gallate (EGCG), which has been suggested to have numerous beneficial health effects [106]. Studies *in vitro* have suggested that EGCG helps in preventing hyperglycaemia by improving the activity of insulin and perchance by averting damage to β-cells [106]. This catechin has been shown to inhibit differentiation in 3T3-L1 cells and adipocyte proliferation, escalate fat oxidation and increase GLUT4 expression in adipose tissue of an animal model. [108].

2.8.2 Class of Antidiabetic Compounds

2.8.2.1 Flavonoids

Flavonoids are dispersed in plants as naturally occurring phenolic compounds [109]. These have anti-oxidant properties as they possess aromatic hydroxyl groups [109]. Flavonoids have been extensively studied due to their beneficial properties in relieving cardiovascular disease [110]. These compounds overturn the glucose level, decrease plasma triglycerides and cholesterol, and induce hepatic glucokinase activity hence increasing the release of insulin from the pancreatic islets [3].

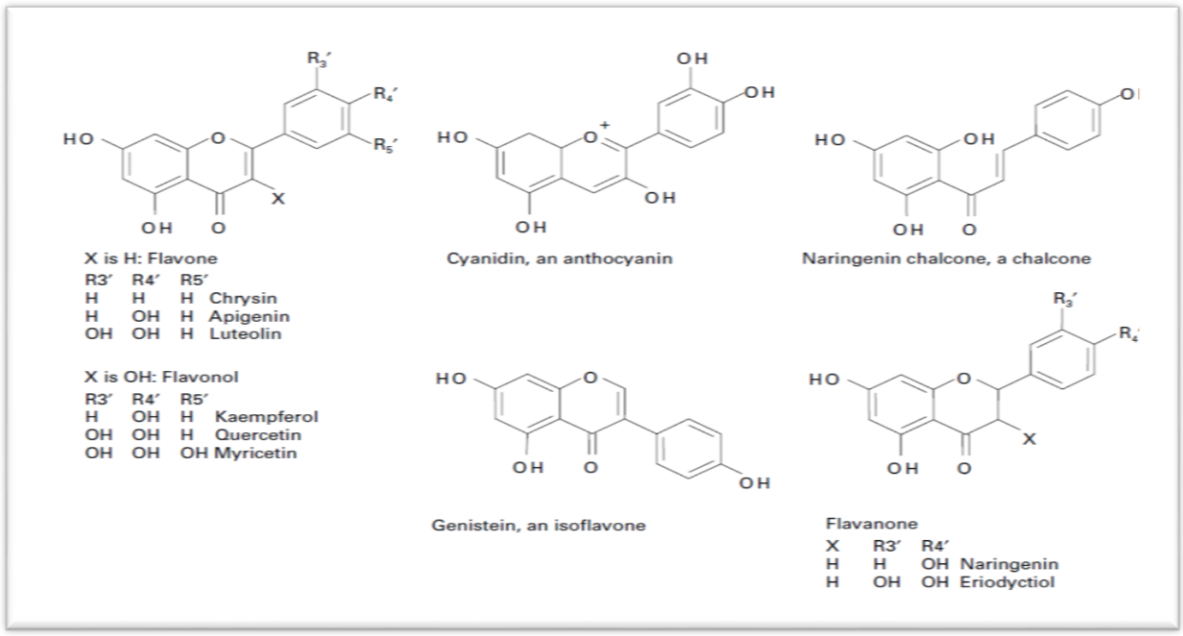


Figure 2.8: Selected flavonoids [111]

2.8.2.2 Alkaloids

Alkaloids are secondary compounds found in several sources including fungi, animals and plants [112]. These compounds have a heterocyclic nitrogen, acting as a H-acceptor and a H-donor [113]. This capability improves the probability of hydrogen bonds, which are critical for acting as therapeutic drugs or bioactive compounds [113]. Alkaloids cause the inhibition of α-glucosidase and reduce transportation of glucose through the intestinal epithelium [3]. Berberine, palmatine, coptisine, and jatrorrhizine are alkaloids that have been confirmed to possess beneficial effects on diabetes and its complications [114].

Jatrorrhizine **Coptisine**

Figure 2.9: Chemical structures of Alkaloids with antidiabetic effect. [115]

2.8.2.3 Saponins

Saponins are naturally produced in plants as glycosides [116]. These compounds contain a sugar moiety usually galactose, glucose, glucuronic acid or methylpentose, linked glycosidically to hydrophobic aglycone such as steroid or triterpenoid (fig 1.5) [116]. Saponins are able to stimulate insulin discharge, blocking glucose formation in the bloodstream [3].

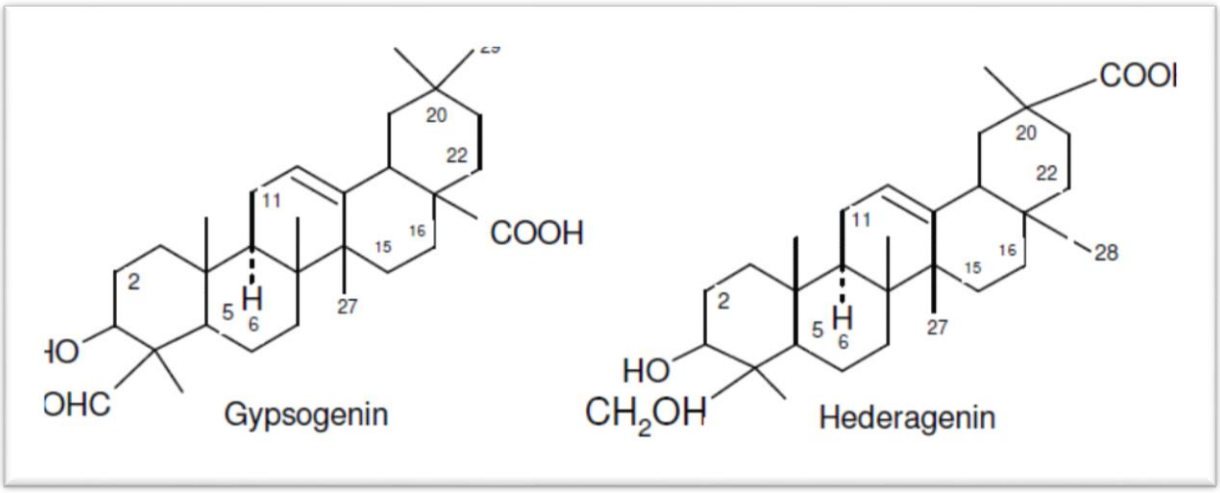


Figure 2.10: **Chemical structures of selected Saponins [117]**

2.9 Selected plant for this study

2.9.1 *Warburgia salutaris*

The genus *Warburgia* belongs to the family Canellaceae, comprising of nine species. The species *W. salutaris* (Bertol. f.) Chiov. is distributed in southern Africa, as a rare constituent in Swaziland, Mozambique, Zimbabwe and South Africa [118]. *W. salutaris* is commonly known as the 'pepper-bark' due to its peppery taste of the leaves and bark [119, 120], also known as 'mulanga' in Venda and 'xibaha' in Tsonga [119]

2.9.1.1 Botanical description

W. salutaris is a medium sized evergreen tree, about 5 to 10 m in height with a stem diameter reaching 30 cm (Fig 2.16). Its trunk is short with an erect, dense canopy. The bark at a young phase is smooth, white to light grey, once it matures it becomes brown. The inner bark is reddish brown in appearance, bitter with a pungent and peppery smell. The leaves have a burning, bitter and aromatic taste when crumpled. The leaves are alternate, simple, dark green above, paler green below and glossy. The flowers are white to greenish in appearance, bisexual, small, solitary and axillary. The flowers grow into round, oval berries, dark green turning purple when ripe and narrowed towards the base. The fruits have a leathery surface, enclosed with glands comprising two or more seeds, during maturation they turn black from purple [119].

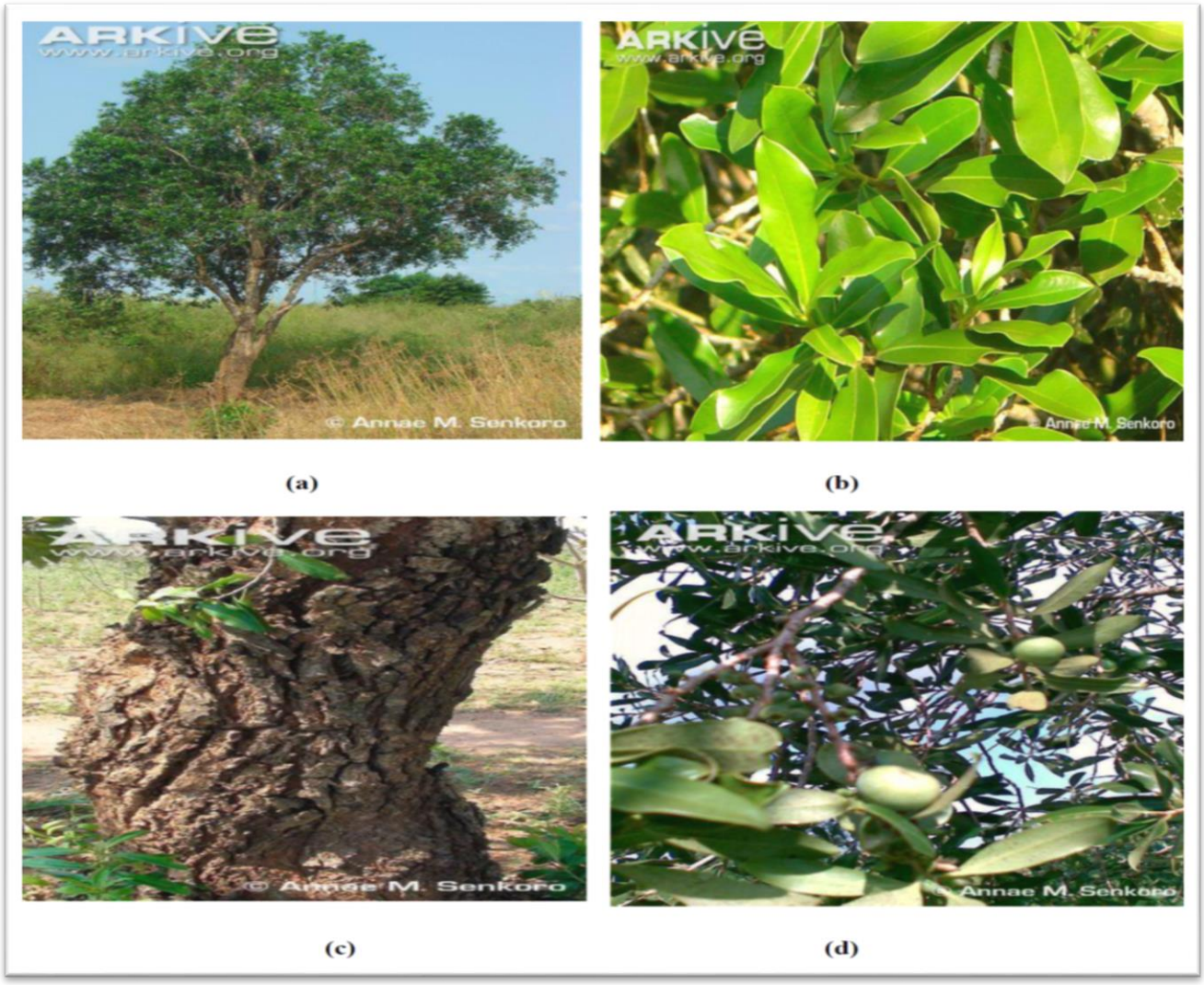


Figure 2.11: Warburgia salutaris: Mature specimen (a), foliage (b), fruit (c) and bark (d). [121]

2.9.1.2 Phytochemistry

Studies have reported numerous drimane sesquiterpenoid compounds isolated from the leaves and bark of *W. salutaris* consisting of biological activities such as antibacterial, antifeedant, antifungal, anticomplemental, phytoyoxic, cytotoxic and molluscicidal activities [122]. These compounds include warburganal, salutarisolide, polygodial, muzigadial, ugandensidial, isopolygodial and mukaadial[120].

2.9.1.3 Medicinal uses

W. salutaris has been used ethnopharmacologically for dermatological pathologies such as sores, urethral inflammation and penile irritation. The powdered bark is applied to sores and inflamed areas. Ointments produced from pounded stalks and leaves, in concoction with the bark and fat, are used for skin irritations and inflammation [122]. A decoction of the bark is usually administered for influenza, colds, sinus [118], as an emetic to clear patches in the lungs and chest infections productive of purulent sputum [123].

2.10 Aim and objectives

2.10.1 Aim

To biologically screen the antidiabetic potential of the plant *W. salutaris.*

2.10.2 Objectives

- To collect the plant material and prepare a voucher specimen
- To isolate, purify and characterize bioactive compound(s) from the plant using chromatographic and spectroscopic techniques.
- To investigate the cytotoxicity of the isolated compound(s).
- To determine *in vitro* the effectiveness of the isolated compound(s) on glucose uptake.
- To investigate *in vitro* the inhibitory action of the crude extract and isolated compound(s) on digestive enzymes.
- To determine *in vitro* the antioxidant ability of the crude extract and isolated compound(s).
- To determine the *in vivo* antidiabetic activity of the crude extract and isolated compound(s).

Chapter 3

Materials and methods

This chapter describes briefly all the methodologies carried out in this study. A list of the significant chemicals, reagents and equipment used in this study is also given. Information pertaining the reagents preparation and details of the methodologies are in Appendix A and B.

3.1 Materials

Chemicals applied in this study were of analytic grade as indicated: Dichloromethane, hexane, ethyl acetate, tween 20, sulphuric acid, ascorbic acid, nutrient agar, potassium dihydrogen orthophosphate, di-potassium hydrogen orthophosphate anhydrous, sodium citrate, sodium chloride, sodium hydrogen phosphate, citric acid sodium dihydrogen phosphate (Merck chemicals (PTY) LTD, Johannesburg South Africa), streptozotocin (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria South Africa), acarbose, starch, alpha glucosidase, P-Nitrophenyl-α-D-glucopyranoside, DNSA, 1,1-diphenyl-2- picrylhydrazyl (Sigma-Aldrich Co., Ltd Steinheim, Germany).

Equipment:

Grinder- Hippo Mills (AC Trading, South Africa)
Rotary evaporator (Heidolph Instruments GmbH & Co, Germany)
UV Light- CL-150 Ultraviolet Fluorescence Analysis Cabinet (Spectronics Co, New York)
Nuclear Magnetic Resonance (Bruker)
Spectrophotometer- Biowave DNA (Biochrome, Cambridge UK)
Spectrostar Nano Plate reader (MBG LABTECH, Germany)
Sprague Dawley rats-Biomedical research unit, University of KwaZulu-Natal

3.2 Methods

3.2.1 Plant collection and preparation

The plant species used in the present study, *Warburgia salutaris* (Bertol. f.) Chiov. (stembark) was collected from the Botanical garden of the University of KwaZulu-Natal, Pietermaritzburg Campus, KwaZulu-Natal, South Africa (29° 37′ 30″ S, 30° 24′ 14″ E), in March 2017. The plant was authenticated and reconfirmed by Mrs Alison Young (horticulturist) and voucher specimen (NU0043932) was obtained at the UKZN's Bews Herbarium (PMB). The plant material was washed and sun dried for one week, weighing 16 kg. The material was thereafter ground into fine powder form using a Hippo-grinder obtained from the botanical garden at UKZN-PMB campus and stored in a brown glass bottle.

3.2.2 Extraction of crude extract

The powered stembark of *W. salutaris* (3.9 kg) was extracted with dichloromethane (DCM) (1:5w/v) for three days successively. Whatman (No.1) filter paper was used to filter the extract and concentrated under a reduced pressure (45°C) to a minimum volume using a Heidolph rotary evaporator. The concentrated extract was desiccated under a fume hood overnight. The resultant DCM crude extract weighed 58g.

