HYPOGLYCEMIC, HYPOLIPIDEMIC AND HEPATOPROTECTIVE EFFECTS OF *Polyscias fulva* ETHANOLIC BARK EXTRACT IN DIABETIC RATS

BY

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been submitted for any academic award in any institution; and shall not be reproduced in part or full, or in any format without prior written permission from the author and/or University of Eldoret.

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To my spouse, children and mama for their encouragement and moral support in pursuing this program.

ABSTRACT

Diabetes Mellitus (DM) has been on the alarming rise worldwide and medicinal plants may play a role in its treatment including *Polyscias fulva* (*P. fulva*) stem bark that is presumed to have anti-diabetic activity but not documented. Efforts to produce synthetic drugs for treating DM is costly with side effects. Therefore, this study examined the effects of crude ethanolic bark extract of *P. fulva* on diabetic rats to elucidate its anti-diabetic potential. General qualitative phytochemicals were analyzed in the extract then male albino rats were randomized into five groups (five rats each) which were 1; normal rats, 2; diabetic-induced untreated rats, 3 and 4; diabetic-induced rats on 200 and 400mg/kg bwt/day of extract, respectively and 5 was diabetic-induced rats on metformin at 100mg/kg bwt/day. The rats received oral treatments daily for 21 days and on normal chow and water *ad libitum*. Fasting blood glucose levels and body weights were determined weekly. After 21 days, animals were sacrificed, blood collected and liver tissues preserved for histopathology and malonedialdehyde (MDA) assays. Serum analysis of lipid profile, liver biomarker enzymes (Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) and proteins (Total proteins-TP, albumin-ALB & globulins-GLB) were performed. Data was expressed as mean ± SEM and statistical analysis done using Student *t*-test and ANOVA with $p<0.05$. *P. fulva* extract showed the presence of tannins, anthraquinones, terpenoids, saponins, flavonoids and steroids and absence of alkaloids. Also, the study showed that *P. fulva* had significant hypoglycemic and hypolipidemic effects in the diabetic rats compared to diabetic untreated rats. Additionally, serum levels of ALP, ALT and AST were significantly lowered in extract-treated diabetic rats while TP and ALB were significantly elevated in the extract treated diabetic rats compared to diabetic untreated rats. The levels of MDA in liver tissues was significantly elevated in diabetic untreated rats compared to normal control rats while the *P. fulva* extract treated rats had significantly lowered levels compared to diabetic untreated group. Results on liver histopathology showed normal hepatocytes in the normal control group but the diabetic untreated group showed liver pathological changes. Upon treatment with *P. fulva* extract at 400mg/kg bwt/day, the pathological changes were reversed in the hepatocytes. In conclusion, the phytochemical–rich extract of *P. fulva* showed hypoglycemic, hypolipidemic, and hepatoprotective effects in streptozotocin (STZ) induced diabetic rats. This study provides insights into the potential of *P. fulva* as an alternative medicine in management of DM.

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LIST OF ABBREVIATIONS

ADA – American diabetes association

AGEs – Advanced Glycated End Products

ALB – Albumin

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AMP – Adenosine Monophosphate

ANOVA – Analysis of Variance

Apo A – Apolipoprotein A

Apo B – Apolipoprotein B-100

AST – Aspartate aminotransferase

ATP – Adenosine Triphosphate

Bwt – Body weight

CAT – Catalase

CHD – Coronary heart disease

CHE – Cholesterol Esterase

CHOD – Cholesterol Oxidase

CHOL-T – Total Cholesterol

CVD – Cardiovascular Disease

DASH – Dietary Approaches to stop Hypertension

DKA – Diabetic Ketoacidosis

DKD – Diabetic Kidney Disease

DM - Diabetes mellitus

DPN – Diabetic Peripheral Neuropathy

DPP-4 – Dipeptyl Peptidase 4

FBS – Fasting Blood Sugars

FBWTS – Fasting body weights

FPG – Fasting Plasma Glucose

GAD – Glutamic acid decarboxylase

GDM – Gestational Diabetes Mellitus

GI – Gastrointestinal

GLB - Globulins

GLP-1 – Glucagon-like peptide 1

GOD – Glucose Oxidase

GPx – Glucose Peroxidase

H_2O_2 – Hydrogen Peroxide

HbA1c – Glycated hemoglobin

HDL-C – High density lipoproteins

HLA – Human leukocyte antigen

IFCC – International Federation of Clinical Chemistry

IGT – Impaired blood glucose

IR – Insulin resistance

KATP – Adenosine Triphosphate Sensitive Potassium Channels

LDL-C – Low density lipoproteins

LPO – Lipid peroxidation

MDA – Malonldialdehyde

MDH – Malate Dehydrogenase

MODY – Maturity Onset Diabetes of the Young

OD – Optical density

OGGT – Oral Glucose Tolerance Test

OH^- - Hydroxyl Radical

PA – Physical Activity

PEG – Polyethylglycol

POD – Peroxidase

R.p.m – Revolutions per minute

SDS – Sodium dodecyl sulphate

SEM – Standard Error of the mean

SGLT2 – Sodium Glucose Cotransporter 2

SOD – Superoxide Dismutase

SPSS – Statistical Package of Social Sciences

STZ – Streptozotocin

SUR 1 – Sulfonylurea Receptor 1

T1DM – Type1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TGs – Triglycerides

TMP – Tetramethoxypropane

TNF – Tumor Necrotic Factor

TP - Total Proteins

TZDs – Thiazolidinedione’s

VEGF – Vascular Endothelial Growth Factor

VLDL – Very low density lipoprotein

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CHAPTER ONE

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a disorder of carbohydrate, fat and protein metabolism, and characterized by absolute or relative lack of insulin, resulting in hyperglycemia (Saini, *et al*., 2017). DM has also been described as a state of physiological stress, during which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin, leading to the elevation in plasma gl (Rai *et al*., 2016). The hyperglycemia results due to compromised insulin secretion or sensitivity, disturbed postprandial metabolism resulting in less efficient uptake and utilization of glucose by the targeted tissues and more utilization of proteins and fats (Li *et al*., 2018). A progressive dysfunction of pancreatic β -cells with a decline in insulin secretion leads to persistent hyperglycemia (Kurup & Mini, 2017). High blood sugar level results either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. Hyperglycemia produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Khan *et al*., 2017). This metabolic disorder is characterized by chronic hyperglycemia coupled with disturbances in carbohydrate, protein and fat metabolism resulting from decrease in circulating concentration of insulin, decrease in the response of peripheral tissues to insulin or both (Couturier *et al*., 2015; Emordi *et al*., 2016).

An endocrine disorder remains the major cause of DM and oral hypoglycemic drugs and/or insulin is used generally for the treatment. As the immune system of the body diminishes, the pancreatic β cells that produce insulin for the regulation of blood glucose

level, get affected, hence, develop type 1 DM (T1DM) (Sheweita *et al*., 2016). In type 2 DM (T2DM), resistance is developed against the use of insulin by cells. As a result, the need for insulin increases and pancreas gradually loses its ability to produce insulin. Although pathogenesis of both T1DM and T2DM are varied, hyperglycemia and its related complications are common to some extent in both types (Rashid *et al*., 2014). Impaired lipid and lipoprotein metabolism, oxidative stress (over production of free radicals and defect in endogenous antioxidant defense system), sub-clinical inflammation, vascular endothelial dysfunction and hypertension are commonly associated with T2DM (Mirmiran *et al*., 2014). These metabolic disorders lead to long-term secondary pathogenic disorders such as micro- and macro-vascular complications including neuropathy, retinopathy, nephropathy, and decreased quality of life and increased mortality rate (Irudayaraj *et al*., 2017).

The most recent estimate of people with diabetes is projected to increase from 415 million in 2015 to 642 million by 2040 indicating an increase in number of diabetic patients by 35.3% in 25 years (Chaudhary *et al*., 2016). This number has doubled over the past 20 years, and notably 80% of people with diabetes live in low and middle income countries due to increased rate of industrialization amongst other risk factors (Emordi *et al*., 2016). Diabetes already contributes significantly to morbidity and mortality in Africa. The highest global age-specific mortality rate is recorded in Africa (Werfalli *et al*., 2014). This number of people with DM worldwide is exponentially increasing and by 2030, it is projected to rise to 552 million (Martín-Timón *et al*., 2014).

According to many researchers, regular consumption of diets rich in vegetables, fruits, whole grains, herbs, nuts, seeds, which contain plenty of phenolic compounds,

terpenoids, phytosterols and organo-sulfur exert their beneficial effect in disease prevention, by regulating several cellular molecular pathways like regulation of inflammation, redox potentials, metabolic disorder, apoptosis, among other regulatory processes (Upadhyay, 2015).

Plants have been used to treat different health related problems in many countries of the world since time immemorial. It has also been reported that the continuous use of synthetic drugs has increased the health- related problems because of their side effects. Therefore, plant-based medicines are becoming popular throughout the world (Sidhu & Sharma, 2014). For instance, many efforts have been made to search for other effective and safe enzyme inhibitors from natural materials in order to control diabetes (Shori, 2015). The tropical rain forests have become an important point of this activity, primarily due to the rich biodiversity they harbor, which promises a high diversity of chemicals with potential novel compounds. However, of this rich biodiversity, only a small portion has been studied for its medicinal potential. Thus, natural plants and herbs can be a robust source of drugs, with fewer side effects and better bioavailability for treatment of some chronic diseases in the future. Besides drugs, several species of plants have been cited in literature as having hypoglycemic activity (Ramadan *et al*., 2013). The alarming rate of increase of T2DM is worsening this disease burden. This condition initially thought to be in well developed countries is no longer the case. Change in lifestyle in developing countries has increased T2DM exponentially (Abdirahman *et al*., 2015). This has warranted researchers to be actively involved in research to try and remedy the situation. *Polyscias fulva (P. fulva)* is one of the many plants widely used in traditional folk

medicine in Kenya and Africa and there is need to scientifically undertake studies to validate claims that it has antidiabetic effects (Sagnia *et al*., 2014).

1.2 Statement of the Problem

The rising number of cases of T2DM and its accompanying complications is alarming. Worse still are the effects felt in the third world initially thought to be a condition of the western world (Werfalli *et al*., 2014). Urbanization has driven dramatic changes in lifestyle and in particular in developing countries. With these rapid transitions come accompanying increases in risk factors for noncommunicable diseases like T2DM (Guariguata *et al*., 2014). Moreover, there is lack of policies governing nutrition and few numbers of nutritionists to advice on the recommended dietary allowances. The incidence of type 1 diabetes, which accounts about 5–10% of all cases of diabetes; continues to increase worldwide and it has serious short-term and long-term implications (Daneman, 2006). Despite availability of many pharmacological interventions including oral hypoglycemic agents and insulin therapy for diabetes management, current evidence shows an alarming rising trend in the occurrence of undesirable complications among patients. In addition, modern remedies have not fully addressed diabetes mellitus and its associated complications and many patients become dependent on anti-diabetic drugs for life (Mirmiran *et al*., 2014). Also, the efforts to produce synthetic drugs for treating diabetic patients is expensive coupled with high level of treatment failures and unpleasant side effects. Researchers have generated an urgent need and desire for alternative treatments and herbal medicine is an alternative to the poor populations particularly in developing countries (Emordi *et al*., 2016). The management of diabetes mellitus is also considered a global problem and successful treatment is not yet available (Kazeem *et al*.,

2013a). DM has been described as an epidemic requiring global attention and urgent action (Herman & Zimmet, 2012). This therefore calls for research in locally available alternative remedies and or plants targeting the regulation of the hyperglycemia and its associated complications.

1.3 Justification of the Study

P. fulva stem, bark and leaves have been used traditionally to treat many conditions (Fern, 2014). In view of this medicinal value attached to *P. fulva* and its abundance in many African countries including Kenya (Orwa, 2010), investigation of hypoglycemic, hypolipidemic and hepato-protective properties of the bark of this plant was carried out in this study because traditional African medicine men have reported that the bark of the plant has been used to treat diabetes mellitus and has also been used to treat obesity (Jeruto *et al*., 2008). To validate this claim, the animal model of choice in this study was the male Wistar albino rat; - 6-8 weeks old and weighing between 140-180g. Since, synteny or relatedness of rats to humans is also close and are easily bred, easily handled, its longevity and availability makes the use of this rat model in biological as well as medical research appropriate. Moreover, normative behavior of Wistar rats can easily be studied or induced pathological process can be investigated, and in which the phenomenon resembles the likelihood phenomenon in humans or other species of animals (Clause, 1993). The rats were diabetically-induced by single intraperitoneal injection of freshly prepared ice cold streptozotozin (STZ) of 50mg/kg bwt dissolved in sodium citrate buffer at pH 4.5 (Ramadan *et al*., 2013). STZ is preferred in diabetes induction due to its similarity in structure to glucose and glucose can compete with STZ, and thus, fasting animals tend to be more susceptible. Moreover, chemically induced diabetes is

appropriate to use when testing drugs or therapies where the main mechanism of action is lowering blood glucose in a non-beta-cell-dependent manner (King, 2012).

The use of ethanol in the extraction of the bark crude extract of *P. fulva* was preferred since it is an organic solvent able to extract whole active ingredients (crude extract) commonly used in bioassays. To monitor anti-diabetic activity of the extract in rats, fasting blood glucose levels was analyzed (Piero *et al*., 2012). Initial analysis of blood glucose was to confirm induction of diabetes mellitus as a good indicator of hyperglycemia. Blood glucose levels was also necessary in monitoring the anti-diabetic effect of *P. fulva.* ethanolic bark extract. To assess whether there were changes in lipid profile following treatment with *P. fulva* ethanolic bark extract, serum levels of total Cholesterol (Chol-T), Triglycerides (TGs), High density lipoprotein-cholesterol (HDL-C) and Low density lipoprotein-cholesterol (LDL-C) were analyzed (Rai *et al*., 2016) because lipid abnormalities leads to non-alcoholic fatty liver disease (NAFLD) which are prevalent in DM due to insulin resistance or deficiency that affects key enzymes and pathways in lipid metabolism (Ozder, 2014). Moreover, serum liver biomarkers; Alkaline phosphatase (ALP), Alaline aminotransferase (ALT), Aspartate aminotransferase (AST), Total proteins (TP), Albumin (ALB), Globulins (GLB) was determined because they are useful predictors of liver injury. The liver is the major source of most the serum proteins. For instance, albumin is the most important protein synthesized by the liver and is a useful indicator of hepatic function (Thapa & Walia, 2007). Aminotransferases are considered as indicators of hepatocellular health since ALT and AST are found primarily in the liver. Hyperglycemia can damage the liver leading to increase in ALT, AST, and ALP in the liver hepatocytes. The liver also plays a role in the maintenance of normal

glucose concentrations during fasting, as well as postprandial glucose levels. The lack of a direct effect of insulin to suppress hepatic glucose production and glycogenolysis in the liver causes enhanced hepatic glucose production. Additionally, the liver is a major site of insulin clearance (Javad *et al*., 2012). Finally, liver tissue histopathological examinations were carried out to assess liver function and any of its histopathological changes following treatment. Furthermore, the level of malondialdehyde as an index of lipid peroxidation in liver tissues was determined to assess if *P. fulva* extract has protective effects against oxidative stress that is common in cases of hyperglycemia (Kazeem *et al*., 2013b; Rai *et al*., 2010b).

1.4 Objectives of the Study

1.4.1 Overall Objective

This study aimed at examining the hypoglycemic, hypolipidemic and hepato-protective effects of ethanolic bark extract *P. fulva* in STZ diabetic induced male Wistar albino rat model (*Rattus norvegicus*).

1.4.2 Specific Objectives

The specific objectives of this study were;

1. To evaluate qualitatively some general classes of phytochemicals present in ethanolic bark extract of *P. fulva.*
2. To study the effects of ethanolic bark extract of *P. fulva* on blood glucose, serum lipids and body weights of streptozotocin diabetic induced rats.
3. To examine the effect of ethanolic bark extract of *P. fulva* on liver lipid peroxidation in streptozotocin diabetic induced rats.

4. To examine the liver function and histopathological changes of streptozotocin diabetic induced rats following treatment with ethanolic bark extract of *P. fulva.*

1.5 Hypothesis

1.5.1 The Null Hypothesis

1. There are no phytochemicals present in ethanolic bark extract of *P. fulva*.
2. There is no effect of ethanolic bark extract of *P. fulva* on blood glucose and lipids in streptozotocin diabetic induced rats.
3. There is no effect of ethanolic bark extract of *P. fulva* on liver lipid peroxidation in streptozotocin diabetic induced rats.
4. There are no changes in liver function serum indices and liver histology following treatment with ethanolic bark extract of *P. fulva* in streptozotocin diabetic induced rat model.

1.5.2 The Alternative Hypothesis

1. Phytochemicals are present in ethanolic bark extract of *P. fulva*.
2. There is an effect of ethanolic bark extract of *P. fulva* on blood glucose and lipids levels in streptozotocin diabetic induced rats.
3. There is an effect of ethanolic bark extract of *P. fulva* on liver lipid peroxidation in streptozotocin diabetic induced rats.
4. There are differences on liver function serum indices and liver histology following treatment with ethanolic bark extract of *P. fulva* in streptozotocin diabetic induced rats.

1.6 Research Questions

1. Are there phytochemicals present in ethanolic bark extract of *P. fulva*?
2. Is there a significant difference on the effect of ethanolic bark extract of *P. fulva* bark on hyperglycemia and hyperlipidemia on streptozotocin diabetic induced rats versus untreated diabetic rats?
3. Is there a significant difference in the effect of ethanolic bark extract of *P. fulva* on liver lipid peroxidation in streptozotocin diabetic induced rats versus untreated diabetic rats?
4. Is there a significant difference on liver function serum indices and liver histology following treatment with ethanolic bark extract of *P. fulva* in streptozotocin diabetic induced rats versus untreated diabetic rats?

1.7 Overall Study Significance

This study provides insightful information for the management of diabetes mellitus using *P. fulva* bark extract in rats. Alternative medicine such as the *P. fulva* bark extract might therefore be beneficial in the management and prevention of diabetes mellitus in humans with potentially low cost and minimal side effects as compared to the conventional drugs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Kharroubi & Darwish, 2015). It is one of the most common non-communicable diseases world-wide. It is the major metabolic disorder characterized by insufficient insulin secretion and/or insensitive target tissues to metabolic actions of insulin (Emordi *et al*., 2016). The role of insulin in the body is to stimulate the fat cells and muscle to remove the glucose from the blood and stimulate the liver to metabolize glucose, causing the blood sugar level to decrease to normal level (Kumar *et al*., 2014a). Diabetes mellitus is characterized by hyperglycemia with classical symptoms of polyuria, polydipsia and polyphagia (Cooke & Plotnick, 2008). Other signs of diabetes may include unusual fatigue, unexplained weight loss, a number of tingling of extremities, blurred vision, dry or itchy skin, recurrent infections and cuts or bruises that take long time to heal.

2.2 Epidemiology of Diabetes mellitus

The global pervasiveness of diabetic patients is projected to increase from 415 million in 2015 to 642 million by 2040 indicating an increase in number of diabetic patients by 35.3% in 25 years (Chaudhary *et al*., 2016). Recent reports illustrate steep increases in the global prevalence of DM over past few decades and projected the trend to keep rising over the next 20 to 40 years (Mattei *et al*., 2015). The rise in DM is noted worldwide, causing 4.8 million deaths and morbidity in 371 million people every year. In recent years, change in patterns have been observed in the age of onset of DM with younger

populations now disproportionately affected (Kanguru *et al*., 2014). This number has doubled over the past 20 years, and notably 80% of people with diabetes live in low and middle income countries. Diabetes already contributes significantly to morbidity and mortality in Africa. The highest global age-specific mortality rate is recorded in Africa (Werfalli *et al*., 2014).

In Kenya, a recent study has shown a prevalence of 4.2% in the general population, with a prevalence of 2.2% in the rural areas and a prevalence of 12.2% in the urban population (Abdirahman *et al*., 2015). The loss of life from premature death among persons with diabetes is greatest in developing countries. In these countries, three-quarters of all people with diabetes are under 65 years old and 25% of all adults with diabetes are younger than 44 years. In contrast, more than half of all people with diabetes in developed countries are older than 65 years, and only 8% of adults with diabetes are younger than 44 years (Abdirahman *et al*., 2015). By 2030 developing countries will face an increase by 69% and industrialized countries by 20% of the number of patients with diabetes compared to diabetes status in 2010. For Africa, more than 18 million and in some estimations, even 24 million diabetic patients are predicted for the year 2030 (Nentwich & Ulbig, 2015). Recent figures suggest the worldwide prevalence of diabetes is 9.2% in women and 9.8% in men (King, 2012).

2.3 Classification of Diabetes mellitus

2.3.1 Type 1 Diabetes mellitus (T1DM)

T1DM accounts for only 5–10% of those with diabetes (Daneman, 2006). It was previously encompassed by the terms insulin-dependent diabetes (IDDM) or juvenile onset diabetes. It results from a cellular-mediated autoimmune destruction of the β-cells

of the pancreas. T1DM has been considered a disorder predominant in children and young adults but studies support a different model in which the disease can occur at any age (Li *et al*., 2004). Specifically, 5–30% of patients initially diagnosed with type 2 diabetes have type 1 diabetes. When treated with oral agents, such patients do not respond to oral hypoglycemic therapy, and are unable to maintain adequate glycemic control (Atkinson & Eisenbarth, 2001). The slow onset and protracted disease course has led to the disease designation of such individuals as latent autoimmune diabetes of adults (LADA). Given the large numbers of patients diagnosed with type 2 diabetes, patients with LADA are likely to comprise about half all patients with type 1 DM (Fourlanos *et al*., 2005)

2.3.2 Type 2 Diabetes mellitus (T2DM)

Type 2 Diabetes mellitus (T2DM) formerly referred to as non-insulin dependent diabetes mellitus (NIDDM) is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance, defective insulin secretion and pancreatic β- cell damage (Irudayaraj *et al*., 2017). It is associated with morbidity and mortality, resulting from its microvascular, macrovascular and neuropathic complications (Chaulya *et al*, 2011b). T2DM accounts for 90–95% of cases and results from a combination of the inability of muscle cells, liver and adipose tissue to respond to insulin properly (insulin resistance) and inadequate compensatory insulin secretion (Colberg *et al*., 2010). The metabolic features of T2DM include insulin resistance, defective insulin secretion and pancreatic β-cell damage (Irudayaraj *et al*., 2017). It is characterized by hyperglycemia, developing insulin resistance, β-cell dysfunction and impaired insulin secretion (Mirmiran *et al*., 2014). This form of diabetes frequently goes undiagnosed for many years because the

hyperglycemia develops gradually and at earlier stages is often not severe enough for the patient to notice any of the classic symptoms of diabetes. Nevertheless, such patients are at increased risk of developing macrovascular and microvascular complications (American Diabetes Association, 2015a).

2.3.3 Gestational Diabetes mellitus (GDM)

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance that is diagnosed for the first time in pregnancy. Pregnancy-related hormonal changes that reduce insulin sensitivity result in glucose intolerance in women with reduced β-cell reserve or with more marked underlying insulin resistance (Markovic *et al*., 2016). Progression in insulin resistance (IR) develops at around mid-pregnancy, and continues during the third trimester. The possible causes of IR in GDM are hormones and adipokines secreted from the placenta, including tumor necrosis factor (TNF)-α, human placental lactogen, and human placental growth hormone. Moreover, increased estrogen, progesterone, and cortisol during pregnancy contribute to disruption of the glucose insulin balance. This leads to increase in insulin secretion from the pancreas to compensate for the peripheral IR during pregnancy leading to development of GDM because the pancreas does not secrete enough insulin to keep up with the metabolic stress of the IR. Additionally, increased maternal adipose deposition, decreased exercise coupled with increased caloric intake contribute to glucose intolerance (Alfadhli, 2015).

Gestational diabetes complicates about 8-9% of all pregnancies, though the rates may double in populations at high-risk for type 2 diabetes. Clinical detection is important, since therapy will reduce perinatal morbidity and mortality (Triplitt *et al*., 2015). Hyperglycemia in pregnancy is responsible for the increased risk for macrosomia (birth

weight ≥ 4.5 kg), large for gestational age births, pre-eclampsia, preterm birth and cesarean delivery due to large babies. Risk factors for gestational diabetes include obesity, personal history of gestational diabetes, family history of diabetes, maternal age, polycystic ovary syndrome, sedentary life, and exposure to toxic factors (Kharroubi & Darwish, 2015).

2.4 Risk Factors of Diabetes mellitus

2.4.1 Obesity

This is number one risk factor for type 2 DM. Greater weight means a higher risk of insulin resistance because fat interferes with the body's ability to use insulin. Body mass index (BMI) is the most frequently used diagnostic tool for the measurement of obesity and is also typically associated with type 2 diabetes (Eckel *et al.*, 2015). Insulin is an important hormone that delivers glucose to cells and its resistance can be improved by weight reduction (King, 2012). Excessive energy intake over energy expenditure gives rise to unwarranted growth of adipose tissue leading to obesity. Because of its rising prevalence and its association with chronic health disorders such as insulin resistance, hyperlipidemia, hypertension, cardiovascular diseases, non-alcoholic fatty liver and osteo-arthritis, obesity has turned out to be a major public health concern in both developed and developing countries. At the cellular level, enlargement of the adipose tissue mass is characterized by an increase in the size (hypertrophy) or number (hyperplasia) of adipocytes. The triglyceride (TG) content in adipocytes reflects the balance between lipogenesis and lipolysis, which is largely related to cell volume. When adipocytes reach a critical size threshold, pre-adipocytes in close proximity to the

adipocytes will respond to positive energy balance by proliferating and then differentiating into adipocytes to store the excess energy (Chen *et al.*, 2008).