3.2.3 Chromatographic analysis

In this study, silica gel column chromatography (60x1000mm; Merck silica gel, 60: 0.063-0.200mm) was performed to isolate the DCM crude extract and the eluent system used was hexane: ethyl acetate. The distillation of hexane and ethyl acetate at 67°C and 77°C respectively was prepared preceding the analysis. The eluent ratio used was 9:1 to 8:2. In each column, 10g of crude extract was used with 300g silica gel.

Thin layer chromatography (TLC) (silica gel 60 aluminium sheets, F254—Merck, Whitehouse Station, New Jersey, USA) was used to monitor the presence of a compound. The TLC spots were fixed with a solution made of 20% H_2SO_4 in methanol, heated with a hair dryer for colour development and visualized under Spectroline UV light (Berg Engineering & Sales, Company, Inc. Rolling Meadows, United States) at 254nm. The fractions were collected per 80ml and

accordingly combined through their TLC profiles. The retention factor value of the visualized compound was 0.6. The fractions were left overnight to dry under a fume hood. A white powder (NN-01) was obtained from the 8:2 ratio of *W. salutaris*.

3.2.4 Structural Elucidation

The structure of the compound was identified using NMR spectroscopy techniques, infrared (IR) and x-ray crystallography at the University of KwaZulu-Natal PMB campus Chemistry department. The NMR techniques conducted in this study are as follows; 1H, 13C, DEPT, COSY, DEPT, HSQC, NOESY and HMBC. Fillezilla 3.21.0 copyright © 2004-2016 Tim Kosse was used to collect the NMR data and process it into spectra. Spinworks 4.0 copyright ©2014, Kirk Marat, University of Manitoba was used to produce the NMR spectrum (See Appendix C).

3.3 *In vitro* studies

3.3.1 Cytotoxicity Evaluation

Cytotoxicity assay was determined at Nelson Mandela Metropolitan University, Department of Biochemistry and Microbiology. L6 cells, originally derived from skeletal muscle of rat were grown in DMEM supplemented with 10% FCS and subcultured by trypsinisation. Cells were seeded into 96-well plates at a density of 5000 cells/well. At approximately 80% confluent the medium was substituted with differentiation medium (DMEM containing 2% horse serum). Cells were incubated for 3-5 days to allow full differentiation.

Spent culture medium was substituted with fresh medium containing the test compounds at the indicated concentrations and treated for 48hr. Spent culture medium was removed and 100µl fix solution (10% formaldehyde in PBS) was added. After removal of the fix solution, 100µl crystal violet was added and incubated at room temperature for 10min. The crystal violet dye was removed and the wells washed three times with tap water and once with distilled water. Plates were left to dry overnight at 37°C and the bound dye solubilised by adding 200µl 10% acetic acid. The absorbance at 595nm was read and the cytotoxicity expressed as a percentage of the untreated control.

3.3.2 Glucose utilisation screening

Glucose utilisation assay was determined at Nelson Mandela Metropolitan University, Department of Biochemistry and Microbiology. Cells were seeded and treated identically as described for the cytotoxicity assay except that after the 48hr treatment and removal of spent culture medium, the cells were washed once with PBS. Fifty micro-litres of 8mM glucose solution (DMEM medium diluted with PBS and supplemented with BSA to a final concentration of 0.1%) was added. For the positive control, 1000ng insulin was added. Plates were returned to the incubator. After 2 hours 5µl was transferred to a new plate and 200ul glucose assay reagent (glucose oxidase/peroxidase colorimetric reagent) was added and incubated at 37° for 10min. Absorbance was measured at 510nm. Glucose utilisation was calculated as the difference between the no cell control and the test sample and expressed as a percentage of the untreated control.

3.3.3 α- Amylase inhibition assays

The inhibition of α- amylase was determined according to the method described by Sathiavelu et al. [124]. A total of 500µl of M. acetate and crude extract at varying concentrations and 500µl of 0.02M sodium phosphate buffer (pH 6.9 comprising 0.006M sodium chloride) containing α-amylase solution (0.5mg/ml) were incubated for 10 minutes at 25°C. Afterwards, 500µl of 1% starch solution in the same buffer was added to each test tube. The reaction mixture was incubated for a further 10 min at 25°C. DNSA colour reagent (1 ml) was added to halt the reaction. The test tubes were incubated in boiling water bath for 5min and cooled to room temperature. 10ml dH_2O was thereafter added to the mixture, followed by absorbance measurement at 540nm. Acarbose was used as a positive control.

3.3.4 α – Glucosidase inhibition assays

The inhibition of α – Glucosidase activity was done according to the assay adopted from Bajpai et al. [125]. Briefly, 10µL of test samples at several concentrations and 50 µL of yeast α-glucosidase, dissolved in 100 mM phosphate buffer (pH 7.0) (containing0.2 g/L NaN3 and 2 g/L bovine serum albumin) were mixed in 96 well micro-plate and absorbance at 595 nm was read at zero time with a micro-plate reader. After an incubation period of 5 min, 50 µL of P-Nitrophenyl-α-D-glucopyranoside (5 mM) in the same buffer (pH 7.0) was used as a substrate

solution and incubated for a further 5 min at room temperature. 80 µL of 0.2 M sodium carbonate solution was added to terminate the reaction. The absorbance was read at 595 nm. The reaction without α-glucosidase was used as a blank, and acarbose at several concentrations was used as a positive control.

Each experiment was conducted in triplicates and the enzymatic inhibition rate was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Control absorption-Sample absorption}}{\text{Control absorption}} \times 100$$

3.4 Antioxidant Activity

3.4.1 1,1-diphenyl-2- picrylhydrazyl scavenging activity

1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging effect of M. acetate was determined by a standard method Brand-Williams et al. [126]. An aliquot of DPPH solution (2mg in 100 ml MeOH) was prepared and 2ml of this solution was added to 2 ml of each sample solution in methanol at varying concentrations. The mixtures were left for 30 min in the dark at room temperature. A spectrophotometer was used to measure the absorbance of each sample at 517 nm. The scavenging effect expressed (%) was calculated using the following formula**:**

$$\text{Scavenging effect (\%)} = 1 - [A_{Sample} / A_{Control}] \times 100$$

3.5 *In Vivo* Study

3.5.1 Ethical clearance

The experiments on animals were approved by the University of KwaZulu-Natal Ethics committee (Ref: AREC/009/017M) (See Appendix D).

3.5.2 Animals

In this study, male Sprague Dawley rats (200-250g) housed and bred at the University of KwaZulu-Natal, Westville campus, Biomedical Resource Unit were used. The animals were maintained under standard laboratory conditions of 12:12 h light/dark cycle and controlled

temperature of 24 ±1°C with relative humidity 40-60%, with water and food (Meadows, Pietermaritzburg, South Africa) freely available.

3.5.3 Preparation of the treatment

The crude extract (1.5mg/kg) and compound M. acetate (0.5, 1.5 & 2.5mg/kg) were prepared by thawing it in tween 20 and bringing it up to the required volume by the addition of dH_2O. Acarbose was used as a positive control (10 mg/kg) [127]. The doses depended on the average weight per group.

3.5.4 Induction of Diabetes Mellitus

Diabetes mellitus was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 60mg/kg) dissolved in freshly prepared cold 0.1 mol/L citrate buffer (pH 4.5). The animals were considered to be diabetic after one week with a blood glucose concentration ≥18 mmol/L [128]. The blood glucose was measured from the tail vein blood using a Roche ACCUCHEK Aviva Nano glucometer (Basel, Switzerland).

3.5.5 Experimental Design

The experiment was carried out on sixty six male Sprague Dawley rats for 21 days. The animals were divided into two main groups (Non-Diabetic and Diabetic), with six animals in each subgroup. The groups were as follows:

Non-Diabetic Groups	Diabetic Groups
Non-treated	Non-treated
Vehicle (Tween 20)	Acarbose (10 mg/kg)
M. acetate (0.5 mg/kg)	M. acetate (0.5 mg/kg)
M. acetate (1.5 mg/kg)	M. acetate (1.5 mg/kg)
M. acetate (2.5 mg/kg)	M. acetate (2.5 mg/kg)
	Crude Extract (1.5 mg/kg)

On the commencement of the experiment, animals were placed in metabolic cages (one per cage) overnight. On Day 0, the baseline measurements and samples were taken (blood glucose

levels, food intake, water intake, weight and urine). The measurements and samples were taken in three day intervals before and after the treatment period, during treatment they were taken daily. The animals were treated orally once per day (900hr) for five sequential days (Day8-12). On Day 21, the animals were sacrificed, blood was collected into pre-cooled heparinised tubes for plasma insulin measurement and EDTA tubes for haematology parameters determination. Harvesting of muscle, liver, pancreas and kidneys was also done for further evaluation. The organs were halved, placed in 10% formalin and -70 bio freezer.

The rat grimace scale [129], from the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs), was used to evaluate whether the animals were suffering during the test. The grimace scale consists of five facial parameters (nose and cheek flattening, orbital tightening, whisker change and ear change) that were used to assess pain sensed by the animals. If an animal was found to be suffering, it was excluded from the experiment via decapitation.

3.5.5.1 Experimental Flow Diagram

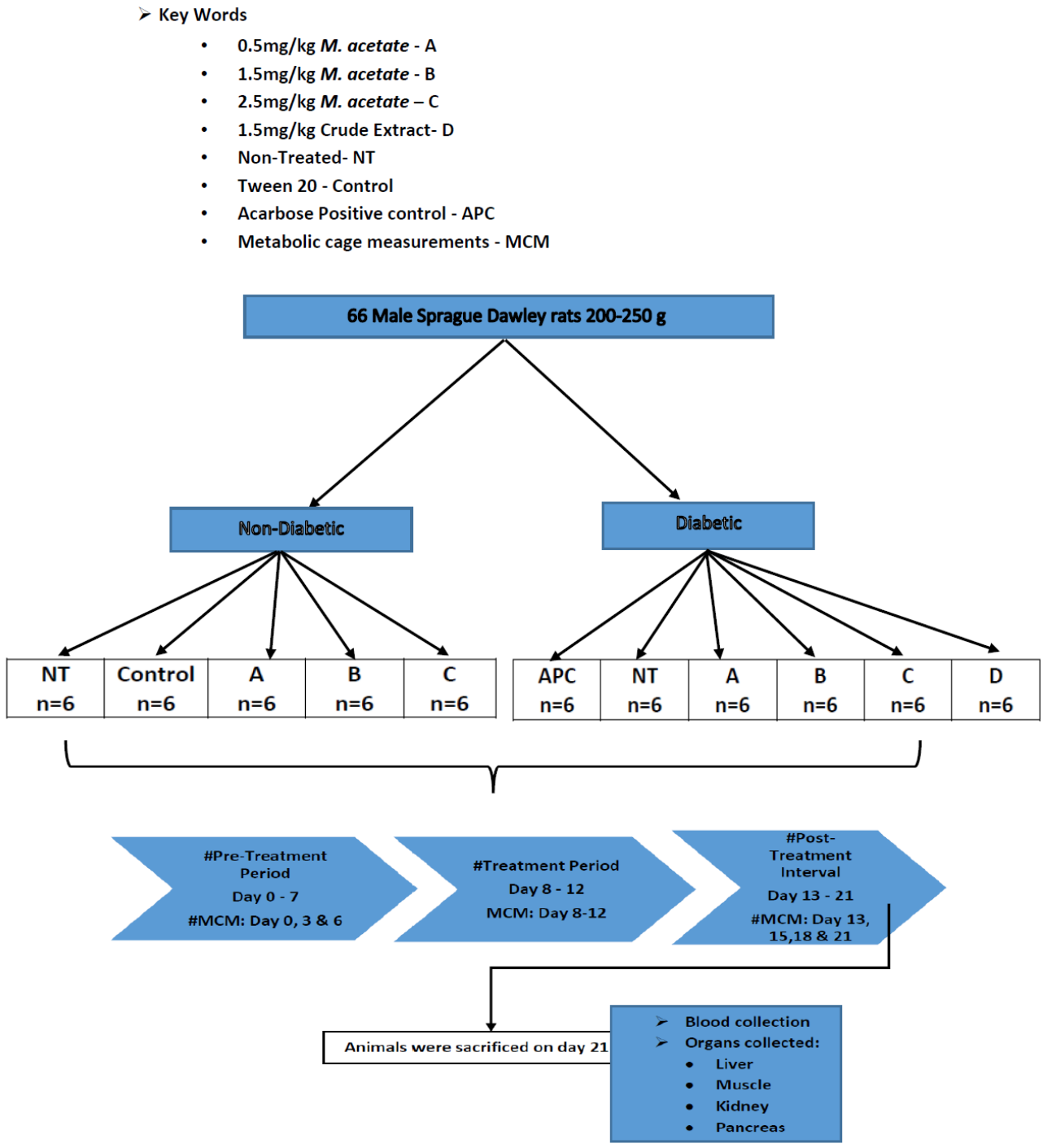


Figure 3.1: In vivo experimental flow diagram

3.5.6 Insulin Assay

Rat Insulin Elisa Kit (Thermo Scientific™ Pierce™, USA), is an enzyme-linked immunosorbent assay which was used to measure rat insulin serum. The procedure was as follows: 100µL of each standard and sample was added into appropriate wells and incubated for 2.5h at room temperature (RT) on an orbital shaker. Thereafter, the solution was discarded and the plate was washed four times with a Wash Buffer. 100µL Biotinylated antibody was added and incubated at RT for 1h. The plate was washed four times, adding 100µL streptavidin-HRP to each well and incubated at RT for 45min. The plate was washed four times, 100µL TMB substrate was added and incubated in the dark at RT for 30min on an orbital shaker. The reaction was terminated by adding 50µL of stop solution. The absorbance was read on an ELISA plate reader at 450nm and 550nm.

3.5.7 Haematology parameters determination

At the end of the experimental study period, on day 21, blood was collected by cardiac puncture into pre-cooled heparinised tubes for plasma insulin measurement. Blood was also collected into pre-cooled EDTA tubes for haematology parameters determination for measurement of haematology parameters (red blood cell count (RBC), haemoglobin (HBG), haematocrit (HCT), neutrophils (NE), basophils (BA), eosinophils (EO), monocytes (MO) and lymphocytes (LY). A calibrated automated Beckman Coulter haematology analyser was used (Climet Instruments Company, Redlands CA United States).

3.6 Statistical analysis

The data was expressed as means ± SEM. Statistical analysis of cytotoxicity and glucose utilisation was measured using Microsoft excel software, 2013. Statistical comparison of the differences between the means of control and experimental groups was performed with GraphPad Prism Software version 5.00, using two-way analysis of variance (ANOVA), followed by Bonferroni posttests. A value of $p < 0.05$ was considered significant.

Chapter 4

Results

Crude extract of the medicinal plant *W. salutaris* was screened for bioactive compounds, antidiabetic and antioxidant activity. The isolated compound M. acetate was also screened for antidiabetic and antioxidant activity. This chapter gives an overview of the findings of the present study.

4.1 Structural Elucidation

The chemical shift of the compound NN-01 (Table 4.1) was studied and it was established that it was a novel compound isolated from the plant *W. salutaris* or any other plant species. The compound was regarded as Mukaadial acetate (Fig 4.1). Low resolution and high resolution Mass spectra (MS) was determined at the University of KwaZulu-Natal PMB campus Chemistry department. Fig 4.2 displays the mass spectra of M. acetate.