2.4.2 Sedentary Life

It refers to time spent in activities with low energy expenditure such as watching television, using a computer, or sitting during communication while working and has been identified as a potential risk factor for diabetes and the metabolic syndrome, independent of physical activity (Rockette-Wagner *et al*., 2015). A sedentary lifestyle is damaging to health and bears responsibility for the growing obesity problems (Wu *et al*., 2014). Inactivity and being overweight go hand in hand towards a diagnosis of types 2 diabetes (Tuomilehto *et al*., 2001). Muscle cells have more insulin receptors than fat cells hence a person can decrease insulin resistance by exercising. More activity lowers glucose by increasing insulin sensitivity in cells and thus aiding in the prevention and management of T2DM but many with this chronic disease do not become regularly active (King, 2012). It is now well established that effective physical activity (PA) is a key element and participation in regular PA improves blood glucose control and can prevent or delay type 2 diabetes, along with positively affecting lipids, blood pressure, cardiovascular events, mortality, and quality of life. Structured interventions combining PA and modest weight loss have been shown to lower type 2 diabetes risk by up to 58% in high-risk populations (Colberg *et al*., 2010). The benefits of PA on diabetes management are realized through acute and chronic improvements in insulin action, accomplished with both aerobic and resistance training (Colberg *et al.*, 2010).

2.4.3 Unhealthy Eating Habits

Undesirable dietary habits, as opposed to adherence to dietary guidelines especially by urban individuals increase the risk of developing type 2 diabetes (Alhazmi *et al.*, 2014). Eating high glycemic index foods tend to create extreme spikes in the blood sugar levels. Accumulation of excess lipids in the liver, heart, skeletal muscle, kidney and pancreas contributes to the pathogenesis of fatty liver, heart failure and insulin resistance (Kavitha, 2018). An increase in fat tissue is associated with a higher risk of insulin resistance which may eventually lead to diabetes and other complications (Arboix, 2015). DM is the most common manifestation of metabolic disorder which occurs in humans due to high consumption of carbohydrates and lipids. High fat diet increase body weight and visceral fat depots, induce alterations in carbohydrate and lipid metabolism leading to insulin resistance and an alteration in adipokines (Kavitha, 2018). Therefore, dietary change which is focused on caloric restriction, increased energy expenditure through increased physical activity together with regular aerobic activity and behavioral changes related to lifestyle leads to reduced incidence of DM. Adherence to Dietary Approaches to Stop Hypertension (DASH) diet high in fruits, vegetables, whole cereal products, low-fat dairy products, fish, chicken, and lean meats designed to be low in saturated fat and cholesterol, moderately high in protein and high in minerals and fiber may have the potential to improve insulin sensitivity and to prevent appearance of type 2 diabetes. In addition, the DASH diet with aerobic exercise and caloric restriction leads to lower glucose levels and improved insulin sensitivity as compared to DASH diet alone (Medina-Remón *et al*., 2018).

2.4.4 Family History and Genetics

To a slight extent people who have family members diagnosed with type 2 diabetes are at greater risks of developing it themselves. An individual with a family history of type 2 diabetes (FH+) with one or more first-degree relatives (FH1) has a 30–70% increased risk of developing the disease (Cederberg *et al*., 2015). Also, the cumulative prevalence of T2DM at 80 years is about 3.5 times higher (38% vs 11%) for people with a first-degree relative with T2DM compared to people without any affected relative (Valdez, 2009). However, large genome-wide association studies have been conducted and can only explain ~10% of the risk. This could be due to unidentified gene variants, epigenetic factors and/or the bacterial metagenome, although it is likely that the environment is a key contributor to diabetes risk (Cederberg *et al*., 2015).

T1DM results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas. Markers of the immune destruction of the β-cell include islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase - GAD (GAD65), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2β. Also, the disease has strong human leukocyte antigen (HLA) associations, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective. Other forms of T1DM have no known etiologies and some patients have permanent insulinopenia and are prone to ketoacidosis with no evidence of autoimmunity. Such individuals with this form of diabetes suffer from episodic ketoacidosis, exhibiting varying degrees of insulin deficiency between episodes (American Diabetes Association, 2010).

2.4.5 High Blood Pressure and High Cholesterol Levels

High blood pressure and high cholesterol levels may damage the heart vessels. Dyslipidemia, high blood pressure, impaired glucose tolerance and abdominal fat accumulation (collectively termed as metabolic syndrome), is a collection of conditions associated with metabolic disorder and increased risk of developing cardiovascular disease (Pitsavos *et al*., 2006). Having metabolic syndrome increases risk factor for heart disease, stroke and diabetes. The risk of myocardial infarction in diabetic patients is similar to that of non-diabetic patients who have already suffered a myocardial infarction. Because saturated fats are the major dietary determinants of serum LDL cholesterol levels, people with diabetes should strive to keep saturated fat consumption to < 7% of total daily calories and to minimize consumption of trans-fatty acids. Cholesterol consumption should be < 200 mg/day (Fowler, 2007).

2.4.6 History of Gestational Diabetes

Gestational diabetes begins when hormones of the placenta makes the mother insulin resistant. Mothers with gestational diabetes most likely develop type 2 diabetes later (Bao *et al*., 2015). GDM is associated with maternal obesity and confers a 4^{th} to 7^{th} fold greater risk of incident type 2 diabetes (DM) later in life (Gunderson *et al*., 2014). In addition, babies born from diabetic pregnancies have an increased risk of developing obesity in childhood, metabolic disturbances in adolescence and type 2 DM in adulthood, linked to the metabolic imbalance experienced *in utero* (Kanguru *et al*., 2014).

2.4.7 Other Risk Factors of Diabetes mellitus

2.4.7.1 Genetic Defects of the Pancreatic β – cells

Several forms of diabetes are associated with monogenetic defects in β-cell function. These forms of diabetes are frequently characterized by onset of hyperglycemia at an early age generally before age 25 years (American Diabetes Association, 2010). Maturity Onset Diabetes of the Young (MODY) is characterized by impaired insulin secretion with minimal or no insulin resistance. MODY can be subtyped into neonatal and MODY-like. Neonatal diabetes usually has an onset in the first 6 months of life and can be transient or permanent. MODY may affect genes important for beta-cell glucose sensing, development, function, and regulation. Genetic inability to convert pro-insulin to insulin results in mild hyperglycemia. Similarly, the production of mutant insulin molecules has been identified in a few families and results in mild glucose intolerance. MODY is most often associated with renal cysts but can rarely cause diabetes. The natural history of MODY is highly dependent on the underlying genetic defect and most typically exhibit mild hyperglycemia at an early age. The disease is inherited in an autosomal dominant pattern (Triplitt *et al*., 2015). Point mutations in mitochondrial DNA have been found to be associated with diabetes and deafness. The most common mutation occurs at position 3,243 in the tRNA leucine gene, leading to an A-to-G transition. Genetic abnormalities that result in the inability to convert proinsulin to insulin have been identified in a few families, and such traits are inherited in an autosomal dominant pattern. The resultant glucose intolerance is mild. Similarly, the production of mutant insulin molecules with resultant impaired receptor binding has also been identified in a few families and is

associated with an autosomal inheritance and only mildly impaired or even normal glucose metabolism (American Diabetes Association, 2010).

2.4.7.2 Diseases of the Exocrine Pancreas

Any process that diffusely injures the pancreas can cause diabetes. Acquired processes include pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma (American Diabetes Association, 2010). The most common causes are pancreatitis, trauma, and carcinoma. Chronic pancreatitis can cause general inflammatory/fibrotic changes in the pancreas which can cause diabetes. Cystic fibrosis causes a well-recognized pancreatic exocrine function insufficiency, but the same thick, viscous secretions cause inflammation, obstruction, and destruction of small ducts in the pancreas, which can lead to insulin deficiency (Triplitt *et al*., 2015).

2.4.7.3 Endocrinopathies

Several hormones for instance growth hormone, cortisol, glucagon and epinephrine antagonize insulin action. Excess amounts of these hormones can cause diabetes. This generally occurs in individuals with preexisting defects in insulin secretion, and hyperglycemia typically resolves when the hormone excess is resolved (American Diabetes Association, 2010). Since growth hormone, cortisol, glucagon, and epinephrine increase hepatic glucose production and induce insulin resistance in peripheral (muscle) tissues; excess production of these hormones can cause or exacerbate underlying diabetes (Triplitt *et al*., 2015).

2.4.7.4 Infections

According to American Diabetes Association (2010), a variety of infections have been etiologically related to the development of diabetes mellitus. Approximately 20% of infants who are infected with the rubella virus at birth develop autoimmune type 2 diabetes later in life. These individuals have the typical type 1 susceptibility genotype, DR3/DR4 (Triplitt *et al*., 2015). In addition, coxsackievirus B, cytomegalovirus, adenovirus, and mumps have been implicated in inducing certain cases of the disease.

2.4.7.5 Drugs

A large number of commonly used drugs have been shown to induce insulin resistance and/or impair pancreatic beta cell function and can lead to the development of diabetes mellitus in susceptible individuals. For instance, HMG-CoA reductase inhibitors (statins) have shown to cause a small increase in the risk of diabetes, though the exact mechanisms of how it may increase the risk of diabetes are not completely understood (American Diabetes Association, 2010; Triplitt *et al*., 2015).

2.5 Pathogenesis of Diabetes mellitus

T1DM results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas. The disease has strong HLA associations, with linkage to the DQA and DQB genes, and it is influenced by the DRB genes. These HLA-DR/DQ alleles can be either predisposing or protective (Kharroubi & Darwish, 2015). In this form of diabetes, the rate of β -cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Insulin therefore has to be replaced by

regular subcutaneous injections, and blood glucose levels must be frequently monitored to manage the risk of hypoglycemia (King, 2012).

In T2DM, insulin resistance is a major pathophysiologic factor influencing glucose homeostasis. Impaired insulin-mediated suppression of hepatic glucose production plays a major role in the pathogenesis of T2DM (Perry *et al*., 2015). T2DM accounts for over 85% of diabetes worldwide and is associated with morbidity and mortality, resulting from its microvascular, macrovascular and neuropathic complications. The basic effect of insulin lack or insulin resistance on glucose metabolism is inefficient glucose uptake and utilization by most cells of the body except those of the brain. As a result, blood glucose concentration increases, utilization of glucose by cells falls leading to increase in utilization fat and protein (Abdirahman *et al*., 2015).

In T2DM, IR increases the mobilization of free fatty acids from adipose tissue. Elevated hepatic production of very low-density lipoproteins is caused by increased lipogenesis, an exacerbation of substrate availability, and decreased apolipoprotein B-100 (ApoB) degradation. These changes leads to lipid profile marked by low high-density lipoprotein cholesterol (HDL-C), high triglycerides (TGs), increased ApoB synthesis and small dense LDL particles (Jung & Choi, 2014). LDL subtype is more inclined to oxidation, playing an important role in atherogenesis. Stronger than LDL cholesterol, a low HDL-C or lonely elevated TGs, atherogenic dyslipidaemia (Low HDL-C and ApoA, elevation of both fasting and post-prandial TGs, elevation of small dense LDL particles, elevation of ApoB) is in T2DM patients a self-determining predictor of cardiovascular risk (Martín-Timón *et al*., 2014). The protective function of HDL may be lost in type 2 diabetics owing to alterations of the protein, resulting in a pro-oxidant, inflammatory phenotype.

DM related to oxidative stress leads to an increased generation of free radicals such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-).

Effect of oxidative stress in the progression of diabetes mellitus is not only by free radical generation but also due to non-enzymatic protein glycation, impaired antioxidant enzyme system and formation of peroxides which may lead to liver disorder (Kazeem *et al*., 2013b). Further, increase in blood glucose levels in diabetic patients cause alteration in utilization of glucose in the vital organs such as adipose tissues and liver. Increase in blood glucose level continuously generate reactive oxygen species (ROS) and superoxide anions, which further aggravate diabetic complications by damaging the proteins, deoxyribonucleic acid and carbohydrates, which lead to increase in the oxidative stress (Kumar *et al*., 2014a).

Several *in vitro* and *in vivo* studies suggest that oxidative stress as shown in Figure 2.1 may be related to liver damage. Thus, it contributes to lipid peroxidation, one of the critical factors involved in the genesis and progression of nonalcoholic steatohepatitis and liver cancer. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotic and aging (Lunardi *et al.,* 2014). Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process. Patients with diabetes have a high prevalence of liver disease and patients with liver disease have a high prevalence of diabetes. Virtually the entire spectrum of liver disease is seen in patients with type 2 diabetes. Abnormal liver enzymes, nonalcoholic fatty liver disease, cirrhosis, hepatocellular carcinoma, and acute liver failure are among the conditions seen in type 2 diabetes. Chronic mild elevations of transaminases are

frequently found in type 2 diabetic patients (Abolfathi *et al*., 2012). The increase in the activities of serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine transaminase (ALT) indicate liver dysfunction in diabetes (Ramadan *et al*., 2013).