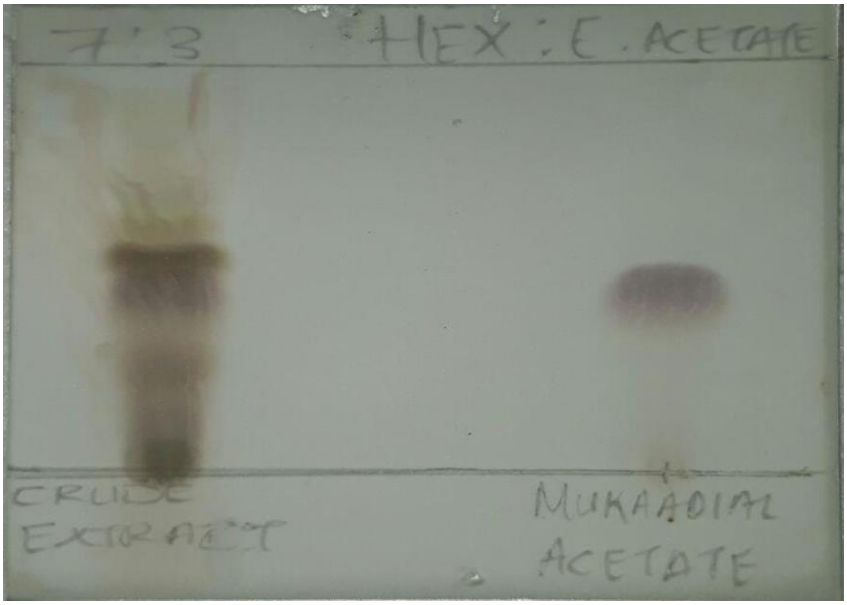


Figure 4.1: Thin layer chromatography plate presenting crude extract and Mukaadial acetate

Table 4.1: ^{1}H- and ^{13}C-NMR chemical shifts (δ, ppm) of M. acetate

position	δc (ppm)	CHn	δH (ppm)
1	66.13	CH	5.91(t, H, J = 4.8 Hz)
2	148.60	CH	7.01(d, H, J = 4.8 Hz)
3	141.00	C	
4	32.56	CH	4.07
5	41.66	C	
6	31.83	CH2	
7	17.70	CH2	
8	44.02	CH2	
9	34.00	C	
10	44.97	CH	2.06(d, H, J = 4.8 Hz)
11	193.01	CHO	9.76
12	201.44	CHO	9.48
13	19.95	CH3	1.34
14	24.75	CH3	1.17
15	21.44	CH3	1.03
16	170.01	COOR	
17	19.95	CH3	2.14

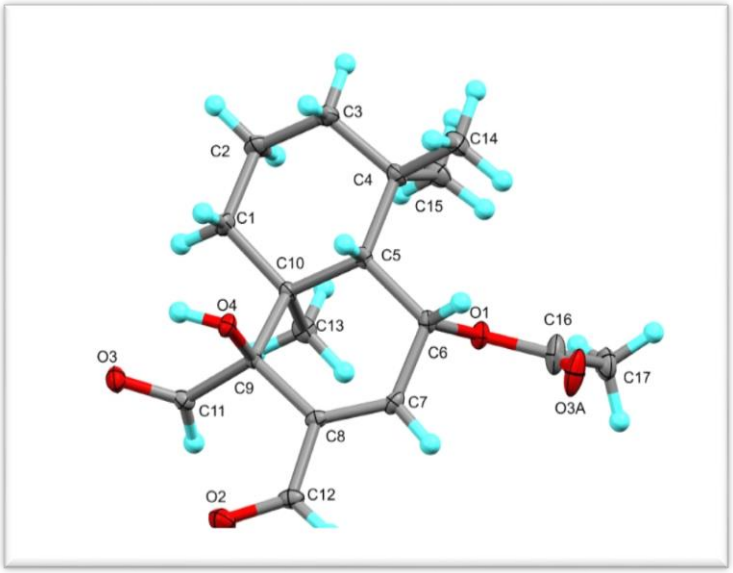


Figure 4.2: X-ray structure of M. acetate

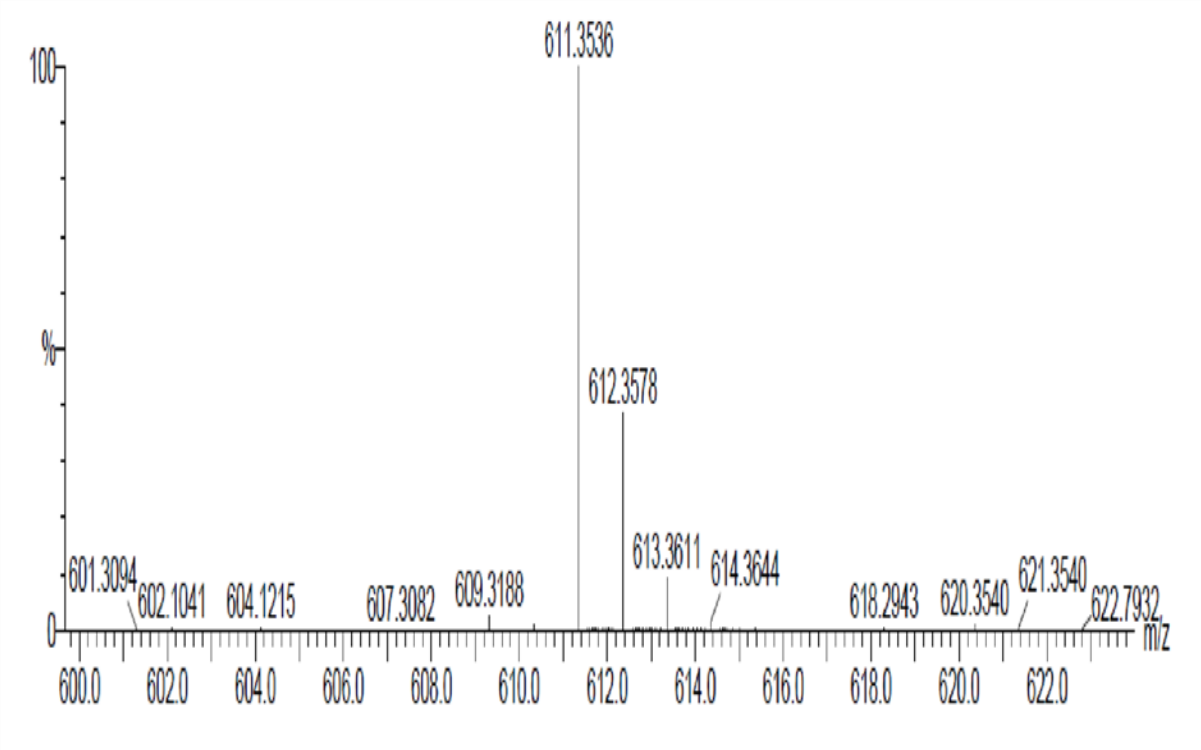


Figure 4.3: Single mass analysis of M. acetate

4.2 *In vitro* studies

4.2.1 Cytotoxicity Evaluation of M. acetate

The cell viability effect of M acetate was highest at 3µg/ml with a percentage of 98.4, with an IC_{50} value of 13.45µg/ml. At concentrations greater than 3µg/ml, cell viability significantly reduced in a dose dependant manner ($p < 0.05$) (Fig 4.4)

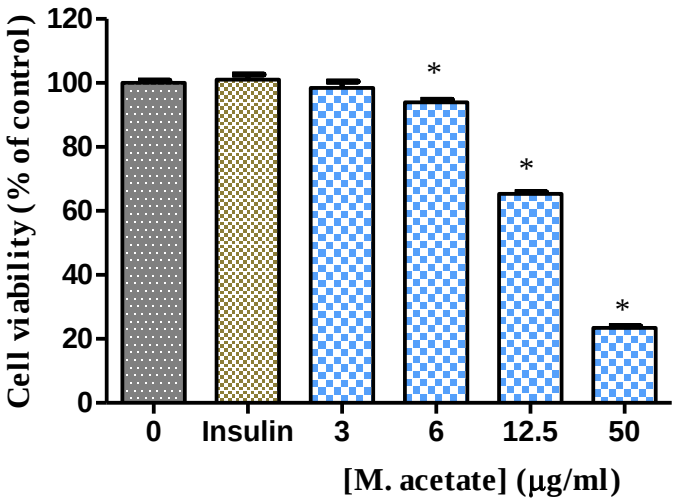


Figure 4.4: Cytotoxicity of L6 cells after 48 hour treatment with M. acetate. Cytotoxicity was determined using crystal violet and data expressed as a percentage of the untreated control. *$p < 0.05$

4.2.2 Glucose utilisation screening of M. acetate

M. acetate significantly ($p<0.05$) and dose dependently increased glucose utilisation up to 215.18% (12.5µg/ml), however, cytotoxicity at the higher concentrations tested severely attenuate cellular glucose utilisation (Fig 4.5).

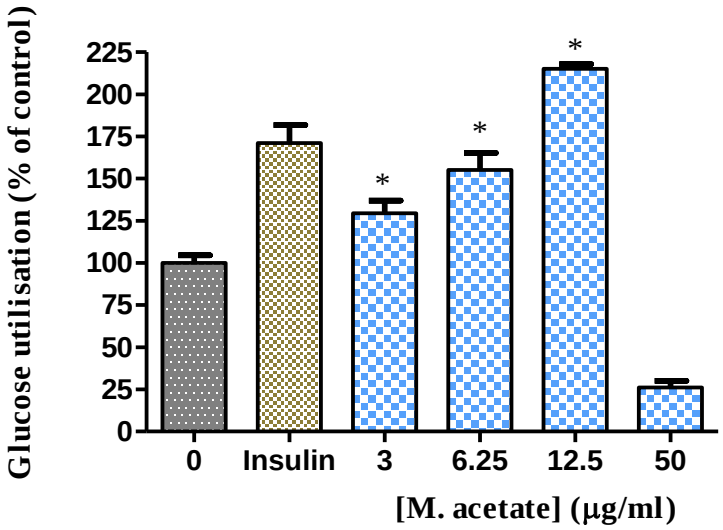


Figure 4.5: Glucose utilisation in L6 cells after 48 hour treatment with M. acetate. Glucose utilisation is calculated as the difference in remaining glucose after 2hr incubation between the no cell control and cells with the respective treatment. Data is expressed as a percentage relative to the untreated control. *$p< 0.05$

4.2.3 Effect of M. acetate and crude extract on α- amylase inhibition

α- Amylase inhibitory activity of M. acetate and crude extract was found to be in a concentration dependent manner. M. acetate showed inhibition of the enzyme with the highest value of 41.05% seen at 50µg/ml compared to the crude extract with a value of 31.22%. (Fig 4.6).

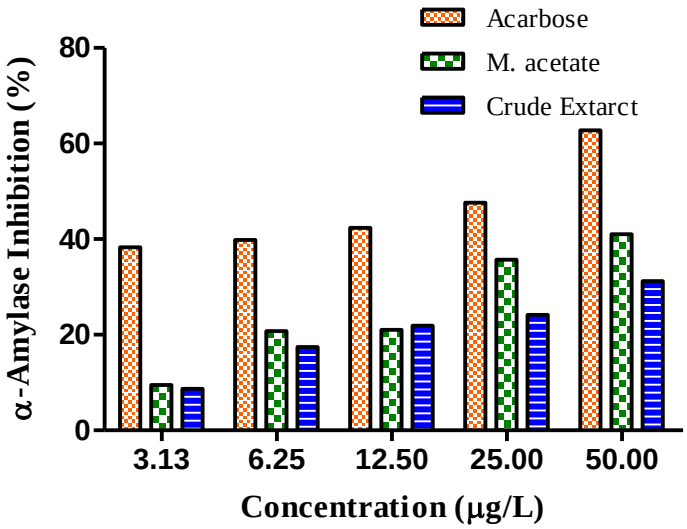


Figure 4.6: Percentage α-Amylase inhibitory effect of standard compound Acarbose, M. acetate and crude extract. Values are presented as means.

4.2.4 Effect of M. acetate and crude extract on α – Glucosidase inhibition

α- Glucosidase inhibitory effect of M. acetate and crude extract was executed against the enzyme in a dose dependent manner. M. acetate showed inhibition of the enzyme with the highest value of 29.61% seen at 50µg/ml compared to the crude extract with a value of 22.17%. (Fig 4.7).

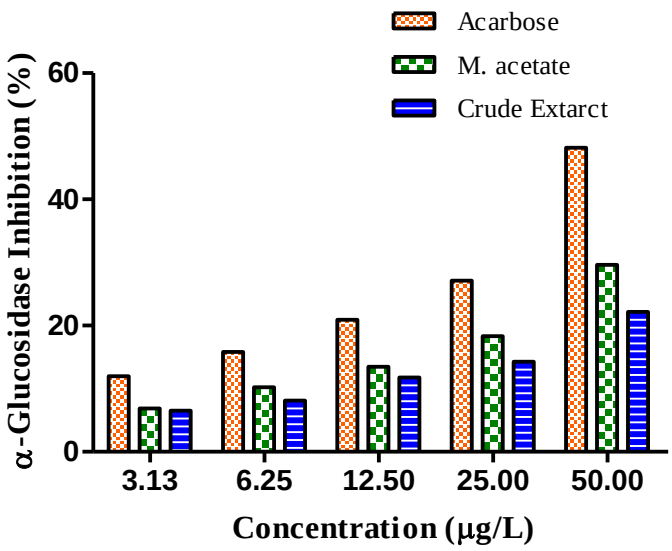


Figure 4.7: Percentage α-Glucosidase inhibitory effect of standard compound Acarbose, M. acetate and crude extract. Values are presented as means.

4.2.5 Effect of M. acetate and crude extract on 1,1-diphenyl-2 picrylhydrazyl

The scavenging activity of M. acetate and crude extract against DPPH was presented in a dose dependent manner. M. acetate displayed a scavenging effect with the highest value of 42.65% seen at 50µg/ml compared to the crude extract with a value of 40.97%. (Fig 4.8).

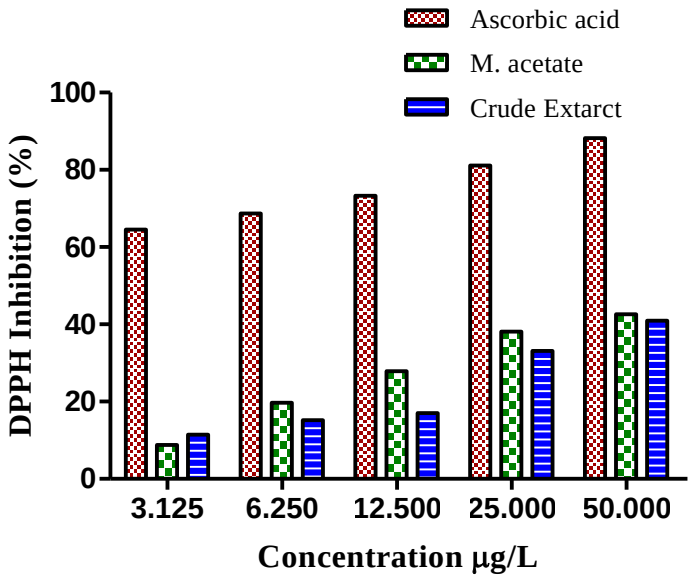


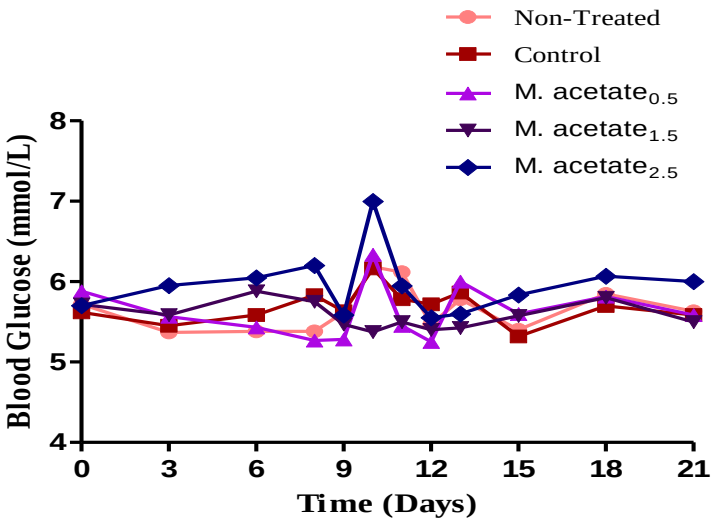
Figure 4.8: Percentage DPPH inhibition activity of standard compound Ascorbic acid M. acetate and crude extract. Values are presented as means.

4.3 *In Vivo* Study

4.3.1 Effect of M. acetate and crude extract on blood glucose and insulin concentrations

M. acetate at the varying concentrations (0.5, 1.5 and 2.5mg/kg) in the non-diabetic group did not significantly affect the blood glucose levels, compared to the control animals (Fig 4.9 (a)). On Day 11 and 13, a significant (**$p<0.01$) reduction in blood glucose levels was presented by the diabetic positive control. A significant increase in blood glucose levels was presented by M. acetate during treatment and post-treatment, particularly at the highest concentrations. The crude extract displayed a significant (*$p<0.05$) increase in blood glucose levels on Day 21 (Fig 4.9 (b)). The plasma insulin concentrations in both non-diabetic and diabetic groups was not significantly affected (Fig 4.10).

(a)



(b)

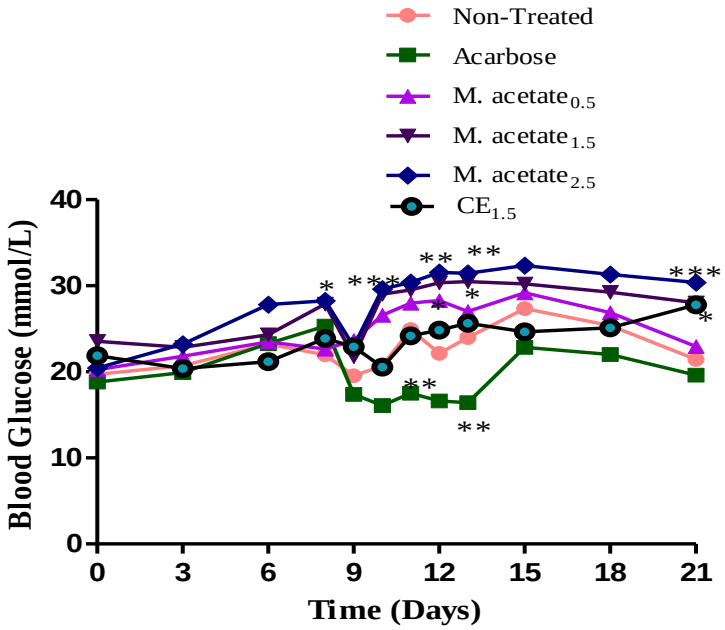
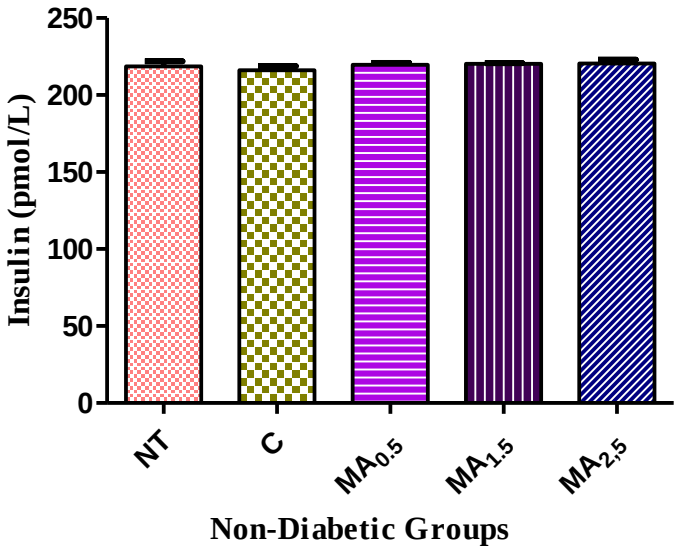


Figure 4.9: Blood glucose responses to M. acetate and crude extract in non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Values are presented as means ±SEM (n = 6 in each group). *p<0.05, **p<0.01, ***p<0.001.

(a)



(b)

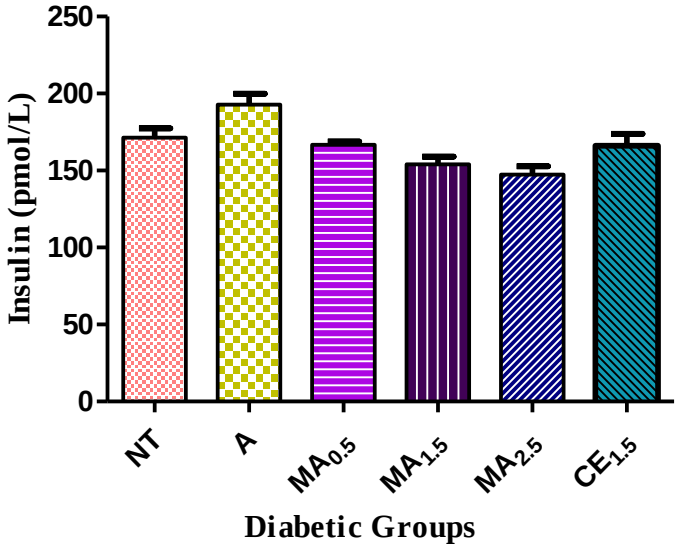


Figure 4.10: Effect of M. acetate and crude extract on plasma insulin concentration of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group).

4.3.2 Effect of M. acetate and crude extract on water and food intake, urine output and body weight changes

Table 4.2 represents the water intake, table 4.3 urine output, table 4.4 food intake and table 4.5 weight change of the non-diabetic and diabetic groups during pre- treatment, treatment and post-treatment. On Day 9, a significant urine output was presented in the non- diabetic control ($p<0.05$) and M. acetate 0.5mg/kg ($p<0.01$) groups.

On Day 9, a significant ($p<0.001$ and 0.01) reduction in food intake in the non-diabetic 2.5 and 1.5mg/kg M. acetate group respectively was observed. On Day 21, a significant ($p<0.05$) increase in food intake in the control and 0.5 mg/kg groups was observed. A significant decrease in weight change Day 9 ($p<0.001$) and 21 ($p<0.05$) were observed in the non-diabetic 1.5mg/kg M. acetate group.

Table 4.2: Effect of M. acetate, crude extract and Acarbose on water intake of non-diabetic and diabetic animal's pre-and post-treatment.

Experimental Groups	Water intake (ml)			
	Pre-Treatment	Treatment		Post-Treatment
	Day 0	Day 9	Day 12	Day 21
Non-diabetic animals				
Non-treated	18.33	14.16	11.67	22.50
Control (Tween 20)	18.33	12.50	17.50	22.50
M. acetate (0.5 mg/kg)	21.67	10.00	16.67	22.50
M. acetate (1.5 mg/kg	21.67	8.33	21.25	25.00
M. acetate (2.5 mg/kg**)**	23.33	7.50	9.75	26.67
Diabetic animals				
Non-treated	67.50	72.50	80.00	87.50
Acarbose ((10 mg/kg)	57.83	60.50	55.00	58.33
M. acetate (0.5 mg/kg)	57.67	81.17	87.50	90.83
M. acetate (1.5 mg/kg	72.50	82.50	91.67	96.67
M. acetate (2.5 mg/kg)	70.83	74.17	93.33	95.83
Crude Extract (1.5 mg/kg	76.67	94.67	86.67	80.83

Values are presented as means ±SEM (n = 6 in each group)

Table 4.3: Effect of M. acetate, crude extract and Acarbose on urine output of non-diabetic and diabetic animal's pre-and post-treatment.

Experimental Groups	Urine output (ml)			
	Pre-Treatment	Treatment		Post-Treatment
	Day 0	Day 9	Day 12	Day 21
Non-diabetic animals				
Non-treated	9.50	11.67	6.66	10.50
Control (Tween 20)	9.66	7.33*	5.66	7.33
M. acetate (0.5 mg/kg)	10.50	6.66**	5.66	7.33
M. acetate (1.5 mg/kg	10.00	8.66	7.00	8.00
M. acetate (2.5 mg/kg**)**	9.83	7.66	7.00	6.66
Diabetic animals				
Non-treated	59.00	67.83	71.67	84.00
Acarbose ((10 mg/kg)	46.50	51.67	55.83	54.33
M. acetate (0.5 mg/kg)	57.33	79.33	89. 67	83.00
M. acetate (1.5 mg/kg	57.33	69.00	83.00	93.00
M. acetate (2.5 mg/kg)	57.67	79.33	93.00	91.67
Crude Extract (1.5 mg/kg	61.67	84.50	83.67	77.00

Values are presented as means ±SEM (n = 6 in each group). *p<0.05, **p<0.01

Table 4.4: Effect of M. acetate, crude extract and Acarbose on food intake of non-diabetic and diabetic animal's pre-and post-treatment.

Experimental Groups	Food intake (g)				Feed conversion ratio (g)	
	Pre-Treatment	Treatment		Post-Treatment		
	Day 0	Day 9	Day 12	Day 21	Day 0	Day 21
Non-diabetic animals						
Non-treated	20.18	14.54	11.05	10.33	1.61	0.19
Control (Tween 20)	18.94	13.67	11.21	16.64*	1.86	0.28
M. acetate (0.5 mg/kg)	21.53	11.10	11.26	15.92	2.01	0.34
M. acetate (1.5 mg/kg	21.12	6.90***	11.10	13.77	2.48	0.40
M. acetate (2.5 mg/kg)	20.05	7.89**	11.75	13.67	2.61	0.31
Diabetic animals						
Non-treated	22.40	20.96	21.25	22.96	2.75	1.03
Acarbose ((10 mg/kg)	18.82	21.75	21.29	20.55	3.06	0.38
M. acetate (0.5 mg/kg)	19.56	21.43	21.27	18.74	3.26	0.57
M. acetate (1.5 mg/kg	20.46	19.10	22.02	24.52	1.71	0.63
M. acetate (2.5 mg/kg)	20.27	22.25	22.54	25.57	2.43	0.64
Crude Extract (1.5 mg/kg	18.19	22.75	21.63	18.98	-6.83	0.54

Notes: Values are presented as means ±SEM (n = 6 in each group). *p<0.05, **p<0.01, ***p<0.001.

Feed conversion ratio was obtained by food intake/weight gain

Table 4.5: Effect of M. acetate, crude extract and Acarbose on body weight change of non-diabetic and diabetic animal's pre-and post-treatment.

Experimental Groups	Body weight (g)				Feed conversion ratio (g)	
	Pre-Treatment	Treatment		Post-Treatment		
	Day 0	Day 9	Day 12	Day 21	Day 0	Day 21
Non-diabetic animals						
Non-treated	12.50	22.00	27.50	53.33	1.61	0.19
Control (Tween 20)	10.17	20.83	24.33	58.83	1.86	0.28
M. acetate (0.5 mg/kg)	10.67	14.83	21.50	45.50	2.01	0.34
M. acetate (1.5 mg/kg	8.50	5.66	-9.00***	34.25*	2.48	0.40
M. acetate (2.5 mg/kg)	7.66	13.50	19.75	43.33	2.61	0.31
Diabetic animals						
Non-treated	8.16	-5.83	7.66	22.17	2.75	1.03
Acarbose ((10 mg/kg)	6.16	24.00	29.83	53.83	3.06	0.38
M. acetate (0.5 mg/kg)	6.00	-2.00	5.50	32.50	3.26	0.57
M. acetate (1.5 mg/kg	11.83	10.50	18.33	38.50	1.71	0.63
M. acetate (2.5 mg/kg)	8.33	7.00	12.83	39.67	2.43	0.64
Crude Extract (1.5 mg/kg	-2.66	6.50	8.66	34.67	-6.83	0.54

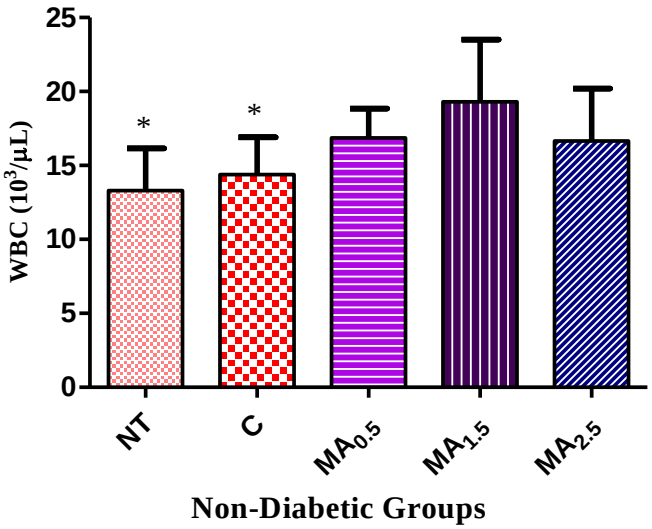
Notes: Values are presented as means ±SEM (n = 6 in each group). *p<0.05, ***p<0.001

Feed conversion ratio was obtained by food intake/weight gain

4.3.3 Effect of M. acetate and crude extract on Haematology profile

Figures 4.11-19 represent the haematological profiles of the non-diabetic and diabetic groups on Day 21. The white blood cell and basophil count of the non-diabetic NT group were significantly ($p<0.05$) increased, conversely monocyte and eosinophil count were decreased (Fig 4.11, 4.17-19). The diabetic positive control significantly ($p<0.05$) reduced haematocrit (Fig 4.14 (b)). The non-diabetic NT group, significantly reduced white blood cell and red blood cell (Fig 4.11 and 4.12). A significant ($p<0.05$) increase in eosinophil was presented in diabetic groups; Acarbose, 1.5mg/kg M. acetate and 2,5mg/kg M. acetate (Fig 4.18 (b))

(a)



(b)

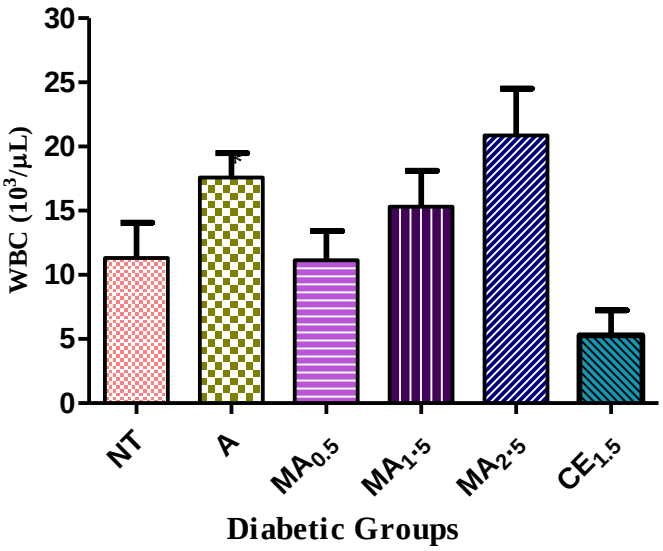
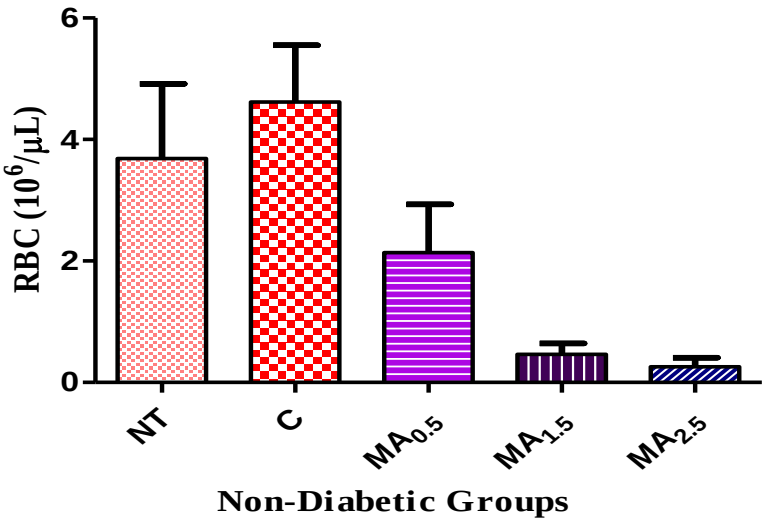


Figure 4.11: Effect of M. acetate and crude extract on White blood cell count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *$p<0.05$.