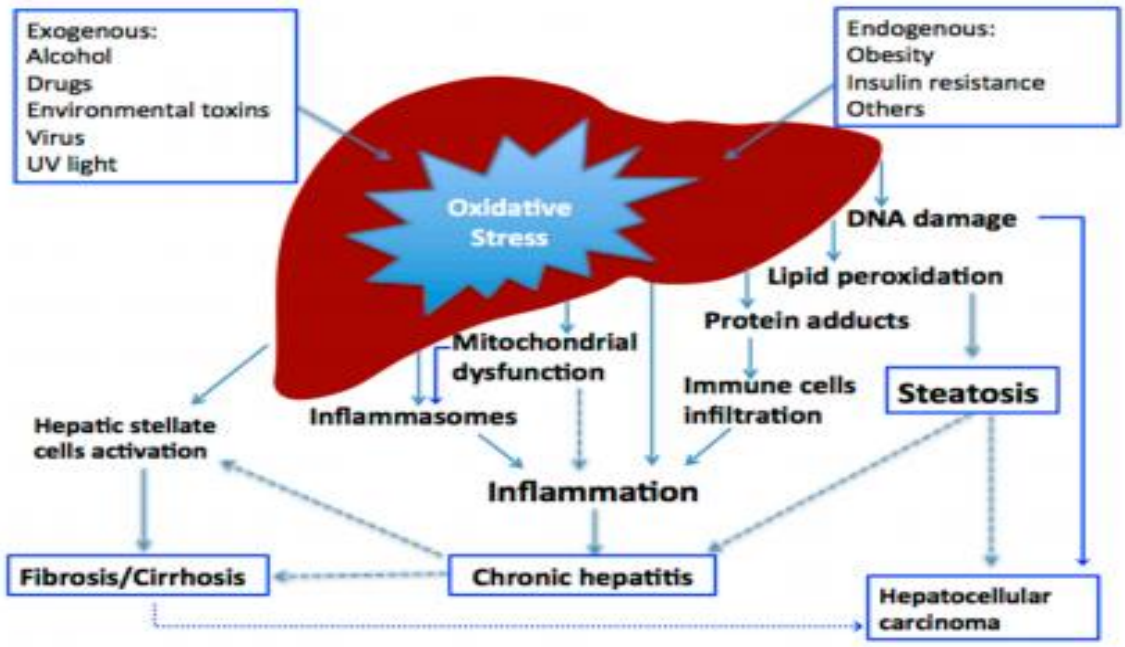


Figure 2.1: The general scheme of oxidative stress mechanisms induced by various factors on liver disease. Source; (Li *et al*., 2015).

2.6 Signs and Symptoms of Diabetes mellitus

According to American Diabetes Association (2010), most common symptoms of diabetes mellitus (DM) are polyuria, polydipsia, and polyphagia, along with lassitude, nausea, and blurred vision, all of which result from the hyperglycemia itself. Polyuria is caused by osmotic diuresis secondary to hyperglycemia. Severe nocturnal enuresis secondary to polyuria can be an indication of onset of diabetes in young children. Thirst is a response to the hyperosmolar state and dehydration. Fatigue and weakness may be caused by muscle wasting from the catabolic state of insulin deficiency, hypovolemia,

and hypokalemia (Chaulya *et al*., 2011a). Muscle cramps are caused by electrolyte imbalance. Blurred vision results from the effect of the hyperosmolar state on the lens and vitreous humor. Glucose and its metabolites cause osmotic swelling of the lens, altering its normal focal length. Symptoms at the time of the first clinical presentation can usually be traced back several days to several weeks. However, beta-cell destruction may have started months, or even years, before the onset of clinical symptoms (Nentwich & Ulbig, 2015). The onset of symptomatic disease may be sudden.

It is not unusual for patients with T1DM to present with diabetic ketoacidosis (DKA), which may occur *de novo* or secondary to the stress of illness or surgery. An explosive onset of symptoms in a young lean patient with ketoacidosis always has been considered diagnostic of T1DM. Over time, patients with new-onset type 1 DM will lose weight, despite normal or increased appetite, because of depletion of water and a catabolic state with reduced glycogen, proteins, and triglycerides. Weight loss may not occur if treatment is initiated promptly after the onset of the disease (Papatheodorou *et al*., 2015). Gastrointestinal (GI) symptoms of type 1 DM are as follows: Nausea, abdominal discomfort or pain, and change in bowel movements may accompany acute DKA. Acute fatty liver may lead to distention of the hepatic capsule, causing right upper quadrant pain. Persistent abdominal pain may indicate another serious abdominal cause of DKA. Chronic GI symptoms in the later stage of DM are caused by visceral autonomic neuropathy. Neuropathy affects up to 50% of patients with type 1 DM, but symptomatic neuropathy is typically a late development, developing after many years of chronic prolonged hyperglycemia. Peripheral neuropathy presents as numbness and tingling in

both hands and feet, in a glove-and-stocking pattern; it is bilateral, symmetric, and ascending (Callaghan *et al.*, 2012).

2.7 Complications Associated with Diabetes mellitus

The main injuries as a result of hyperglycemia is injury to vasculature, classified into small vascular injury (microvascular disease) or injury to large blood vessels of the body (macrovascular disease) (Fowler, 2011). There is growing evidence that the underlying mechanisms in the pathogenesis of diabetic complications include oxidative stress created by the overproduction of reactive oxygen species (ROS) and defects in the insulin signal transduction pathway in which ceramide, a bioactive sphingolipid, may have an important inhibitory effect (Papatheodorou *et al*., 2015).

2.7.1 Microvascular Complications

Diabetes and its complications create a severe health care crisis worldwide. Persistent hyperglycemia leads to the development of microvascular and macrovascular complications (Kurup & Mini, 2017). The risk of developing diabetic microvascular complications of diabetes depends on both the duration and the severity of hyperglycemia (Behnam-Rassouli *et al*., 2010). There are several modes leading to microvascular complications. Aldose reductase may participate in the development of diabetes complications. Aldose reductase is the initial enzyme in the intracellular polyol pathway. This pathway involves the conversion of glucose into glucose alcohol (sorbitol). High glucose levels increase the flux of sugar molecules through the polyol pathway, which causes sorbitol accumulation in cells. Osmotic stress from sorbitol accumulation has been postulated as an underlying mechanism in the development of diabetic microvascular complications (Zhou *et al*., 2015).

Cells are also thought to be injured by glycoproteins. High glucose concentrations can promote the none-enzymatic formation of advanced glycosylated end products (AGEs) (Papatheodorou *et al*., 2015). Oxidative stress may also play an important role in cellular injury from hyperglycemia. High glucose levels can stimulate free radical production and reactive oxygen species formation (Fiorentino *et al*, 2013). Antioxidant enzymes (SOD, CAT, and GPx) play important role in the maintenance of physiological concentrations of oxygen and hydrogen peroxide by enhancing the dismutation of oxygen radicals and mopping up organic peroxides. SOD mop up superoxide radicals by converting them to H_2O_2 and oxygen while both CAT and GPx are involved in the elimination of H_2O_2. Decrease in the activities of SOD, CAT, and GPx in the liver of diabetics may be due to the rise in generation of ROS such as superoxide (O^{2-}) and hydroxyl (OH^-) radicals (Kazeem *et al*., 2013b).

2.7.1.1 Diabetic Neuropathy

One of the most common complications of diabetes is diabetic peripheral neuropathy (DPN). Diabetic neuropathy represents a clinically diverse group of disorders having differing anatomic distribution, clinical course, and underlying pathophysiology, but ultimately thought to reflect metabolic and microvascular factors that result in axonal degeneration of large- and small-nerve fibers (Martin *et al*., 2014). Not only can chronic hyperglycemia have various negative effects on the central nervous system, but it can also cause gastroparesis. The presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes become confirmative after the exclusion of other causes. The risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycemia, and some individuals may possess genetic attributes that

affect their predisposition to developing such complications. The precise nature of injury to the peripheral nerves from hyperglycemia is not known but is likely related to mechanisms such as polyol accumulation, injury from AGEs, and oxidative stress (Callaghan *et al*., 2012).

2.7.1.2 Diabetic Nephropathy

Diabetic kidney disease (DKD) is a complication that take place in 20% to 40% of all diabetics. It is the major cause of end-stage renal disease, although its pathogenesis is not fully understood (Gheith *et al*., 2016). Emerging evidence suggests that epigenetic modifications and some micro-RNAs may play a role in the pathogenesis of diabetic retinopathy by altering the expression of several genes and controlling certain intracellular pathways. Proteinuria or micro-albuminuria is a characteristic in diabetic nephropathy. Without intervention, diabetic patients with micro-albuminuria typically progress to proteinuria and overt diabetic nephropathy. This progression occurs in both type 1 and type 2 diabetes (Gheith *et al*., 2016; Wada & Makino, 2013).

2.7.1.3 Diabetic Retinopathy

Current global prevalence of diabetic retinopathy is estimated to be 126 million of the 382 million people with diabetes. People who develop diabetic retinopathy are also at high risk of other vascular complications, including diabetic nephropathy and cardiovascular disease (Jenkins *et al*., 2015). The importance of adequate glycemic control in the prevention of diabetic retinopathy is well established. However, other factors may play a role in the pathogenesis of these complications of diabetes. Growth factors, including vascular endothelial growth factor (VEGF), growth hormone, and transforming growth factor β, have also been postulated to play important roles in the

development of diabetic retinopathy. VEGF production is increased in diabetic retinopathy, possibly in response to hypoxia (Yau *et al*., 2012). Main reasons for loss of vision in patients with diabetes mellitus are diabetic macular edema and proliferative diabetic retinopathy. Progression of these potentially blinding complications can be greatly reduced by adequate control of blood glucose and blood pressure levels. Additionally, regular ophthalmic examinations are mandatory for detecting ocular complications and initiating treatments such as laser photocoagulation in case of clinical significant diabetic macular edema or early proliferative diabetic retinopathy (Nentwich & Ulbig, 2015).

2.7.2 Macrovascular Complications of Diabetes mellitus

Atherosclerotic cardiovascular disease remains the principal cause of death and disability among patients with diabetes mellitus. Approximately two-thirds of deaths in people with diabetes are due to cardiovascular disease with 40% from ischemic heart disease, 15% from other forms of heart disease, principally congestive heart failure, and about 10% from stroke (Wang *et al*., 2016). The presence of diabetes is accompanied by a greater prevalence of established atherosclerotic risk factors including hypertension, hypertriglyceridemia, decreased levels of high-density lipoprotein (HDL) cholesterol, small dense low-density lipoprotein (LDL) particles and abdominal obesity. In combination with dysglycemia, the clustering of these risk factors is likely to contribute to the elevated vascular risk observed in individuals with diabetes (Fowler, 2008). This is supported by findings from clinical trials that lowering LDL cholesterol and blood pressure are associated with reductions in cardiovascular events. The up-regulation of inflammatory pathways associated with propagation of atherosclerosis in patients with

diabetes is also likely to promote an increase in cardiovascular risk (Kataoka *et al*., 2013).

According to Papatheodorou *et al*. (2015), atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries. Angiotensin II may promote the oxidation of such particles. Monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation (Rafieian-Kopaei *et al*., 2014). The net result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of the lesion leads to acute vascular infarction (Bornfeldt, 2014; Papatheodorou *et al*., 2015). Diabetes increases the risk that an individual will develop cardiovascular disease.

Although the precise mechanisms through which diabetes increases the likelihood of atherosclerotic plaque formation are not completely defined, the association between the two is profound. CVD is the primary cause of death in people with either type 1 or type 2 diabetes. In fact, CVD accounts for the greatest component of health care expenditures in people with diabetes (Scirica *et al*., 2013). Diabetes is also a strong independent predictor of risk of stroke and cerebrovascular disease, as in coronary artery disease (Arboix, 2015). Patients with type 2 diabetes have a much higher risk of stroke, with an increased risk of 150–400%. Risk of stroke-related dementia and recurrence, as well as stroke-related mortality, is elevated in patients with diabetes. Among diabetic patients who

survive a cerebrovascular disease event (stroke), approximately 50% will have long-term disability. Many preventive and treatment strategies in diabetes are identical to those for non-diabetic patients. New evidence is rapidly informing improved care for stroke patients with diabetes (Phipps *et al*., 2012).

2.8 Diagnostic Methods and Criteria for Diabetes mellitus

The diagnostic methods used for DM are fasting plasma glucose (FPG) which is a measure of glucose concentrations after the person has refrained from eating or drinking anything other than water for 12 hours, random blood sugar, oral glucose tolerance test (OGTT) which measures the changes in blood glucose after a fixed amount of glucose (75g) has been administered and blood glucose estimated half hourly over a period of two hours. Spectrophotometric methods and use of a glucometer are some of the techniques used to determine blood glucose levels. Additionally, HbA1c which is determined using an HbA1c analyzer does not directly measure blood glucose but represents the average amount of glucose in the blood in the past 2–3 months (American Diabetes Association, 2015a).

Currently, ADA recommends the use of any of the following criteria for diagnosing diabetes;

2.8.1 Pre-diabetes

Individuals with pre-diabetes do not meet the criteria of having diabetes but are at high risk to develop type 2 diabetes in the future. According to the ADA Expert Committee, individuals are defined to have prediabetes if they have either impaired fasting plasma glucose (IFG) levels between 100-125 mg/dL (5.6-6.9 mmol/L) or impaired glucose tolerance test (IGT) with 2-h plasma glucose levels in the oral glucose tolerance test (OGTT) of 140-199 mg/dL (7.8-11.0 mmol/L). The World Health Organization (WHO) still adopts the range for IFG from 110-125 mg/dL (6.1-6.9 mmol/L). ADA also recommends the use of an HbA1c (5.7%-6.4%) to diagnose prediabetes (Kharroubi & Darwish, 2015).

2.8.2 Diabetes mellitus

Diabetes mellitus is diagnosed using either the estimation of plasma glucose (FPG or OGTT) or HbA1c. Estimation of the cut off values for glucose and HbA1c is based on the association of FPG or HbA1c with retinopathy. Fasting plasma glucose of ≥ 126 mg/dL (7.0 mmol/L), plasma glucose after 2-h OGTT ≥ 200 mg/dL (11.1 mmol/L), HbA1c ≥ 6.5% (or higher) or a random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) along with symptoms of hyperglycemia is diagnostic of diabetes mellitus. In addition to monitoring the treatment of diabetes, HbA1c has been recommended to diagnose diabetes by the International Expert Committee and endorsed by ADA, the Endocrine Society, the WHO and many scientists and related organizations all over the world (Kharroubi & Darwish, 2015; Colberg *et al*., 2010). This blood test indicates the average blood sugar level for the past two to three months of an individual. It works by measuring the percentage of blood sugar attached to hemoglobin, the oxygen-carrying protein in red

blood cells. The higher the blood sugar levels, the more hemoglobin is attached with sugar. An HbA1c level of 6.5 percent or higher on two separate tests indicates you have diabetes.

2.8.3 Gestational Diabetes

Undiagnosed type 2 diabetes is tested at the first prenatal visit in those with risk factors, using standard diagnostic criteria. GDM is tested at 24–28 weeks of gestation in pregnant women not previously known to have diabetes. Women with a history of GDM should have lifelong screening for the development of diabetes or prediabetes at least every 3 years. Women with a history of GDM found to have prediabetes should receive lifestyle interventions or metformin to prevent diabetes (American Diabetes Association, 2015b).

2.9 Prevention and Management of Diabetes mellitus

Technological improvements in insulin pumps and continuous glucose monitors help patients with T1DM manage the challenge of lifelong insulin administration (Atkinson *et al*., 2014). All patients with T1DM need age-appropriate care, with an understanding of their specific needs and limitations. Regardless of age, an individualized care plan with ongoing education and support, ongoing assessment for acute and chronic complications, and access to medical providers is key in addressing type 1 diabetes (Chiang *et al*., 2014). Several studies have shown that healthy eating and regular physical activity, used with medication if prescribed, can help control health complications from type 2 diabetes or can prevent or delay the onset of type 2 diabetes (Control, 2012). Medical nutrition therapy is the main part of type 2 diabetes management; estimation of energy and nutrients requirements, carbohydrate counting as well as glycemic index and glycemic load, recommendation for dietary fats and cholesterol and protein intakes, explanation of

the foods exchange list for patients and common important recommendations for a healthy diet (Mirmiran *et al.*, 2014).

Several mechanisms reduce the risk of obesity, including reduced energy/food intake, decreased intestine adsorption, suppressed lipogenesis, enhanced lipolysis and fat oxidation, increased energy expenditure and inhibited preadipocyte proliferation and differentiation (Chen *et al*., 2008). Based on the multiple biological properties of functional foods and their bioactive compounds, a functional foods-based diet has been hypothesized as a novel and comprehensive dietary approach for management of type 2 diabetes and prevention of long-term complications (Mirmiran *et al*., 2014).

Type 2 diabetes is a chronic illness that requires continuous medical care and ongoing patient self-management education and support to prevent acute complications and to reduce the risk of long-term complications. The standards of care are intended to provide clinicians, patients, researchers, and other interested individuals with the components of diabetes care, general treatment goals, and tools to evaluate the quality of care (American Diabetes Association, 2010). Main aim of treatment is to lower the blood glucose and strictly maintaining an acceptable range to reduce symptoms and the risk of complications. The normal range for blood glucose levels is 4-7 mmol/L. Treatment majorly includes diet and exercise, self-monitoring, oral medications, injected medications, insulin pumps, and continuous glucose monitoring (Fowler, 2007).

Balancing postprandial blood glucose levels is extremely important, since postprandial hyperglycemia is considered more dangerous than fasting blood glucose. α-Glucosidase can release glucose by hydrolyzing linear and branched iso-maltose oligosaccharides,

resulting in postprandial hyperglycemia. Therefore, identifying and characterizing the inhibitors of α-glucosidase that can be used therapeutically is important (Zhang *et al*., 2014).

2.10 Treatment of Diabetes mellitus

Diabetes has rapidly become a physical and mental burden that lowers the quality of life and results in high rates of mortality and disability (Zhang *et al*., 2014). A cornerstone of diabetes treatment is attention to lifestyle. Unhealthy lifestyles, such as lack of physical activity and excessive eating, initiate and propagate the majority of type 2 diabetes (Fowler, 2007). An endocrine disorder is the major cause of diabetes mellitus (DM) and oral hypoglycemic drugs and/or insulin is used generally for the treatment (Rashid *et al*., 2014). Although no cure is yet available for type 2 diabetes, oral hypoglycemic agents have been developed and are widely used. Current medications, however, are not adequately effective in maintaining long-term glycemic control in most patients, even when used in combination, leaving diabetics susceptible to developing life threatening and debilitating complications. Therefore, there is an urgent need for more potent and safe therapeutic agents with noble mechanisms of action (Yuan *et al.*, 2012).

2.10.1 Pharmacological Therapy for Type 1 Diabetes

Most people with type 1 diabetes should be treated with multiple-dose insulin injections (three to four injections per day of basal and prandial insulin) or continuous subcutaneous insulin infusion therapy. Insulin suppresses the inflammatory process not only by controlling hyperglycemia but also by directly modifying key inflammatory molecules involved in pathophysiology of diabetes. Insulin induces expression of endothelial nitric oxide (NO) synthase through the activation of phosphoinositide-3kinase (PI3K) and Akt

kinase (protein kinase B), resulting in the generation of NO that can increase blood flow and inhibit platelet aggregation (Kothari *et al*., 2016). People with type 1 diabetes should be educated on how to match prandial insulin dose to carbohydrate intake, pre-meal blood glucose, and anticipated physical activity. People with type 1 diabetes should use insulin analogs to reduce hypoglycemia risk. For patients with frequent nocturnal hypoglycemia and/or hypo-glycemic unawareness, a sensor-augmented low glucose threshold suspend pump may be considered (American Diabetes Association, 2015b).

2.10.2 Pharmacological Therapy for Type 2 Diabetes

Treatment options for type 2 diabetes are becoming increasingly complex with people often prescribed multiple medications, and may include both oral and injectable therapies. For T2DM patients not achieving an agreed HbA1c target despite intensive therapy with oral hypoglycemic drugs, insulin is considered an essential component of the treatment strategy (Inzucchi *et al*., 2015).

2.10.2.1 Biguanides

Biguanides such as metformin (N',N'-dimethylbiguanide) is prescribed as a first-line medication for newly diagnosed T2DM patients. Antihyperglycemic effects of metformin includes down regulation of hepatic gluconeogenesis, improvement in insulin sensitivity and significant reduction in insulin resistance. Metformin interferes with mitochondrial respiratory chain complex 1 by increasing AMP/ATP ratio, which promotes the activation of AMP kinase. Metformin-induced AMP kinase activation leads to transcriptional inhibition of hepatic gluconeogenesis (Singh *et al.*, 2016).

2.10.2.2 Sulfonylureas

Sulfonylureas are the most common classes of oral antidiabetic agents being prescribed either alone or in combination. The commonly used second generation drugs are; glimepiride, glibenclamide (glyburide), gliclazide and glipizide. Sulfonylureas stimulate insulin secretion by binding to sulfonylurea receptor 1 (SUR1) protein. This interaction depolarizes the cell membrane of pancreatic beta cells by closing ATP-sensitive potassium (KATP) channels. Subsequent effect of depolarization leads to Ca^{2+} influx which trigger an enhanced insulin secretion from beta cells in a glucose-independent manner (Song *et al*., 2017).

2.10.2.3 Thiazolidinediones (TZDs)

These are insulin sensitizers which promote uptake of glucose by tissue and skeletal muscles and down regulate glucose output from liver. Examples are rosiglitazone and pioglitazone. TZDs primarily bind with the peroxisome proliferator-activated receptor γ (PPARγ) in adipose tissue and affect their metabolism. On binding with PPARγ, TZDs stimulate adipocytes differentiation and decrease plasma glucose level in T2DM patients (Capuano *et al*., 2013).

2.10.2.4 Meglitinides

They act by inhibiting KATP channel promoting insulin secretion. Both sulphonylureas and meglitinides inhibit the activity of KATP channel by binding at two different sites of the SUR1 subunit. Meglitinides have shorter duration of action and more rapid onset as compared with sulphonylureas. Repaglinide (a benzoic acid derivative) and nateglinide (a derivative of d-phenylalanine) stimulate early secretion of insulin. Due to their short

action, a potential adverse effect of meglitinide is to induce hypoglycemia (Singh *et al*., 2016).

2.10.2.5 Dipeptidyl peptidase 4 inhibitors (DPP-4)

Glucagon-like peptide-1 (GLP-1), an incretin hormone stimulates insulin secretion, inhibits glucagon secretion, delays gastric emptying, induces satiety, and may increase islet cell mass but is rapidly inactivated by the enzyme dipeptidyl peptidase-4 (DPP-4). The use of compounds that inhibit the activity of DPP-4 improves glycemic control (Ahrén *et al*., 2004). The incretin hormones are also responsible for reduction in circulating plasma glucose levels by interrupting glucagon secretion and subsequently improve beta cell sensitization by glucose. DPP-4 inhibitors inhibit function of DPP-4 enzyme, thus prolonging action of the incretin hormones and reduces glucagon secretion. Drugs in this category include sitagliptin, vildagliptin and saxagliptin (Capuano *et al*., 2013).

2.10.2.6 Sodium Glucose Cotransporter 2 (SGLT2) Inhibitors

SGLT2 inhibitors are the newest oral approach for the management of T2DM. SGLT2 is a low-affinity, high-capacity cotransporter highly expressed in the proximal renal tubule responsible for the bulk of reabsorption of filtered glucose. The blocking SGLT2 activity by inhibitors induces therapeutic glycosuria (Ferrannini *et al*., 2014). These inhibitors include dapagliflozin, canagliflozin, empagliflozin, and ipragliflozin drugs. SGLT2 inhibitors increase glucosuria, reduce hyperglycemia (without inducing hypoglycemia), promote weight loss, and exert a modest diuretic effect with blood pressure reduction (Kothari *et al*., 2016).

2.11 Alternative Medicines for Management of Diabetes mellitus

Overwhelming evidence from epidemiological, *in vivo*, *in vitro*, and clinical trial data indicates that the plant-based extracts can reduce the risk of chronic diseases such as diabetes due to presence of biologically active plant compounds or phytochemicals (Upadhyay, 2015). The World Health Organization (WHO) has estimated that more than 75% of the world's total population depends on herbal drugs for their primary healthcare needs (Sagnia *et al*., 2014). Moreover, WHO estimates that up to 80% of the world's population, mostly in developing countries, relies on traditional medicine practices for its health care needs. A number of modern drugs have also been synthesized from these natural medicinal plants. These drugs are based on the indigenous medicinal information of plants (Abdirahman *et al*., 2015).

Traditionally, medicinal plants are used to control diabetes and have been investigated with the aim of discovering potential hypoglycemic agents. WHO has also recommended that traditional plants can be an excellent candidate for the treatment of DM because of its hypoglycemic activity, are nontoxic with little or no side effects (Irudayaraj *et al*., 2017). Thus, the investigation and usage of natural products from plant origin in treating various diseases have gained much attention (Baskaran *et al*., 2015). There has been an increase in demand by patients in the use of natural products and other dietary modulators with anti-diabetic activity because insulin cannot currently be taken orally, and repeated injections of insulin have many undesirable adverse effects. Additionally, certain oral hypoglycemic agents are not effective for reduction of the blood glucose level in chronic diabetic patients.

An estimated 25% of prescription drugs and 11% of drugs considered essential by the WHO are derived from plants and a large number of synthetic drugs are obtained from precursor compounds originating from plants. Therefore the documentation of the traditional therapeutic know-how could lead to the discovery of new drugs as well as contribute to the conservation, sustainable management and use of plant resources (Simbo, 2010). Several phyto-constituents possessing anti-diabetic activity have been isolated and studied from many medicinal plants, but still scientists continue their research on medicinal plants to bring good anti-diabetic lead or drugs to the healthcare community (Sabitha *et al*., 2011). The potential of medicinal plant extracts in diabetes management has been acknowledged because natural products are considered effective alternative for the treatment and management of diabetes (Krishnasamy *et al*., 2016).