(a)



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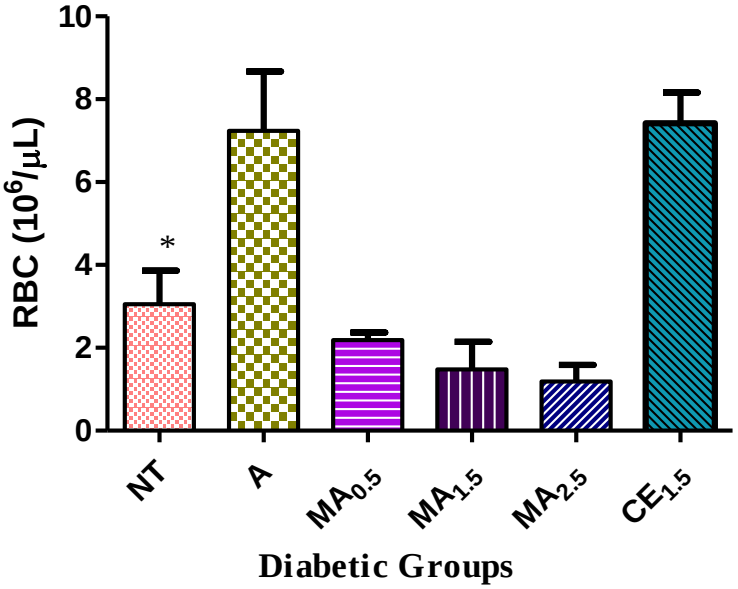
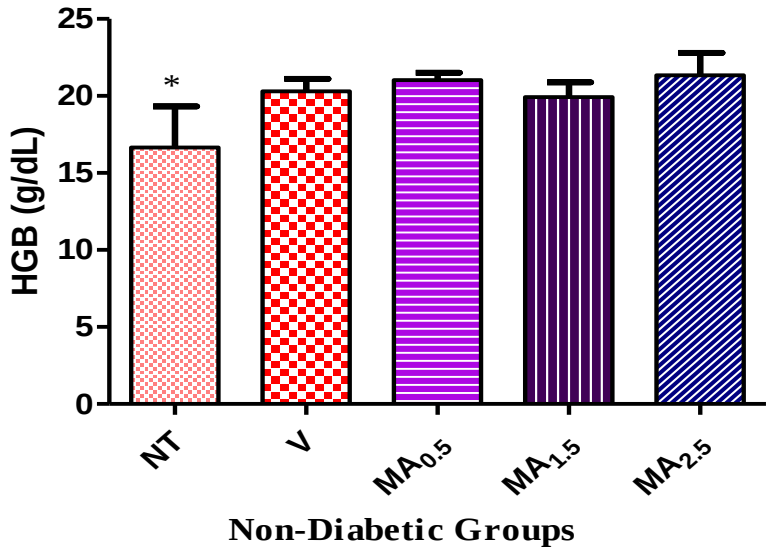


Figure 4.12: Effect of M. acetate and crude extract on Red blood cell count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *p<0.05.

(a)



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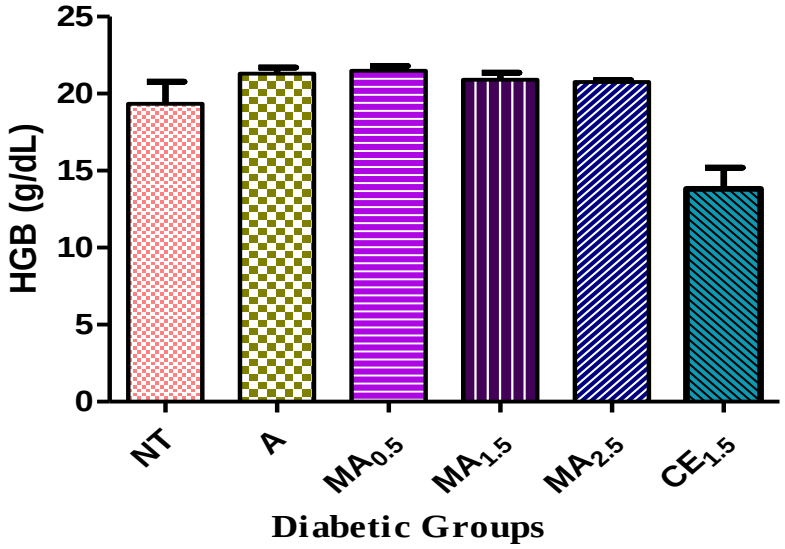
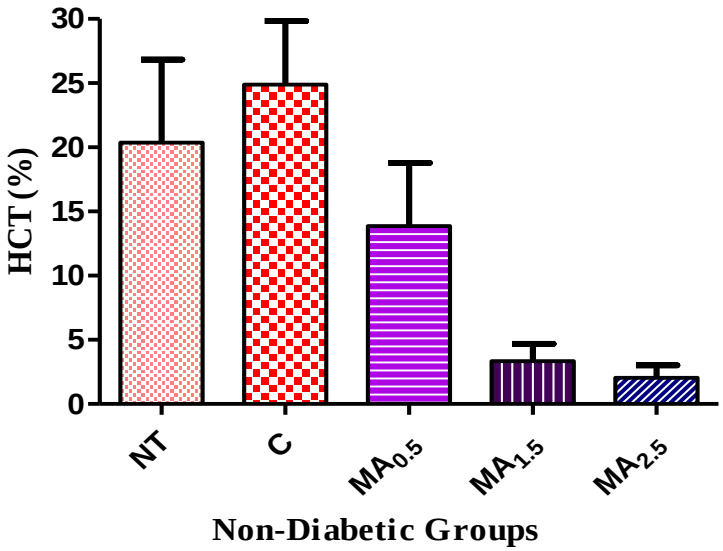


Figure 4.13: Effect of M. acetate and crude extract on Haemoglobin count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *$p<0.05$.

(a)



(b)

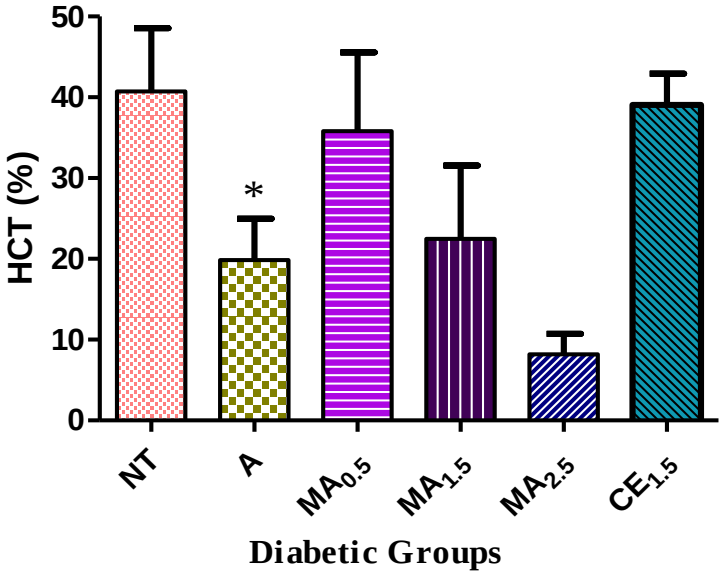
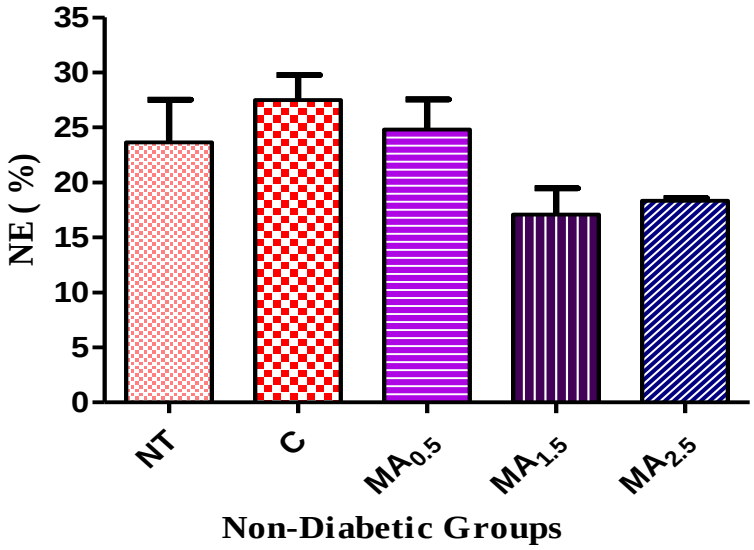


Figure 4.14: Effect of M. acetate and crude extract on Haematocrit count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *$p<0.05$.

(a)



(b)

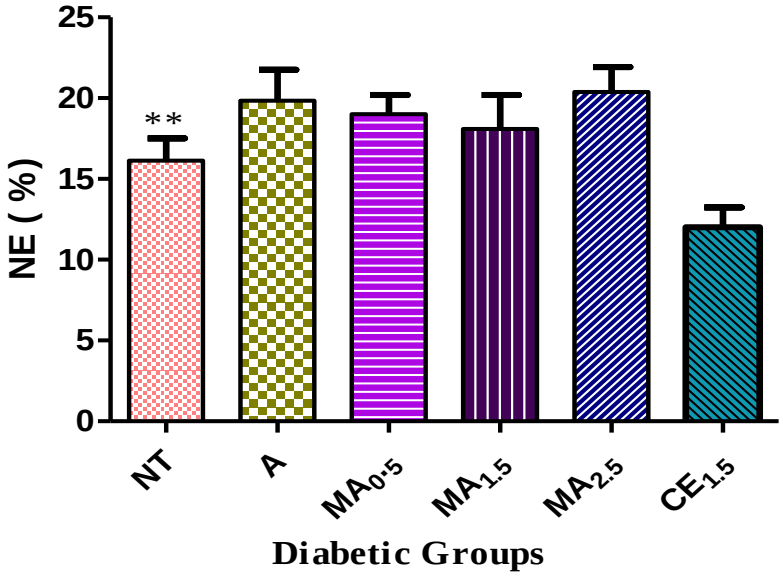
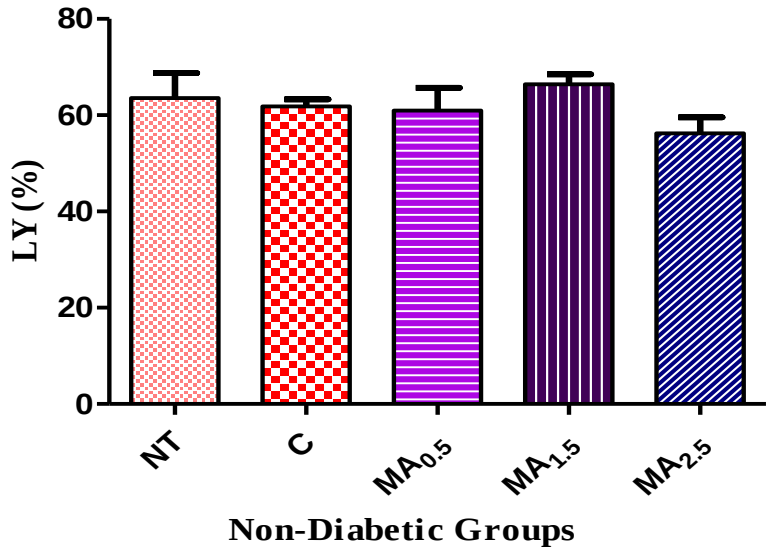


Figure 4.15: Effect of M. acetate and crude extract on Neutrophil count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). **p<0.01.

(a)



(b)

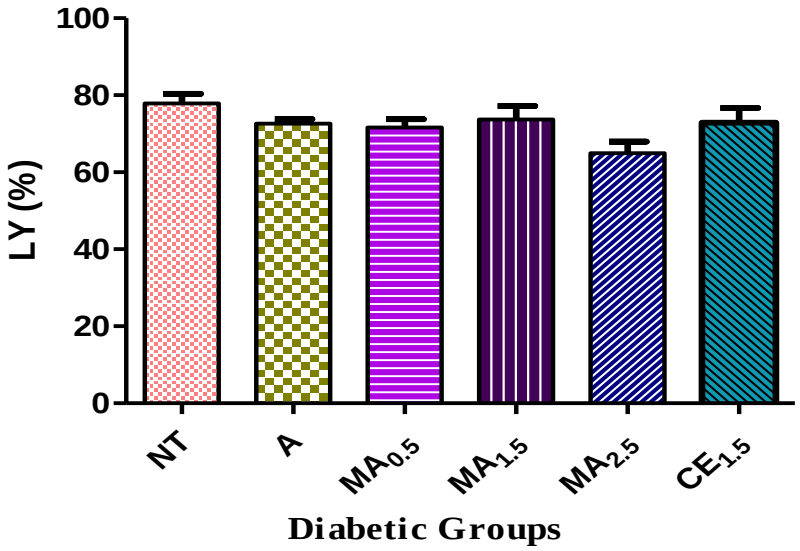
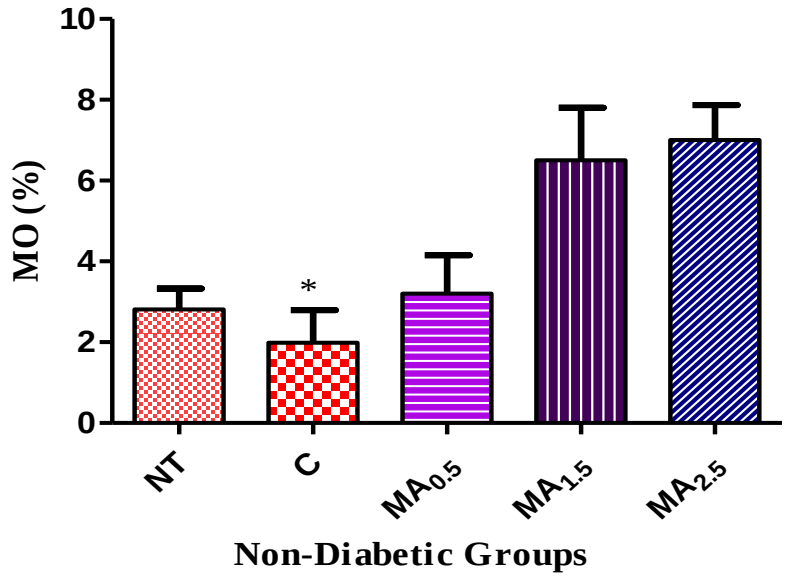


Figure 4.16: Effect of M. acetate and crude extract on Lymphocyte count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group).