2.12 *Polyscias fulva*

Approximately 800 plants studied worldwide have shown antidiabetic potential and they provide a great source of drugs and biological compounds (Feyzmand *et al*., 2018). The use of herbal products to treat DM has greatly increased during the past decades. Plants used in folk medicine to treat DM represent a viable alternative for the control of this disease (Chaulya *et al*., 2011a). Of these plants, Traditional African Medicine practitioners claim that *P. fulva* bark extract is used to treat DM and hence this study sort to evaluate the antidiabetic claims. Moreover, *P. fulva* infusions or decoctions are used as traditional medicine in DR Congo; barks are taken for the treatment of fever and malaria, as an enema to treat colic, and as a purgative. Bark maceration is applied as drops to the nostrils to treat mental illness. Pulverized bark is snuffed as anodyne, and to treat cough, hemoptysis and tuberculosis. In Cameroon the bark is used in mixtures with

other plants to treat epilepsy. Leaf decoctions are taken to treat intestinal complaints including those caused by parasites, whereas pounded leaves are applied externally to treat fractures and internally against peptic ulcers (Lemmens *et al*., 2009).

The plant *P. fulva* commonly known as parasol tree (Figure 2.2) grows to 25-30 m, with a regular branching pattern and a clear, straight trunk with branches developing high up, forming a narrow crown and resembling the spokes of a parasol or an umbrella; no thorns or buttresses; bark is smooth and grey in color; bole is branched, and young stems are marked with prominent leaf scars (Orwa, 2009). Common names of the plant include; Hiern (Harms) and parasol tree in English. Local names include; Setala in Luganda in Uganda and Soyet in Nandi in Kenya.

Scientifically, *P. fulva* plant is classified as follows;

Kingdom - Plantae,

Phylum – Tracheophyta

Class -Magnoliopsida

Order - Apiales

Family -Araliacea

Genus - Polyscias

Species - *Polyscias fulva*

Figure 2.2: *Polyscias fulva.* Source: (Hyde, 2016)

P. fulva is distributed in the highland forests into the bamboo zone. It grows in afro-montane forests and undifferentiated afro-montane forests (broad-leaved forest, Podocarpus forest), often in clearings and regrowth. It also occurs in rainforests, lowland

forests, riverine forests and mountain grasslands. It requires light and may be abundant at forest edges. In Kenya the species grows around Elburgon, north of Mt Elgon, west of Mt Kenya and north of the Nandi forests. It is usually found in wetter highland areas like Kakamega Forest in Kenya, often occurring in tea growing districts. A few remnant trees can be found in the Nairobi area (Orwa, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

Wire cages of rack type that were used to house the albino rats in the study were constructed in Estates Department, University of Eldoret. Cryovials for storage of processed blood samples were bought from Chemlabs Limited, Nairobi. Needles, syringes (2mls), microfine needles (0.5mls), dissecting kit, glucometer (Gluco Rx type), glucose strips (Gluco Rx strips) and automatic pipettes and pipette tips were bought from Chemoquip Limited, Nairobi. Rat pellets (chow) were commercially bought from Maraba Agro Vet in Eldoret. Streptozotozin; STZ was obtained from KEMRI (Sigma Aldrich) while Thiobarbituric acid; TBA (Sigma Aldrich) and Tetramethoxypropane; TMP (PubChem) was bought from Kobian Scientific, Nairobi. All other commercial reagents used were of analytical grade.

3.2 Ethical Considerations

Ethical clearance for this study was granted by the University of Eastern Africa, Baraton's Research Ethics Committee (Reference; REC: UEAB/14/3/17) as shown in Appendix 1V. Further, the experiment was done as per Organization of Economic Cooperation and Development (OECD) guidelines for the care and use of laboratory animals (No, 2008).

3.3 Sample Size Determination

The formula for calculation of sample size for comparison of two groups was used (Charan & Kantharia, 2013).

Considering a sample population with the variance of 40, the standard deviation (SD) will be around 6.4. Therefore,

Sample size = $2SD^2 (1.96 + 0.842)^2 / d^2$; where,

SD = Standard deviation

d = the difference between the hyperglycemic rats for instance with an average of 15 mmol/L to those expected to be normal after treatment for example with an average of 4 mmol/L. (15 - 4 = 11)

Therefore the sample size in this experiment with five groupings was as follows

Sample size = $2(6.4)^2 (1.96+0.842)^2/11^2$

$= 82(7.851)/121$

$= 5.3$ rats.

The groupings therefore contained 5 rats per cage.

3.4. Plant Identification

P. fulva barks were collected in December, 2017 in Timboroa forest (within the forest station) located at Uasin Gishu County, Kenya with permission from the Kenya Forest Service. The plant material was identified and authenticated by Mr. Bernard Wanjohi, a plant taxonomist at University of Eldoret.

3.5 Plant Extract Preparation

The stem barks of *P. fulva* were harvested and peeled off while still fresh, cut into small pieces and then dried at room temperature for two weeks. The stem bark was ground when it had completely dried using an electric mill from the School of Agriculture, University of Eldoret. The powdered plant material was kept at room temperature away

from direct sunlight in closed dry plastic bags for one week awaiting ethanol extraction (Piero *et al*., 2012). The course powdered plant material (about 500 g) was extracted with 95% hydroethanol by maceration with frequent stirring for 3 days. The extract was then filtered through a Whatman no.1 filter paper. The filtrate was evaporated to dryness at 50^0C under reduced pressure using a rotary evaporator (Rotavapor type EL 30 model AG CH-9230 from Germany) in the Department of Chemistry and Biochemistry. The residue obtained constituted the crude extract and it was stored at 4^0C for further use in the study.

3.6 General Qualitative Phytochemical Analysis

The extract was subjected to various qualitative phytochemical tests to determine the general classes of phytochemicals present in the crude stem bark ethanolic extract of *P. fulva* (Ahmed *et al*., 2014). The general phytochemical screening of the bark extract were done using standard procedures as detailed in the subsequent sub-sections (Chaulya *et al*., 2011a; Usunobun & Ngozi, 2016).

3.6.1 Tannins

To the crude bark extract of *P. fulva*, 0.5g was mixed with 10mls of water in a test tube and boiled in a digital water bath type RE300DB from Bibby Scientific Limited, United Kingdom for 5 minutes and then filtered. A few drops of 0.1% ferric chloride was added and the solution observed for brownish green or a blue-black coloration. Hydrolysable tannins give a complex with ferric chloride which can be bluish-black color or precipitate and condensed tannins gives brownish-green ones. If the test is carried on an extract that contains both types of tannins, a blue color is produced which changes to olive-green as more ferric chloride is added. (Baskaran *et al*., 2015a).

3.6.2 Anthraquinones

One gram of the crude extract was boiled in 5mls of 10% hydrochloric acid for 5 minutes in a water bath then filtered and allowed to cool for 10 minutes. The filtrate was shaken with 5mls of chloroform for one minute. Few drops of 10% ammonia were then added to the mixture and heated until a color change was noted. Rose-pink color formation indicates the presence of anthraquinones. Anthraquinone glycosides upon hydrolysis yield aglycone, which are di, tri or tetra hydroxyl-anthraquinone or derivatives of these compounds. The free anthraquinone aglycone exhibits little therapeutic activity. The sugar residue facilitates translocation and absorption of aglycone at the site of action.

3.6.3 Alkaloids

One gram of the crude extract was warmed in 5ml of 2% H_2SO_4 for 3 minutes. The solution was filtered and two drops of Mayer's reagent (prepared by dissolving a mixture of mercuric chloride (1.36g) and of potassium iodide (5.00 g) in 100ml water). Formation of cream colored precipitate indicate presence of alkaloids. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (potassium-mercuric iodide solution) to give a cream colored precipitate (Baskaran *et al*., 2015a).

3.6.4 Terpenoids

Zero point five grams of the crude extract was mixed with 2ml of chloroform. 1ml of acetic anhydride was added followed by 3ml of 6M sulphuric acid. An interference which is reddish in color between interphases confirms the presence of terpenoids.

3.6.5 Tests for Saponins

Two grams of the powdered sample was boiled in 20ml of distilled water in a water bath for 10 minutes and filtered. 10ml of the filtrate was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. Saponins in plants cause persistent foam when aqueous solution is agitated. This ability to foam is caused by the combination on non- polar sapogen and water soluble side chains (Baskaran *et al*., 2015a).

3.6.6 Flavonoids

Five grams of the crude bark extract was dissolved in 5ml of ethanol (95 % v/v) then few drops of concentrated hydrochloric acid and 0.5g of magnesium metal added. The presence of flavonoids was confirmed by pink, crimson or magenta color which developed within two minutes. The reaction involves conversion of the flavonoid to the corresponding anthocyanin. The structure of the resulting anthocyanin contains an extended system of conjugated π-electrons, which constitute a chromophore. As it happens, the electrons of molecules containing conjugated π systems have energy level transitions corresponding to absorbance in the visible (and also ultraviolet) region of the electromagnetic spectrum. This accounts for their perceived color (Chen *et al*., 2012).

3.6.7 Steroids

To crude bark extract, 1g of the extract was dissolved in 10ml of chloroform and equal volume of concentrated H_2SO_4 was added by the sides of the test tubes. Reddish upper layer and yellowish sulphuric acid layer with deep green color indicate the presence of steroids (Baskaran *et al*., 2015a).

3.7 Animal Studies

3.7.1 Animals

Wistar albino rats were bred in the animal house in the Department of Biological Sciences, University of Eldoret after which selection was done. Twenty five (25) healthy male albino rats aged 6-8 weeks and weighing between 140g – 180g were used for this study. The male albino rats were kept in the animal house in wire cages (5 animals per cage) of rack type and maintained in standard conditions (12 h dark and 12 h light circle; 25 ±5°C; 40-60% humidity). The animals were fed with standard pellet feed (chow) and water was also provided *ad libitum.* The rats were acclimatized for one week before experimentation.

3.7.2 Induction of Diabetes mellitus

Twenty five (25) male albino rats were fasted for 18 hours after which fasting body weights (FBWTS) were determined using a weighing balance (Uni- bloc model UW420H) for each rat. Diabetes was then induced for the experimental groups (20 rats) excluding 5 normal rats by single intraperitoneal injection of freshly prepared STZ (50 mg/kg body weight) in100µl ice cold 0.1M sodium citrate buffer (pH 4.5) in less than 5 minutes of dissolving using insulin micro fine (0.5mls) needle. The normal control rats were injected intraperitoneally with 100µl of 0.1M sodium citrate buffer only (Rai *et al*., 2010a). The STZ induced experimental groups were given 5% glucose solution overnight to overcome drug - induced hypoglycemia and were under normal diet and drinking water *ad libitum*. STZ enters into the pancreatic β-cells via GLUT2 transporter. Single dose of STZ cause selective necrosis of pancreatic β-cells in rats and mice as a model of

diabetes (Akash *et al*., 2013). Due to STZ similarity in structure to glucose, they compete with STZ, and thus, fasting animals tend to be more susceptible (King, 2012).

Hyperglycemia was confirmed after 3 days after diabetes induction by estimating random blood sugar (RBS) levels with a value of ≥11.1mmol/L being considered as diabetic (American Diabetes Association, 2010) using a glucometer and glucose strips that uses the principle of glucose oxidase peroxidase (GOD/POD) method (Irudayaraj *et al*., 2017). The principle of glucose determination involves enzymatic oxidation of glucose in the presence of glucose oxidase (GOD). The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminoantipyrine to form a red violet quinoneimine dye as indicator. Before randomization and treatment of the rats, the fasting blood glucose was determined on the 5th day after induction. The tail was sterilized using surgical spirit and the tip cut using surgical blade. The blood from the tail of the rat was placed on the glucometer strip inserted in the glucometer and the fasting blood glucose level of each rat was displayed on the screen of the glucometer. The value displayed in the glucometer in mmol/L was recorded. Rats that had fasting blood glucose ≥13.9 mmol/L were considered diabetic and included in the study (Chaudhary *et al.*, 2016; Furman, 2015).

3.7.3 Experimental Design

After successful diabetes induction, the male albino rats were then randomly placed in 5 experimental groups of 5 animals each as described in the Table 3.1.

Table 3. 1: Categorization of rats into experimental groups

Experimental Groups	Categories of Treatment
1	Normal control with no treatment but administered with 100µl distilled water orally (vehicle)
2	STZ induced diabetic rats untreated but administered with 100µl distilled water orally (vehicle)
3	STZ induced diabetic rats treated with 100µl of 200mg/kg bwt/day of *P. fulva* ethanolic bark extract orally
4	STZ induced diabetic rats treated with 100µl of 400mg/kg bwt/day of *P. fulva* bark ethanolic extract orally
5	STZ induced diabetic rats treated with 100µl of 100mg/kg bwt/day orally of metformin

3.7.4 Treatment of Diabetic-induced Albino Rats with *P. fulva* ethanolic bark extract

Experimental groups as indicated in section 3.7.3 were treated orally for 21 days as per their treatment categories. The crude bark extract of *P. fulva* were weighed according to the specified concentrations of 200mg/kg bwt/day and 400mg/kg bwt/day per rat. Metformin at the concentration of 100mg/kg bwt/day was also weighed. The treatments were all dissolved in water. Crude bark extract suspension of *P. fulva* (100mµ) at a concentration of 200mg/kg bwt/day, 400mg/kg bwt/day and metformin (100mg/kg bwt/day) were orally administered per rat daily for 21 days using an automatic

micropipette. Day 1 was regarded as the first day of treatment. Any detectable irritation, restlessness, or adverse effect (respiratory distress, abnormal locomotion, and catalepsy) was observed and recorded in all the rats under study after the *P. fulva* crude bark extract and metformin administration. Fasting body weights for each albino rat was taken on day 0, 7, 14 & 21 to confirm whether there were changes before and after the treatments during the study period and dosing were adjusted weekly as per the weekly body weights recorded. FBS levels were also determined on day 0, 7, 14 & 21 to ascertain the effect of the treatments on FBS**.**

3.7.5 Sacrifice and Sample Collection

After 21 days of the treatment, the rats were fasted overnight and humanely sacrificed under mild anesthesia of chloroform slightly sprayed in cotton wool in a desiccator for 3 minutes. The rat was removed from the desiccator, mounted on dissection bench and dissected using dissecting kit. The blood was collected through cardiac puncture into vacutainer tubes with clotting activators (with red cap, 5mls), left to stand until whole blood clotted and serum was separated immediately by centrifugation (using a centrifuge model EBA- 21 from Hettich Company Limited, Germany) for 10 minutes at 3000 revolutions per minute. The resultant supernatant (serum) was stored in cryovials and frozen at -20^0c for analysis of lipid profile and liver function serum indices. The liver was excised, freed of surrounding tissues, washed with ice cold 0.9% normal saline and divided into two portions. The 1^{st} portion was stored in simports for all the groups and stored at –20^0C for lipid peroxidation analysis. The 2^{nd} portion of the liver for all the groups were fixed in 10% formalin for histopathological analysis.

3.7.6 Serum Analysis of Lipid Profile and Liver Function Indices

For lipid profile analysis, Total Cholesterol, LDL cholesterol, HDL cholesterol and Triglycerides were analyzed in serum and for liver function tests, serum ALP, AST and ALT, total proteins (TP), albumin (ALB) and globulins (GLB) were analyzed. These tests were done according to standard operating procedures of Cobas Integra 400 plus auto-analyzer from Roche Company at Moi Teaching and Referral Hospital, Eldoret. Serum samples that were obtained as described in section 3.7.5 were allowed to thaw at room temperature for 30 minutes after removal from -20^{0}C. From each serum sample, 150µL was pipetted into a sample cup, labeled and put in a sample rack. The reagents for each test were in form of cassettes arranged in racks inserted into the autoanalyzer. The sample rack with serum samples was inserted into the auto analyzer and commanded through a software system to run the lipid profile parameters and liver function tests. The manufacturer's protocol was followed for all the test estimations. Printouts of the results were generated after 30 minutes. The serum analyses using the Cobas Integra 400 plus auto analyzer is based on the principles described in the subsequent paragraphs for each of the tests performed.

For cholesterol analysis, cholesterol esterases (CHE) hydrolyze the cholesterol esters into free cholesterol and fatty acids. Cholesterol oxidase (CHOD) catalyzes the cholesterol into cholest-4-en-3-one and hydrogen peroxide. Hydrogen peroxide reacts with a mixture of 4-aminoantipyrene and phenol in the presence of peroxidase enzyme (POD) and converts the reactants into a red colored quinoneimine dye. The color intensity of the quinoneimine dye formed is directly proportional to cholesterol concentration which is determined by measuring the increase in absorbance at 520 nm (Parimoo *et al*., 2014).

Serum Triglycerides (TGs) are hydrolyzed by lipoprotein lipase (LPL) to glycerol and fatty acids. Glycerol is then phosphorylated to glycerol-3-phosphate by ATP in a reaction catalyzed by glycerol kinase (GK). The oxidation of glycerol-3- phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase (POD), hydrogen peroxide causes the oxidative coupling of 4- chlorophenol and 4-aminophenazone to form a red colored quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of TGs in sample (Rai *et al*., 2010a).

In the presence of magnesium sulfate and dextran sulfate, water soluble complexes with LDL, VLDL and chylomicrons are formed which are resistant to Polyethylglycol (PEG)-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to 4-cholestenone and hydrogen peroxide. The color intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration which is determined by measuring the increase in absorbance at 583 nm (Rai *et al*., 2010a).

The automated method for the direct determination of LDL-cholesterol takes advantage of selective micellary solubilization of LDL-cholesterol by nonionic detergent and the interaction of a sugar compound and lipoproteins (VLDL and chylomicrons). When a detergent is induced in the enzymatic method for cholesterol determination (cholesterol esterase and cholesterol oxidase coupling reaction), the relative reactivity of cholesterol

in the lipoproteins fraction increase in the order; HDL< chylomicrons < VLDL< LDL. In the presence of Mg^{2+}, a sugar compound markedly reduces the enzymatic reaction of cholesterol measurement in VLDL and chylomicrons. The combination of sugar compound with detergent enables the selective determination of LDL-cholesterol in serum. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to 4-cholestone and hydrogen peroxide. The color intensity of blue quinoneimine dye formed is directly proportional to the LDL-cholesterol concentration which is determined by measuring the increase in absorbance at 583 nm (Maki *et al*., 2012)

For Alkaline Phosphatase (ALP), the test principle is according to the reference method of the International Federation of Clinical Chemistry (IFCC). ALP hydrolyzes colorless 4- nitrophenylphosphate to 4-nitrophenoxide and phosphate. The product of enzyme hydrolysis, 4-nitrophenoxide, has a yellow color. 2-Amino-2- Methyl-1-propanol (AMP) functions as the phosphate acceptor and buffer. The rate of 4-nitrophenoxide formation is directly proportional to the catalytic ALP activity. This is determined by measuring the increase in absorbance at 409 nm (Chaudhary *et al*., 2016).

For Aspartate Aminotransferase (AST), the method is according to IFCC but without pyridoxal-5'-phosphate. AST in the sample catalyzes the transfer of an amino group between L-aspartate and 2-oxyglutarate and L-glutamate. The oxaloacetate then reacts with NADH, in the presence of malate dehydrogenase (MDH) to form NAD^+. The rate of NADH oxidation is directly proportional to the catalytic AST activity. This is then determined by measuring the decrease in absorbance at 340 nm (Parimoo *et al*., 2014).

The principle of analysis of Alanine Aminotransferase (ALT) is according to IFCC, but without pyridoxal-5'-phosphate. ALT catalyzes the reaction between L-alanine and 2-

oxoglutarate. The pyruvate formed is reduced by NADH in a reaction catalyzed by Lactate dehydrogenase (LDH) to form L-lactate and NAD^+. The rate of NADH oxidation is directly proportional to the catalytic ALT activity. This is determined by measuring the decrease in absorbance at 340 nm (Chaudhary *et al*., 2016; Parimoo *et al*., 2014).

3.7.7 Lipid Peroxidation Assay (LPO) in Liver Tissues

Determination of malondialdehye, an index of lipid peroxidiation in liver tissues after extract treatment was determined using the method of Okhawa and as described by Alam *et al*. (2013). Liver tissues initially frozen at -20^0C as described in section 3.7.5 were removed from the freezer and allowed to stand for one hour to attain the room temperature. One gram of the liver tissue in 9mls of 1.15% of cold potassium chloride (KCl) was homogenized with mortar and pestle then centrifuged at 2000 r.p.m for 10 minutes. The resultant supernatant (0.1ml) was then mixed with 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 8% thiobarbituric acid (TBA). The volume of the mixture was made to 4 mL with distilled water and then heated at 95^oC on a water bath for 60 minutes using boiling chips. After incubation the tubes were cooled to room temperature and final volume made to 5 mL in each tube. Five ml of butanol: pyridine (15:1) mixture was added and the contents vortexed thoroughly for 2 minutes. After centrifugation at 4000 rpm for 10 minutes, the upper organic layer was taken and its optical density measured using a spectrophotometer (Spectronic 21D from Milton Roy Limited) at 532nm.

The malondialdehyde levels were obtained from a standard calibration curve generated using hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) as a standard against which readings of the samples were determined. MDA standard was prepared as a hydrolyzed

product of TMP as follows; stock solution of 6.03 M TMP was used to make 100 mM stock by diluting 1.666 mls in 100mls distilled water. This solution was used to make 100 µM stock solutions by taking 50 µL of 100 mM solution by diluting to 50ml with distilled water. The resultant solution was used to make series of 0.5 µM, 1 µM, 2 µM, 3 µM, 4 µM, 5 µM, 7.5 µM and 10 µM solutions. Finally, the respective micromolar solutions were used to make nanomolar (nM) concentration of standards (5-100nM) as indicated in Appendix III by pipetting 0.1 ml of each µM solution and diluted to 10 ml after addition of the above reagents as used in the sample. The reference blank was 0.1 ml of 1.15% KCl treated in the same way as the samples and standards by addition of the same reagents. The level of malondialdehyde in samples was finally expressed in µM. This method depends on the formation of MDA as an end product of lipid peroxidation which reacts with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), a pink chromogen, which can be measured at 532nm.

3.7.8 Histopathological Analysis of Liver Tissues

3.7.8.1 Tissue Preparation and Processing

The liver tissues that were initially fixed in 10% formalin as indicated in section 3.7.5 were used for analysis of histopathological changes. Processing of tissues was done according to the standard operating procedures of STP 120 automatic tissue processor at Moi Teaching and Referral Hospital. Automatic tissue processor was put on and the arrow pointing upwards pressed making the carousel move up and tissue basket loaded. The arrow facing downwards was pressed to lower the carousel and the start key pressed. The program was selected by pressing the start to initiate the process. An alarm blink on the main screen indicated the end of tissue processing. Stop key was then pressed and the

arrow pointing upwards pressed, allowing the carousel to move up and well processed sections removed from the processor. It was then left for 2 minutes to let the wax drip off and the tissue basket removed.

3.7.8.2 Tissue Embedding

Embedding, which is the preparation of the impregnated tissues by use of a mold containing the embedding medium and allowing the medium to solidify to obtain a block of tissue was then performed. The purpose of embedding was to obtain a solid block containing the tissues. The metal mould base was smeared slightly with glycerin and placed in the thermal consol chamber so as to warm. The mould was filled with paraffin wax and a pair of warmed blunt nose forceps used to transfer the tissue from the embedding cassettes to the mould. Forceps was warmed again and used to orientate the tissue to lie in the desired plane. The corresponding embedding cassette was put against the mould. The mould was then transferred to a cold plate (5^0C) and the tissue blocks transferred to a freezer.

3.7.8.3 Microtomy

This involves trimming and sectioning of blocked tissue to obtain thin films or sections (2-5 microns in thickness) for microscopic examinations. This made sure that the sections produced were one cell thick, stainable and lied flat on the slides for ease of diagnosis. First trimming using a semi-automated microtome of Model BK 3358 was done to expose the tissue by chopping off extra wax on the face of the block. The knife was fixed firmly in the microtome knife holder and the tissue block fixed on the rotary arm and then adjusted using course adjustment moving into the knife. The wheel was moved to start sectioning and the sections were picked using clean slides with the aid of an applicator

stick and ensuring the sections lay flat on the slides. Twenty percent of alcohol was poured through the slide of the sections to flatten them.

3.7.8.4 Haematoxylin and Eosin Staining

Staining of the sectioned tissues was then performed for the purpose of demonstrating the general tissue structure. The sections were de-waxed in 3 changes of xylene at an interval of 5 minutes each. Xylene was then removed in three changes of absolute alcohol at an interval of 1 minute each followed by washing with distilled water. The sections were then stained using haematoxylin for 7 minutes and washed with running water. Differentiation in 1% acid alcohol was done until the sections were pale. The sections were then blued in Scotts water substitute until the sections turned blue (for 30 seconds). This was followed by staining the sections in 1% eosin for 7 minutes then dehydrated in alcohol (3 changes) and finally cleared in 3 changes of xylene.

Mounting was done in Distyrene Plasticizer and Xylene (D.P.X). Alum acts as mordant and hematoxylin containing alum stains the nucleus light blue. This turns red in presence of acid, as differentiation is achieved by treating the tissue with acid solution. Bluing step converts the initial soluble red color within the nucleus to an insoluble blue color. The counterstaining is done by using eosin which imparts pink color to the cytoplasm (Rai *et al*., 2010b). The hepatocyte lesion was analyzed for the thickness under a light microscope model CX21FSI from Olympus Corporation, Japan at X40. Examination under a light microscope was followed by taking photomicrographs using Redmi Note 4 model 2016102 phone from Xiaomi communication Company Limited, China.