(a)



(b)

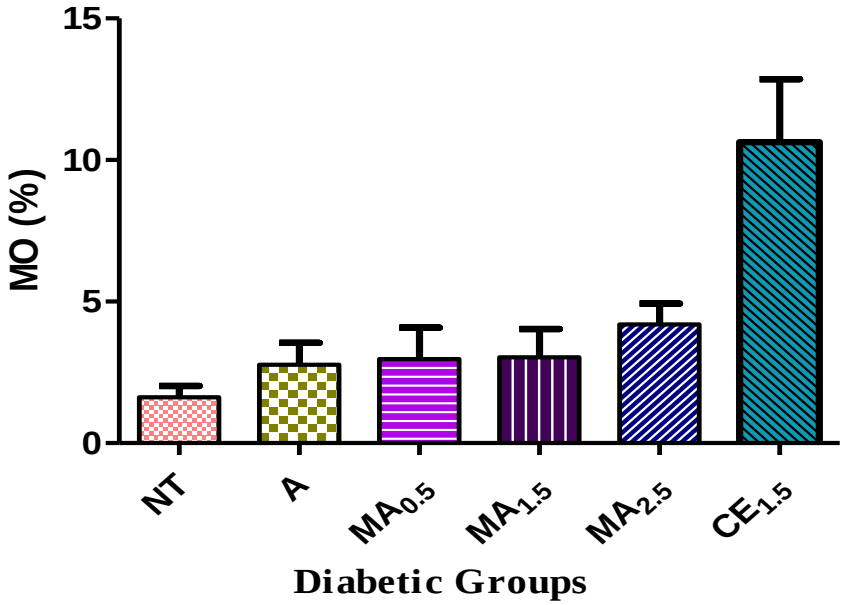
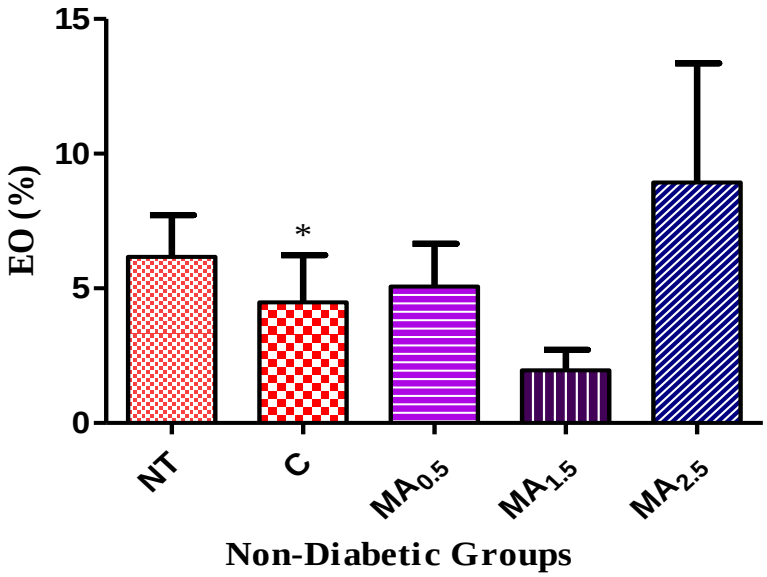


Figure 4.17: Effect of M. acetate and crude extract on Monocyte count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *$p<0.05$.

(a)



(b)

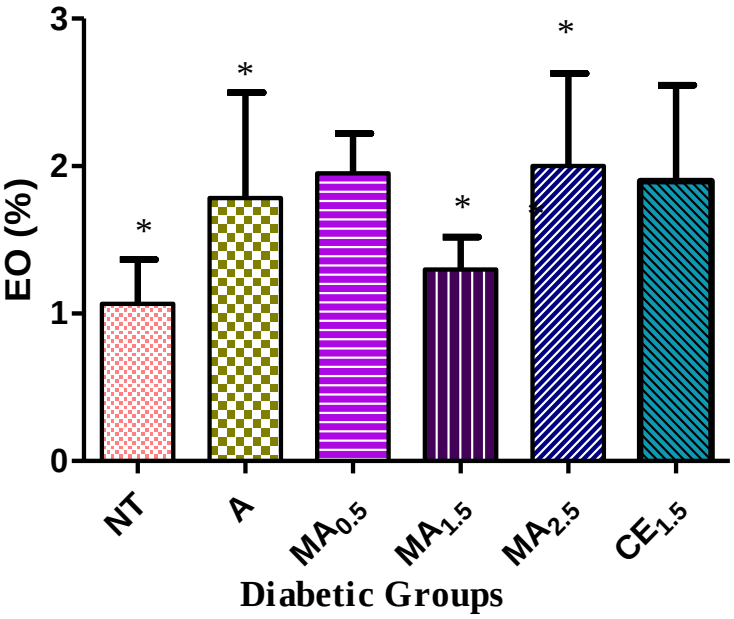
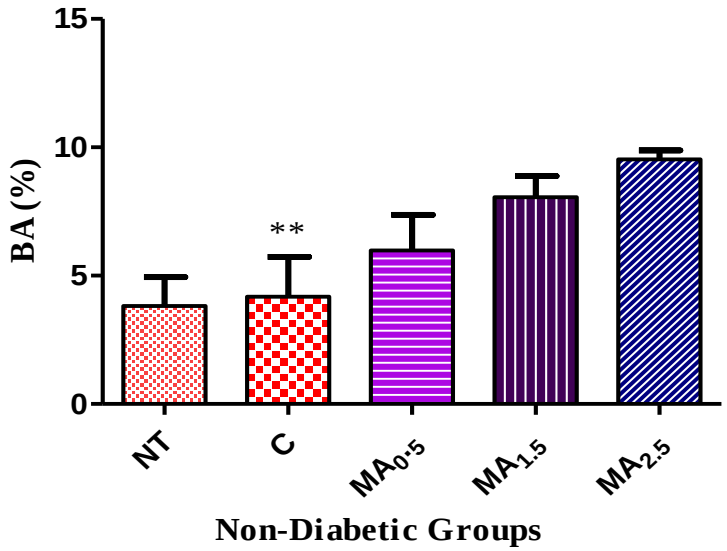


Figure 4.18: Effect of M. acetate and crude extract on Eosinophil count of non-diabetic (a) and crude (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). *$p<0.05$.

(a)



(b)

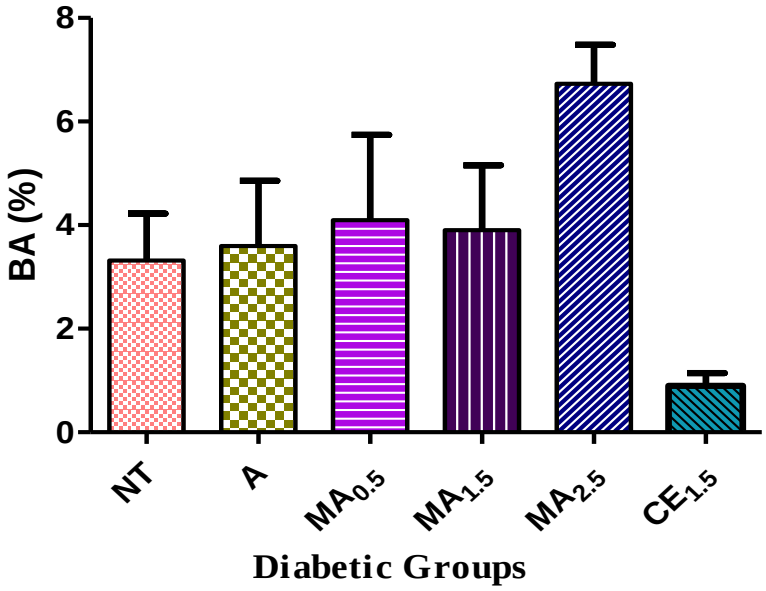


Figure 4.19: Effect of M. acetate and crude extract on Basophil count of non-diabetic (a) and diabetic (b) Sprague-Dawley rats with respective control groups. Non-treated (NT), Control (C), Acarbose (A), 0.5mg/kg M. acetate ($MA_{0.5}$), 1.5mg/kg M. acetate ($MA_{1.5}$), 2.5mg/kg M. acetate ($MA_{2.5}$) and 1.5mg/kg crude extract ($CE_{1.5}$). Values are presented as means, and vertical bars indicate SEM (n = 6 in each group). **p<0.01.

Chapter 5

Discussion

5.1 *In vitro* studies

5.1.1 Glucose utilisation screening and Cytotoxicity Evaluation

Plants have always been a good source of molecules with therapeutic potential, and currently represent an important pool in ethnopharmacology for the discovery of novel drugs [130]. The use of medicinal plants are presently directed on lowering and controlling blood glucose levels in treating diabetes [131]. Thus, medicinal plants have been proven to enhance glucose uptake by GLUT4 translocation using *in vitro* glucose model [132]. In the present study, L6 cell line originally derived from rat skeletal muscle was used. This immortalized myoblast cell line is popular as a model for glucose uptake since the cells differentiate with high reliability into a myotube muscle cell phenotype that expresses the GLUT4 glucose transporter protein naturally [133].

The glucose utilisation of M. acetate at differing doses was found to be prominent over the control in a concentration dependant manner. The L6 cell enhanced the glucose utilisation by 129.41% (3.125 µg/ml), 155.22% (6.25 µg/ml) and 215.18% (12.5 µg/ml). Results were compared to insulin, an anabolic hormone which presented a percentage of 171.15 %. A study by Kawabata et al. [134] previously established that triterpernoids isolated from *Z. jujuba* have the ability to effectively enhance glucose utilisation through the translocation of GLUT4. A defect in GLUT4 expression and translocation has been stated to be the main metabolic irregularity in diabetic skeletal muscle [135]. Furthermore, at higher doses M. acetate attenuated glucose utilisation. Reduction of cell viability was seen at higher concentrations which may contribute to this observation. M. acetate was observed to be non-toxic at 3 µg/ml in L6 cell. In cytotoxicity evaluation, the highest concentration for a test agent should be less than 1000 µg/ml to be considered non-toxic [136]. These findings suggest that M. acetate demonstrated a considerable hypoglycaemic effect by enhancing the rate of glucose utilisation in the L6 cell. The translocation of GLUT 4 may have resulted in the mediation of this deduction and may be helpful to manage hyperglycaemia associated with type 2 DM.

5.1.2 α- Amylase and α – glucosidase inhibition assay

A major goal in the treatment of DM is to maintain near normal blood glucose levels. One of the therapeutic approaches is to decrease postprandial hyperglycaemia by suppressing the gastrointestinal tract production or absorption of glucose by inhibition of either α-amylase or α-glucosidase enzymes [75]. These are the main enzymes involved in the production of glycation end products which may lead to elevated postprandial hyperglycaemia when in excess [137]. The capability of plants to supress the production and absorption of glucose has demonstrated to be an attractive therapeutic approach in managing DM [49]. In the present study, M. acetate and crude extract exhibited an inhibitory effect in a concentration dependent manner on both enzymes. The inhibitory effect of M. acetate was more effective than the crude extract. At the highest concentration 50 µg/ml, M. acetate showed an inhibitory effect on α-amylase and α-glucosidase by 41.01 and 29.61% respectively. It has been shown in literature that plants have the ability to inhibit the enzymes, α- amylase and α-glucosidase, *in vitro* [49, 75, 125, 138]. Based on these findings, *W. salutaris* can be considered to possess hypoglycaemic effect.

5.1.3 Antioxidant Activity

The malicious effects of diabetes have been established to be mediated through oxidative stress which arises from the formation of free radicals. Antioxidant compounds from plants can reduce the generation of free radicals, thus, alleviating diseases triggered by oxidative stress [139]. The antioxidant activity of M. acetate and crude extract was carried out using DPPH free radical scavenging assay, it is apparent that *W. salutaris* has the ability to scavenge free radicals. Similarly, a study by Ai-Dabbas and collaborators [140], showed that the ethanolic extracts and compounds isolated from *V. iphionoides* presented DPPH radical-scavenging activity. Furthermore, a study by Frum et al. [122], reported that the methanol and aqueous extracts of *W. salutaris* exhibited promising antioxidant activities. Therefore, these observations confirm that *W. salutaris* possesses antioxidant activity and may provide protection against oxidative damage in diabetic patients by neutralizing free radicals.

5.2 *In vivo* studies

5.2.1 Effect of M. acetate and crude extract on blood glucose and insulin levels, body weight change and metabolic parameters

Streptozotocin is a beta cytotoxin used to induce DM by selectively cytotoxicity damaging pancreatic beta cells. Thus, affecting endogenous insulin release, resulting in increased blood glucose level [141]. In the present study, STZ was employed to induce diabetes in the experimental animals. The plasma insulin concentrations were lower in the STZ-induced diabetic animals compared to the non-diabetic animals, apparently because of the destructed pancreatic β-cells by STZ [142]. Previous studies have shown the antidiabetic activity of plants by potentiating insulin resulting in decreased blood glucose levels [143, 144]. The treatment of M. acetate and crude extract in the STZ-diabetic animals were incapable of reducing the blood glucose levels, with no apparent increase in insulin levels compared to the standard drug acarbose. However, the lowest concentration of M. acetate (0.5 mg/kg) presented lower blood glucose levels compared to the higher concentrations. In the experiments *in vitro*, at higher concentrations M. acetate attenuated cellular glucose utilisation, giving a probable explanation for the observed results.

By comparison with non-diabetic animals, STZ-diabetic animals had reduced body weight gain with higher water intake throughout the experimental period. Polydipsia and increased water intake resulting from enhanced proteolysis associated with hyperosmotic dehydration has been revealed to be related with weight losses in STZ-induced diabetic animals [145]. Treatment with M. acetate and crude extract in diabetic animals displayed greater body weights compared to diabetic control but not significantly. Thus, indicating that *W. salutaris* may have a protective effect by stabilizing body weight of STZ-induced animals.

5.2.2 Effect of M. acetate and crude extract on haematology parameters

Evaluation of various haematological parameters could be used to determine the deleterious effect of foreign compounds including phyto-extracts on the blood constituents of experimental animals [146, 147]. The incidence of anaemia in diabetic condition, is due to an increase in the non-enzymatic glycosylation of RBC membrane proteins that related with an increase of glucose level [147, 148]. In this study, a reduction in the RBC count in the diabetic animals was observed which might be attributed to the infections on the normal body systems. This

result agrees with report of Sellamuthu et al. [142] who reported the effect of mangiferin isolated from *S. chinensis* on STZ-induced diabetes. It has been established that the deformity of RBC causes anaemic disorder in humans [149]. Following treatment with M. acetate and crude extract, the RBC count of the diabetic animals was not altered. This indicates that M. acetate and crude extract could not stimulate the secretion or formation of erythropoietin, which promotes the production of RBC by stimulating stem cells in the bone marrow [146].

The induction of STZ is also known to suppress the immune system by destructing WBC and certain organs in the body [146]. A reduction of the WBC count in the diabetic control animals was observed in the present study. The reduction could be associated with the destruction of leucocytosis from the bone marrow which could account for poor defensive mechanisms against infection [150]. Following treatment with M. acetate, WBC count was elevated at 1.5 and 2.5 mg/kg. Furthermore, M. acetate reduced HCT percentage in STZ-diabetic animals, elevation of HCT has been associated with several risk factors of diabetes [151]. However, the mechanisms responsible for the affected haematological parameters were not evaluated. The measurement of plasma cytokines might possibly provide mechanisms by which these parameters were affected.

Chapter 6

Conclusion

The present study aimed to scientifically validate the use of the plant, *W. salutaris*. The pure compound, M. acetate was isolated from the plant and evaluated for the first time for bioactivity against diabetes mellitus. The study revealed considerable cytotoxicity of the compound with a significant effect on glucose utilisation *in vitro*. Thus, the translocation of GLUT 4 may have resulted in the mediation of the significant glucose utilisation. It was further observed that the compound prevented the delivery and absorption of carbohydrates *in vitro*, by inhibiting the key carbohydrate metabolising enzymes. M. acetate also displayed a promising scavenging effect on free radicals *in vitro.* Therefore, *in vitro*, M. acetate demonstrated a good therapeutic efficacy as an antidiabetic and antioxidant.

In vivo, hypoglycaemic activity was not observed in STZ-diabetic animals treated with M. acetate and crude extract. Although, M. acetate showed appreciable glucose utilisation *in vitro.* Furthermore, M. acetate was capable of improving some haematological parameters affected by induction of STZ. These findings conclude that M. acetate was displayed as a promising hypoglycaemic agent *in vitro*, with no apparent hypoglycaemic effect *in vivo.* Therefore, further studies are required to confirm the therapeutic efficacy of M. acetate.

6.1 Recommendation for future studies

- Antidiabetic activity investigation of M. acetate at lower dosages, *in vivo.*
- Elucidate the mechanism of action of M. acetate.
- Structural modification studies of M. acetate.
- Evaluation of M. acetate for other pharmacological activities.

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Appendix A
Preparation of reagents

A.1 **20% sulphuric acid**

20 ml of sulphuric was added in 80ml methanol

A.2 Phosphate buffer (pH 7.0)

The buffer contained 13.6g potassium dihydrogen orthophosphate, 17.4g di-potassium hydrogen orthophosphate anhydrous, 5.8g sodium chloride, 2g bovine serum albumin and 0.2g sodium azide, in 1L dH_2O.

A.3 Sodium phosphate buffer (pH 6.9)

The buffer contained 0.29g potassium dihydrogen orthophosphate, 0.34g di-potassium hydrogen orthophosphate anhydrous and 0.11g sodium chloride, in 1L dH_2O.

A.4 Citrate buffer (pH 4.5)

The buffer contained sodium citrate and citric acid, in 0.5L dH_2O.

A.5 Sodium carbonate solution

The solution contained 0.2g sodium carbonate in 10ml dH_2O.

A.6 10% Formalin

The buffer contained 100ml formaldehyde, 4g sodium dihydrogen phosphate, 6.5g sodium hydrogen phosphate and 900ml dH2O.

Appendix B
Details of methodology

B.1 Chromatographic analysis

Chromatography is a method based on the separation of mixtures into individual components. Column chromatography is a technique that consists of a column in which the stationery phase of particulate material such as silica or alumina, and the mobile phase is a liquid. The column was prepared as follows: cotton was added at the base, with the addition of hexane to avoid air bubbles; a thin layer of sea sand; 300g of silica gel mixed with hexane followed; another layer of sea sand was added to protect the shape of the extract and lastly 10g of each crude extract. The eluent was slowly passed through the column to advance the crude extract.

Thin layer chromatography is a technique involving a particulate sorbent spread on a sheet of glass, plastic, or aluminium foil. The sample is spotted on the sorbent and the mobile phase is allowed to move upwards the plate, carrying the sample. The TLC spots were fixed with a solution made of 20% H2SO4 in methanol, heated with a hair dryer for colour development and visualized under Spectroline UV light at 254nm. The fractions were collected per 80ml and accordingly combined through their TLC profiles.

B.2 Structural Elucidation

B.2.1 NMR

NMR spectroscopy is the study of interaction of radio frequency (RF) of the electromagnetic radiation with unpaired nuclear spins in an external magnetic field to obtain structural data on a given sample.

B.2.1.1 One Dimensional NMR

Proton nuclear magnetic resonance (^{1}H NMR) is an application which identifies hydrogen atoms within the molecules of a substance, in order to determine the structure of its molecules. Carbon-13 nuclear magnetic resonance (^{13}C NMR) is an application that identifies carbons in

an organic molecule. The NMR measurements were conducted operating at 500 MHz for ^{1}H (Fig C1) and 125 MHz for ^{13}C (Fig C2) using $CDCL_3$.

B2.1.2 Two dimensional NMR

Distortionless enhancement of polarisation transfer (DEPT) is an application where the nuclei of interest are carbon -13 nuclei, the signals from which are enhanced by the transfer of magnetisation from ^{1}H nuclei. DEPT was operated at 125 MHz using $CDCL_3$. Correlation spectroscopy (COSY) is a 2D NMR application for determining which signals arise from neighbouring protons. NMR measurement were conducted at 400 MHz using $CDCL_3$, both axes show ^{1}H-NMR of the compound (Fig C4)

Nuclear overhauser effect spectroscopy (NOESY) is an application used to determine the intra- or inter-molecular distances. NOESY was operated at 500 MHz using $CDCL_3$, it shows which protons are nearer to each other in space by drawing a straight line from any of the dark spots to each axis of the plot (Fig C5). Heteronuclear single-quantum correlation spectroscopy (HSQC) is an application that shows correlation between directly bonded 1H and an X-heteronuclei, usually ^{13}C and ^{15}N. HSQC was operated at 500 MHz using $CDCL_3$ (Fig C6).

Heteronuclear multiple bond correlation (HMBC) is an application that gives correlations between carbons and protons that are separated by two, three and sometimes conjugated systems, four bonds. HMBC was operated at 500 MHz using $CDCL_3$, the abscissa of the spectrum shows the proton signals, and the ordinate shows the carbon signals. The spots in the spectrum indicate which protons signal is attached to which carbon and the nature of the signals (Fig C7).

B3 Mass Spectroscopy (MS)

MS is an application that generates charged particles (ions) from the molecules of the analyte. The ions are thereafter analysed to provide information on the molecular weight of the compound and its structure.

B4 Infrared spectroscopy

Infrared Spectroscopy is the analysis of infrared light interacting with a molecule. It measures the vibrations of atoms, and based on this it is possible to determine the functional groups.

B5 X-ray crystallography

X-ray crystallography is an instrument used to determine the atomic and molecular structure of a crystal. The crystalline atoms cause a beam of incident X-rays, diffracting into numerous precise directions. The compound Mukaadial Acetate was recrystallized using the solvent hexane as follows; it was simmered in the solvent for 5-10 min and thereafter, left to evaporate at room temperature to obtain cryst.

Appendix C
Spectra

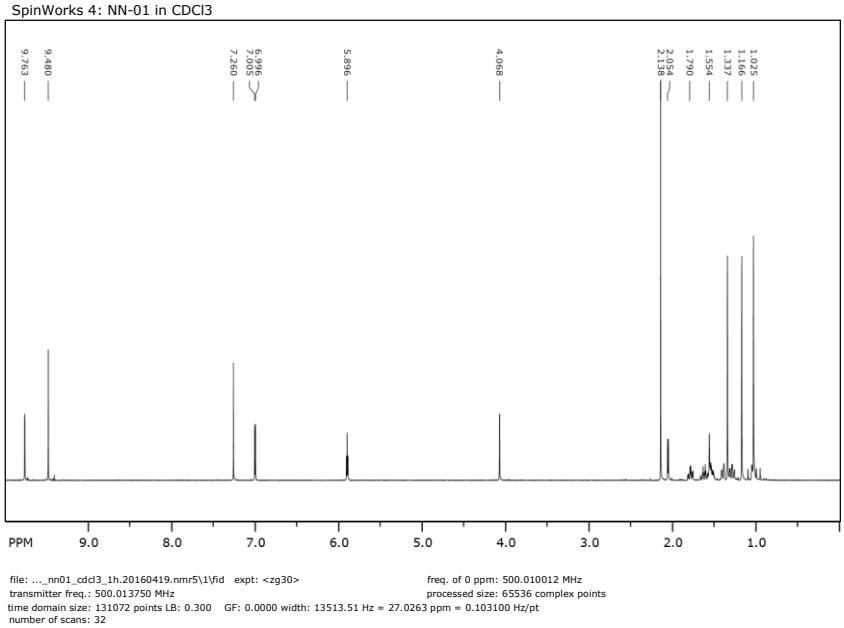


Figure C1: ^{1}H Spectrum of Mukaadial acetate in $CDCL_3$.

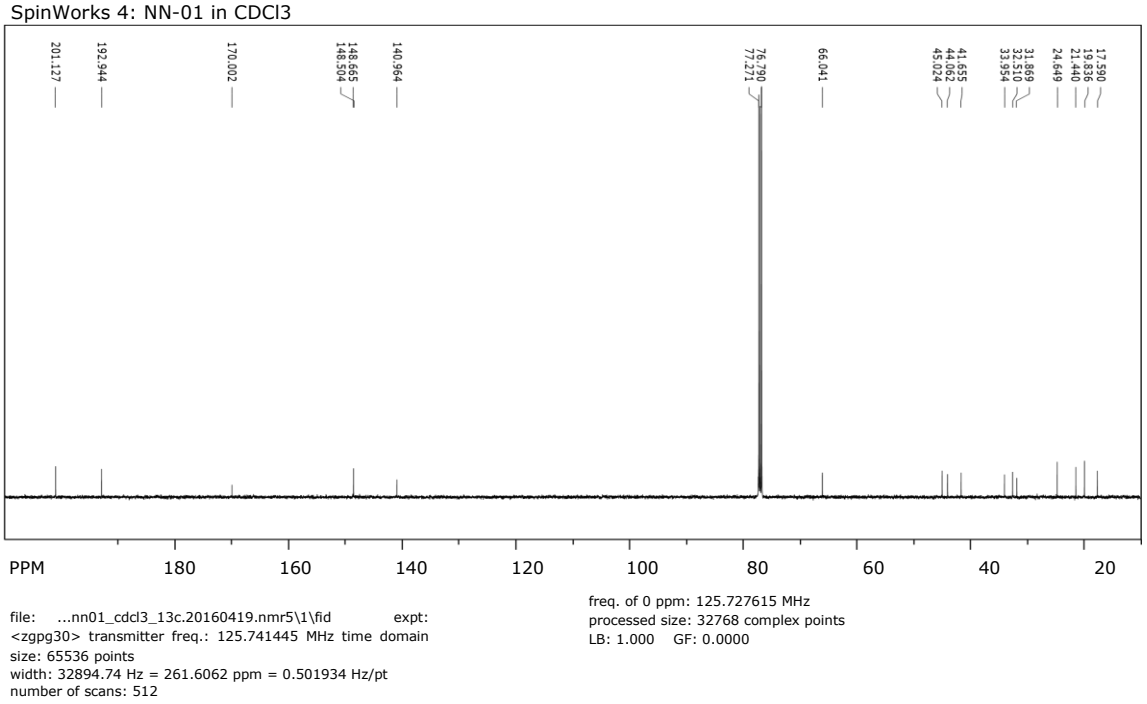


Figure C2: ^{13}C Spectrum of Mukaadial acetate in $CDCL_3$.

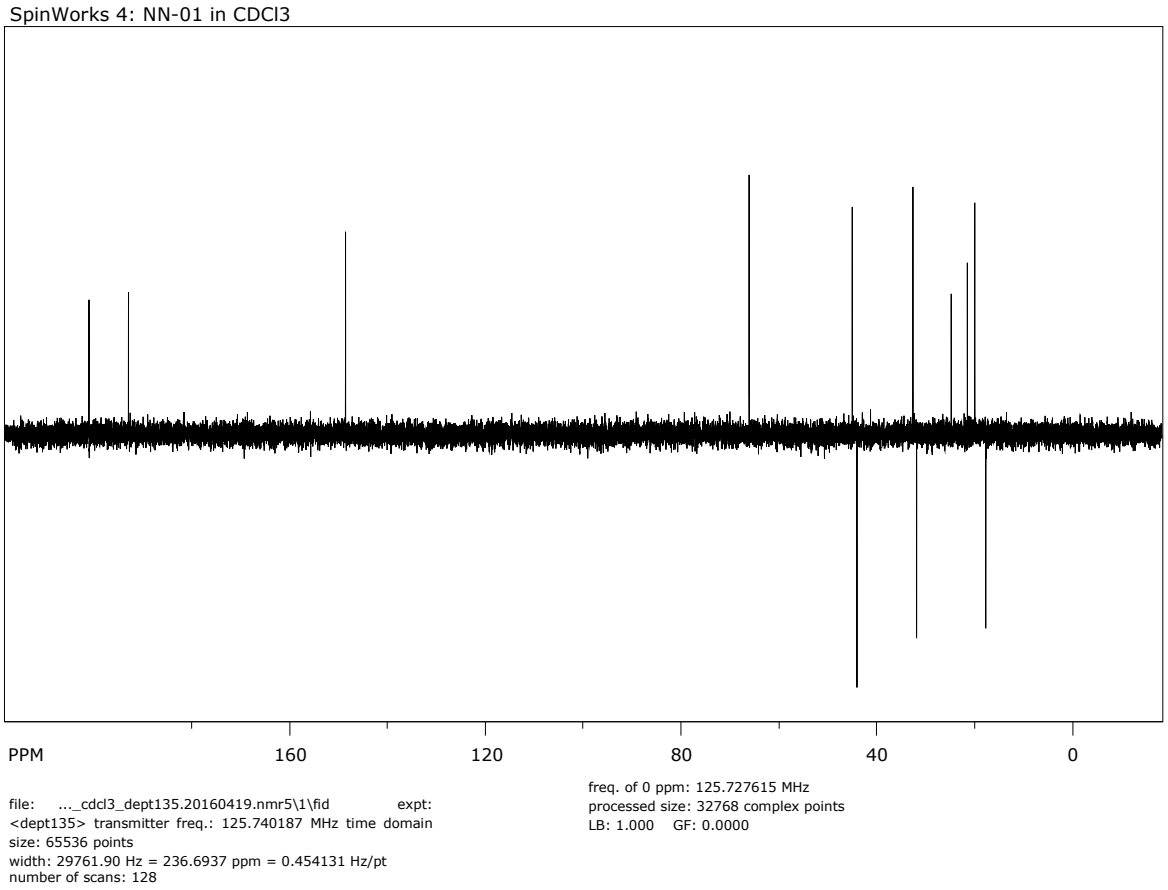


Figure C3: DEPT Spectrum of Mukaadial acetate in $CDCL_3$.

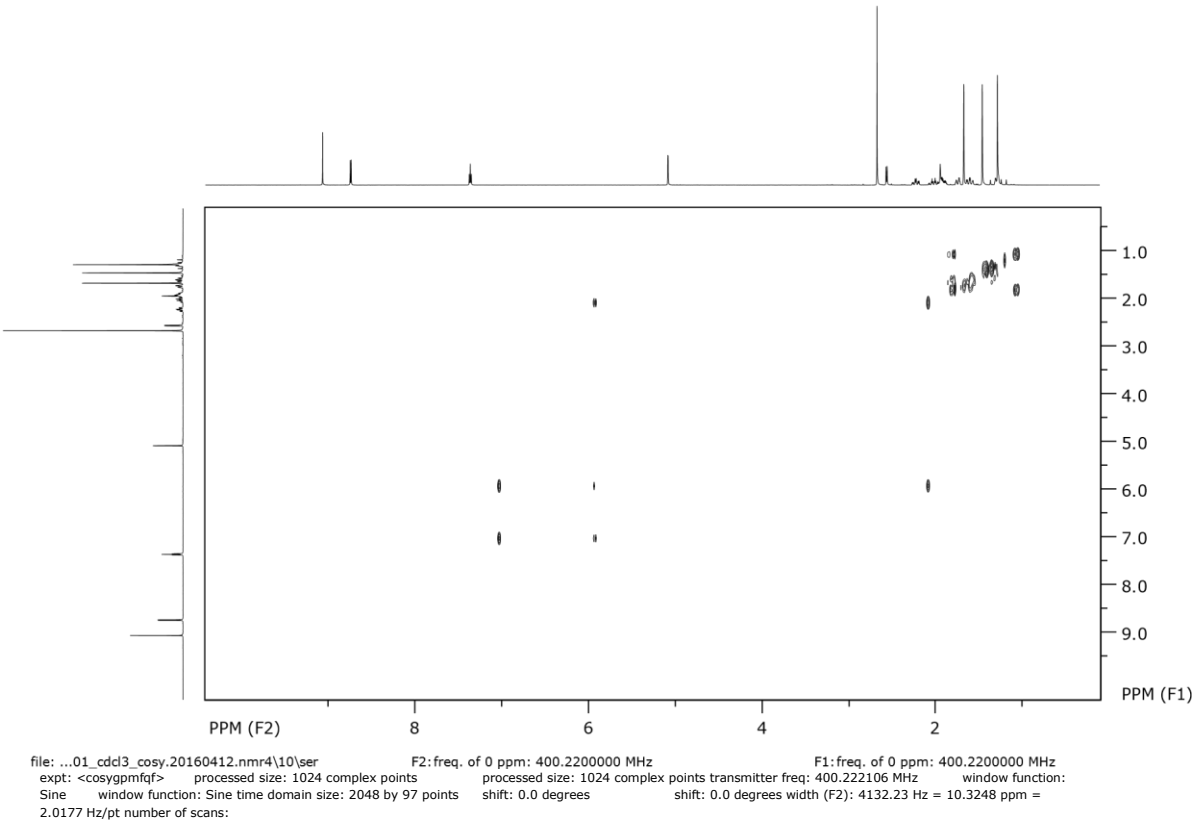


Figure C4: COSY Spectrum of Mukaadial acetate in $CDCL_3$.

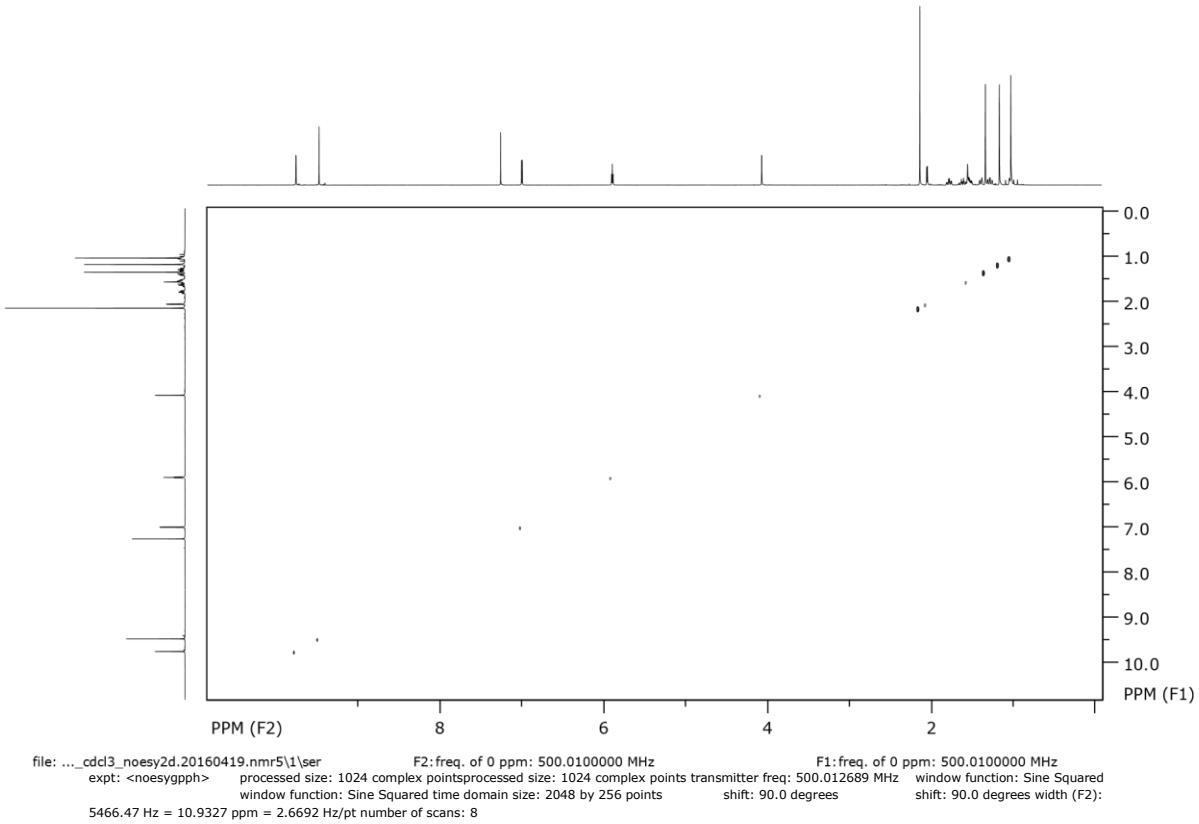


Figure C5: NOESY Spectrum of Mukaardial acetate in $CDCL_3$.

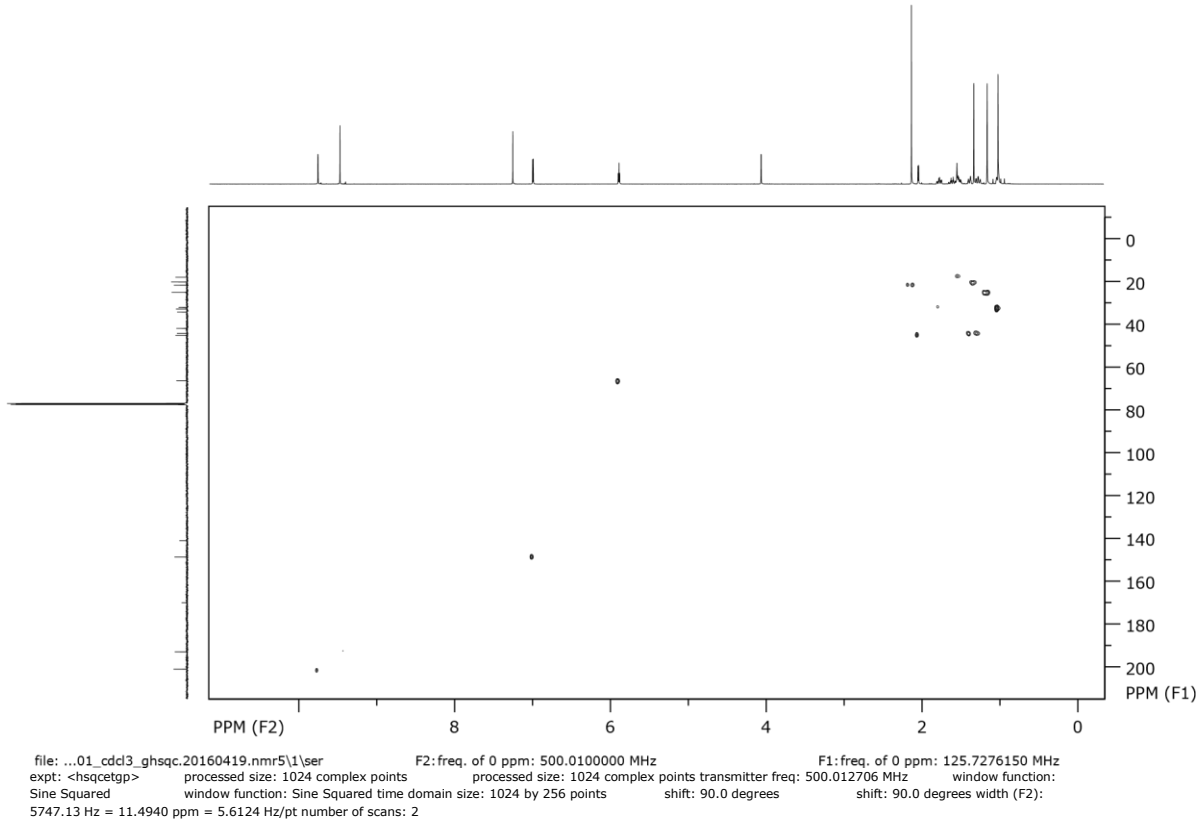
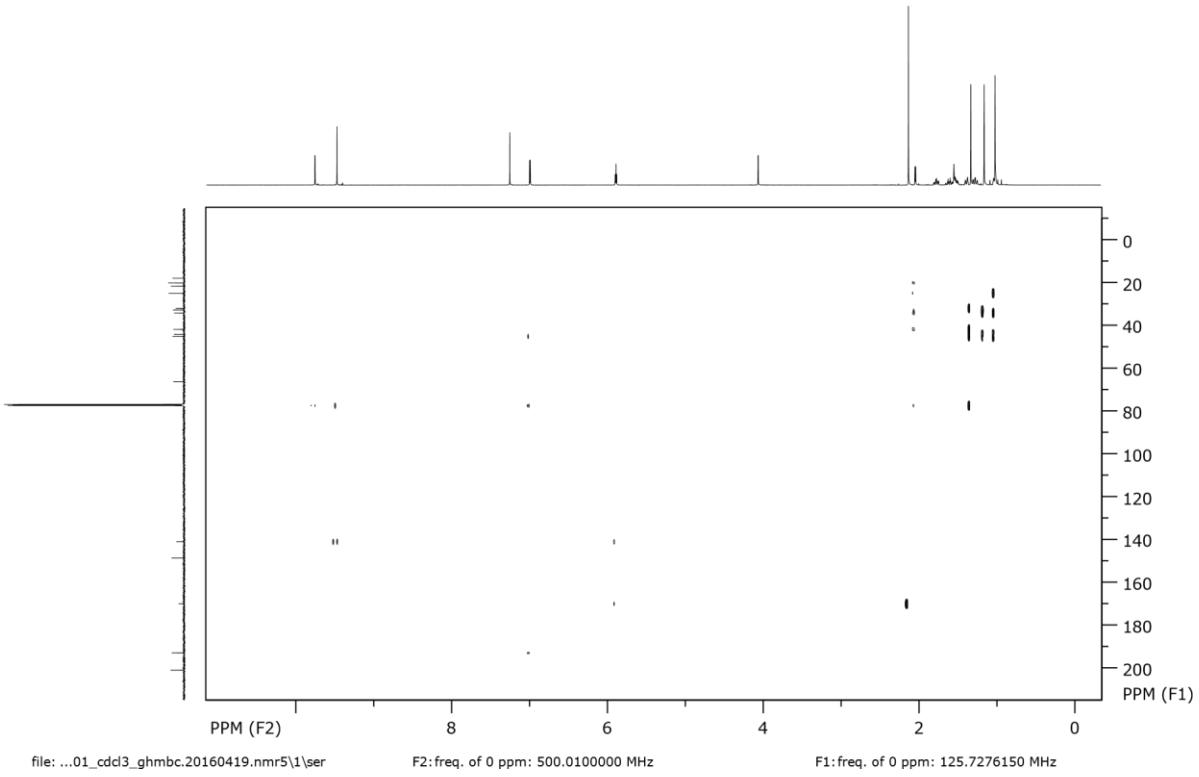


Figure C6: HSQC Spectrum of Mukaadial acetate in $CDCL_3$.



expt: <hmbcgplpndqf> processed size: 2048 complex points processed size: 1024 complex points transmitter freq: 500.012706 MHz window function: Sine window function: Sine time domain size: 4096 by 128 points shift: 0.0 degrees shift: 0.0 degrees width (F2): 5747.13 Hz = 11.4940 ppm = 1.4031 Hz/pt number of scans: 8

Figure C7: HMBC Spectrum of Mukaadial acetate in $CDCL_3$.

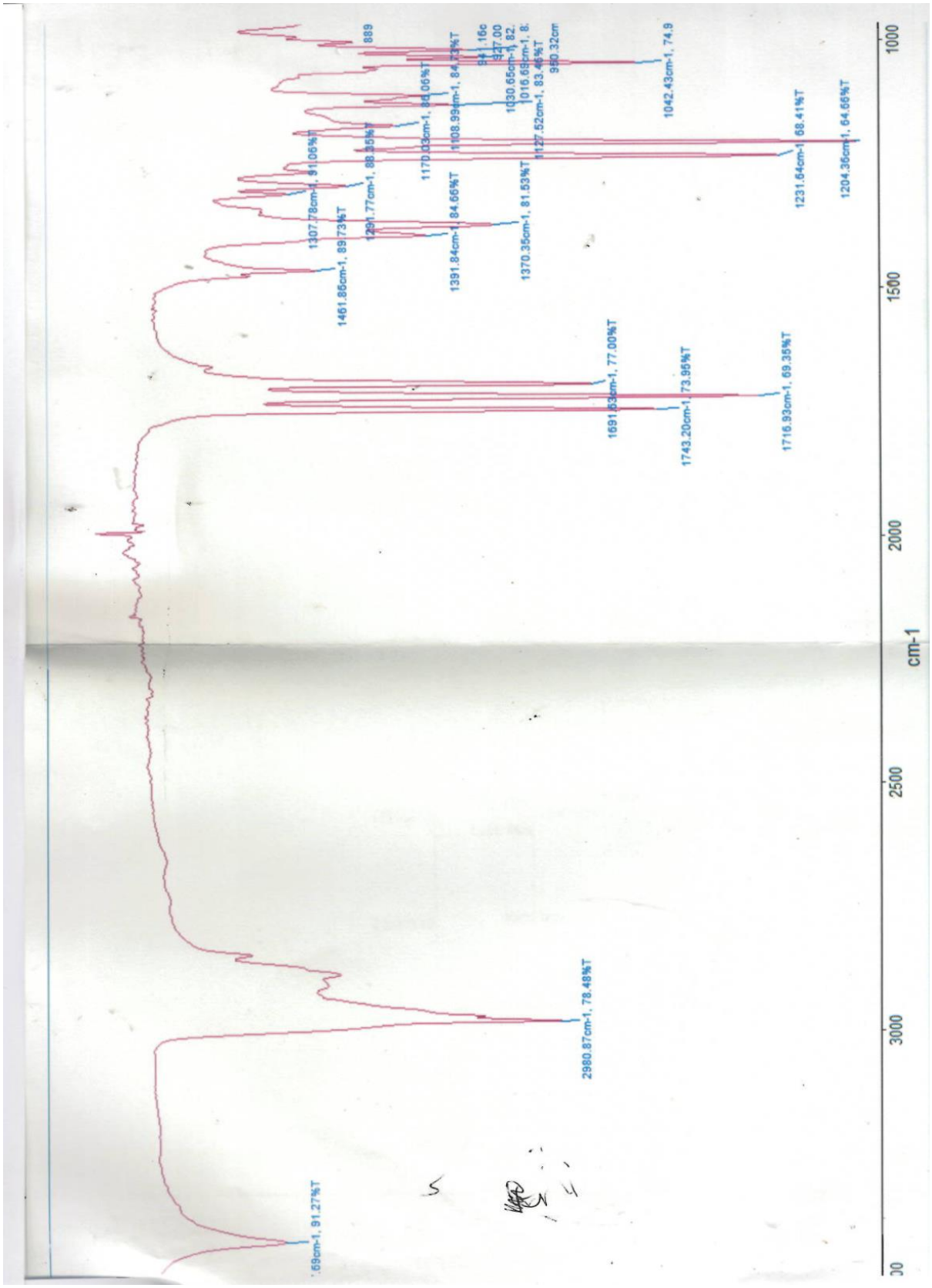


Figure C8: IR spectra for Mukadial acetate

Appendix D
Ethical approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

26 May 2016

Ms Nontokozo Zimbili Msomi (215075542)
School of Life Sciences
Pietermaritzburg Campus

Dear Ms Msomi,

Protocol reference number: AREC/009/017M
Project title: Effects of W.*salutaris* in insulin-resistant state *in vivo*

Full Approval – Research Application

With regards to your revised application received on 18 April 2017 and 19 May 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following condition:

CONDITION:
All veterinary procedures must be conducted by a SAVC registered Vet or a SAVC authorized person or under their assistant supervision.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 26 May 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Dr Mthokozisi BC Simelane
Cc Academic Leader Research: Dr Mogie Singh
Registrar: Mr Simon Mokoena
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Appendix E
Contribution to Knowledge

Chapter 10

Olea europaea subsp. *africana* (Oleaceae)

Nontokozo Z. Msomi and Mthokozisi. B. C Simelane

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/65725

Abstract

Background: Medicinal plants have been used as a key source for medication and they remain to provide new therapeutic remedies to date. Extracts of *Olea europaea* subsp. africana *Oleaceae* (leaf, bark and root) are used extensively in Africa to treat various diseases traditionally. Phytochemistry has identified phenols, terpenoids and coumarins in different parts of the plant. However, little pharmacological studies have been done on *Olea europaea* subsp. *africana*. The present review aims to compile available information on the ethnobotany, phytochemistry, pharmacology and toxicology of *Olea africana*.

Materials and methods: Information available pertaining *Olea europaea* subsp. *africana* was collected through electronic search using (Google Scholar, PubMed and Science Direct).

Results: *Olea africana* has been used throughout Africa traditionally for various ailments. Phytochemical studies have led to the isolation of compounds, namely oleuropein, esculin, ursolic acid, scopolin and oleanolic acid. Studies have shown that the leaf extract contains antihypertensive, diuretic, anti-atherosclerotic, antioxidant, antidiarrhoeal and hypoglycaemic activities. Conclusion: *Olea africana* has been used expansively for treating ailments traditionally, but pharmacological studies are seldom published. Further research is required to extend existing therapeutic potential of the African olive.

Keywords: *Olea europaea* subsp. *africana*, ethnobotany, phytochemistry, pharmacology, toxicology

1. Introduction

Medicinal plants are defined as any plant containing substances which can be used for curative purposes in one or more parts of its organ, which are precursors for the production of useful

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