3.7.9 Data Management and Statistical Analysis

Data entry and management was done in Microsoft Excel 2013. All quantitative data was expressed as Mean ± Standard error mean (SEM). Statistical analysis was by Paired Student *t*-test using Microsoft Excel 2013 for data on serum lipid profile, liver biomarker serum enzymes, proteins, and liver MDA levels. Two way analysis of variance (ANOVA) was used to analyze data on FBS and FBWTS using Statistical Package for Social Sciences (SPSS) version 20. The value of $p < 0.05$ after analysis of controls verses the treatments was considered to be statistically significant.

CHAPTER FOUR

RESULTS

4.1 General qualitative phytochemical analysis of *P. fulva* ethanolic bark extract

The ethanolic extraction of *P. fulva* bark yielded a crude extract which upon general phytochemicals analysis showed the presence of tannins, anthraquinones, terpenoids, saponins, flavonoids and steroids (Table 4.1). However, alkaloids were not detected in the ethanolic bark extract of *P. fulva*.

Table 4.1: Qualitative phytochemical analysis of *P. fulva* ethanolic bark extract

Phytochemicals	Test	Presence (+) /Absence (-)
Tannins	Ferric chloride test	+
Anthraquinones	Borntranger's test	+
Alkaloids	Mayer's test	-
Terpenoids	Salkowski's test	+
Saponins	Frothing test	+
Flavonoids	Shinoda test	+
Steroids	Lieberman-Burchand test	+

4.2 Effect of *P. fulva* ethanolic bark extract on fasting blood sugar (FBS) in normal and STZ induced diabetic rats

Determination of FBS levels was done weekly for the study period (Day 0, 7, 14 and 21) as shown in Table 4.2. Before treatment (day 0), all STZ induced rats were diabetic with FBS levels in the range of 23.22±2.56 to 30.22±1.02 mmol/L as compared to the normal control rats which had normal mean FBS levels of 4.68±0.17 mmol/L. However, treatment with 200mg/kg bwt/day and 400mg/kg bwt/day of *P. fulva* crude bark extract as well as 100mg/kg bwt/day of metformin for 21 days significantly reduced the mean FBS levels from 25.64 to 15.76 mmol/L, 25.54 to 9.70 mmol/L and 30.22 to 11.08 mmol/L respectively while the diabetic untreated group's FBS levels remained elevated at 25.35±1.14 mmol/L. In addition, ANOVA results indicated a significant effect on treatment, duration and interaction on FBS (Appendix I).

Table 4. 2: Effect of *P. fulva* bark extract on FBS in normal & STZ induced diabetic treated rats

Experimental group	Duration (Day)	FBS (mmol/L)
Normal control with no treatment	0	4.68±0.17a
	7	4.44±0.21a
	14	4.92±0.32a
	21	4.48±0.24a
STZ induced diabetic rats untreated	0	23.22±2.56e
	7	24.18±1.85e
	14	25.38±1.78e
	21	25.35±1.14e
STZ induced diabetic rats treated with 200mg/kg/day of *P. fulva* bark extract	0	25.64±1.70e
	7	20.30±1.52d
	14	17.54±1.04c
	21	15.76±0.45c
STZ induced diabetic rats treated with 400mg/kg/day *P. fulva* bark extract	0	25.54±1.75e
	7	17.96±0.86c
	14	15.24±0.63c
	21	9.70±0.69b
STZ induced diabetic rats treated with 100mg/kg/day metformin	0	30.22±1.02f
	7	20.48±0.81d
	14	15.88±1.23c
	21	11.08±0.81b

Values are expressed as mean ± Standard Error of Mean (SEM); n=5. Mean values with different letters in columns show statistically significant differences (p < 0.05, ANOVA). STZ-Streptozotocin, FBS – Fasting Blood Sugar

4. 3 Effect of *P. fulva* bark extract on fasting body weights (FBWTS) of rats

Determination of mean FBWTS was done weekly and the results is as presented in Table 4.3. The mean FBWTS for the normal control rats increased significantly from day zero (151.18g) to the 21st day (165.56g). In contrast, diabetic induced untreated rats showed a significant reduction in FBWTS from 160.88g to 142.27g after 21 days of this study. Also, significant decreases in FBWTS was recorded in the diabetic induced rats treated with 200mg/kg bwt/day *P. fulva* extract from 158.50g to 144.58g, and 161.20g to

148.82g in the diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* bark extract as well as in the 100mg/kg/day metformin-treated group that FBWTS decreased from 166.78g to 151.52g after 21 days of treatment. FBWTS reduction in the treatment groups had no significant difference when compared to the diabetic untreated rats. ANOVA results indicated a significant difference on both treatment and duration. However, there were no interaction between treatment and duration (Appendix II).

Table 4.3: Effect of *P. fulva* bark extract on FBWTS of rats

Experimental group	Duration (Day)	FBWTS (g)
Normal control with no treatment	0	151.18±5.54a
	7	154.20±5.32a
	14	158.60±4.85a
	21	165.56±4.47b
STZ induced diabetic rats untreated	0	160.88±5.33b
	7	150.74±5.06a
	14	147.52±4.87a
	21	142.27±4.49a
STZ induced diabetic rats treated with 200mg/kg/day of *P. fulva* bark extract	0	158.50±3.76a
	7	149.06±3.45a
	14	146.46±3.29a
	21	144.58±3.16a
STZ induced diabetic rats treated with 400mg/kg/day *P. fulva* bark extract	0	161.20±2.31b
	7	152.96±3.52a
	14	149.16±3.47a
	21	148.82±3.55a
STZ induced diabetic rats treated with 100mg/kg/day metformin	0	166.78±6.16b
	7	158.74±6.26a
	14	155.24±6.05a
	21	151.52±5.54a

Values are expressed as mean ± Standard Error of Mean (SEM); n=5. Mean values with different letters in columns show statistically significant differences ($p < 0.05$, ANOVA). FBWTS- Fasting body weights, STZ- Streptozotozin

4.4 Effect of *P. fulva* Bark Extract on Serum Lipid Profile Parameters

Effect of *P. fulva* bark extract on serum lipid profile parameters was determined and results are shown in Figure 4.1. CHOL-T levels for the normal control rats were significantly higher (1.53mmol/L) than diabetic induced untreated group (1.31mmol/L). In contrast, after treatment, a significant reduction in CHOL-T (1.11mmol/L) was recorded in diabetic induced rats treated with 200mg/kg bwt/day of *P. fulva* when compared to the diabetic untreated group (1.31mmol/L). However, this mean value of 1.11mmol/L from diabetic group treated with 200mg/kg bwt/day was not significantly different from that of diabetic induced rats treated with metformin at a dose of 100mg/kg bwt/day (1.01mmol/L). Induced diabetic rats treated with 400mg/kg bwt/day of *P. fulva* recorded cholesterol level of 1.32mmol/L which was not significantly different from those of diabetic induced untreated rats.

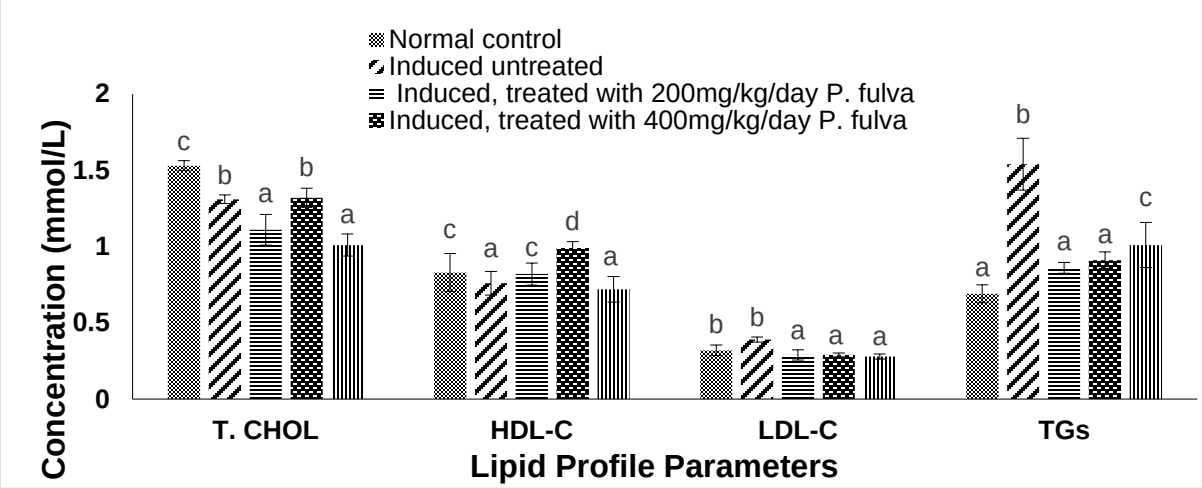
As shown in Figure 4.1, the diabetic induced untreated rats had a significant decrease in HDL-C levels (9.52%) compared to the normal control rats. Highest significant increase in HDL-C (0.99 mmol/L) was recorded in the diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* compared to the diabetic induced untreated rats (0.76 mmol/L). However, this mean of 0.99mmol/L elicited by the the diabetic rats treated with 400mg/kg bwt/day of *P. fulva* was not significantly different ($p>0.05$) when compared to diabetic induced rats treated with 200mg/kg bwt/day *P. fulva* bark extract (0.82mmol/L). Lowest HDL-C levels (0.72 mmol/L) was observed in STZ induced diabetic rats treated with 100mg/kg bwt/day metformin. Nevertheless, this lowest mean from the diabetic rats treated with 100mg/kg bwt/day metformin was not significantly diferrent when compared

to diabetic induced rats treated with 200mg/kg bwt/day *P. fulva* and the diabetic induced unreated rats (0.76mmol/L).

Mean values for LDL-C levels of the experimental groups after *P. fulva* treatment are as shown in Figure 4.1. There was no statistical difference between the means of LDL-C in the normal control rats (0.32 mmol/L) and the STZ diabetic induced untreated rats (0.39 mmol/L). On the other hand, treatment with 200mg/kg bwt/day and 400mg/kg bwt/day of *P. fulva* as well as 100mg/kg bwt/day metformin recorded a significant decrease in LDL-C levels to 0.29, 0.29 and 0.28mmol/L respectively when compared to diabetic induced untreated rats that had LDL-C levels of 0.53mmol/L.

Results on TGs levels in Figure 4.1 shows that diabetic induced untreated rats recorded a significant higher levels of TGs (1.74mmol/L) as compared to normal control rats (0.69mmol/L). Significant lower levels of TGs (0.86 mmol/L) were recorded in diabetic induced rats treated with 200mg/kg bwt/day *P. fulva* when compared to diabetic untreated rats (1.74mmol/L). However, this was not significantly diffferent from diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* (0.91 mmol/L) and diabetic induced rats treated with 100mg/kg bwt/day metformin (1.01 mmol/L).

Figure 4. 1: Effect of *P. fulva* bark extract on serum lipid profile parameters



Bars are expressed as mean ± Standard Error of Mean (SEM); n=5. Mean values with different letters in bar graphs show statistically significant differences (p < 0.05, Student t-test). CHOL – T – Cholesterol total; HDL – C – High density Lipoprotein-cholesterol; LDL – C – Low density lipoprotein-cholesterol; TGs – Triglycerides

4.5 Effect of *P. fulva* Bark Extract on Liver Function Serum Enzymes Indices

Effect of *P. fulva* bark extract on serum liver biomarker enzymes was evaluated and results are shown in Figure 4.2 for the measured parameters (ALP, AST and ALT). After treatment, the diabetic induced untreated group showed significant higher ALP levels (425.1 U/L) as compared to normal control rats (191.8 U/L). Among the treated groups, significant ($p<0.05$) lowest mean of ALP (233.04 U/L) was recorded in diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* compared to diabetic induced untreated rats (425.05U/L). On the other hand, highest ALP levels of 453.63 U/L was recorded in STZ induced diabetic rats treated with 100mg/kg bwt metformin (standard drug) when compared to diabetic induced untreated rats (425.05 U/L). However, the mean of ALP levels for the diabetic induced untreated rats (425.05 U/L) was not significantly different

from the mean (415.3 U/L) of diabetic induced rats treated with 200mg/kg bwt/day of *P. fulva.*

AST levels were increased significantly (341.93 U/L) for the diabetic induced untreated rats when compared with the normal control group (145.28 U/L). However, when treated with 200mg/kg bwt/day and 400mg/kg bwt/day of *P. fulva* as well as 100mg/kg bwt/day metformin, AST levels were reduced significantly; (223.33 U/L, 158.78 U/L and 159.38 U/L, respectively) compared to the diabetic induced untreated rats (341.93 U/L).

Findings on ALT levels show that diabetic induced untreated rats had significantly higher levels (252.02 U/L) as compared to the normal control rats (72.72 U/L). When treated with 200mg/kg bwt/day and 400mg/kg bwt/day of *P. fulva* extract as well as 100mg/kg bwt/day of metformin, significant reduction in ALT levels were recorded (184.25 U/L, 69.14 U/L and 126.6 U/L, respectively) compared to the diabetic induced untreated rats (252.02 U/L).

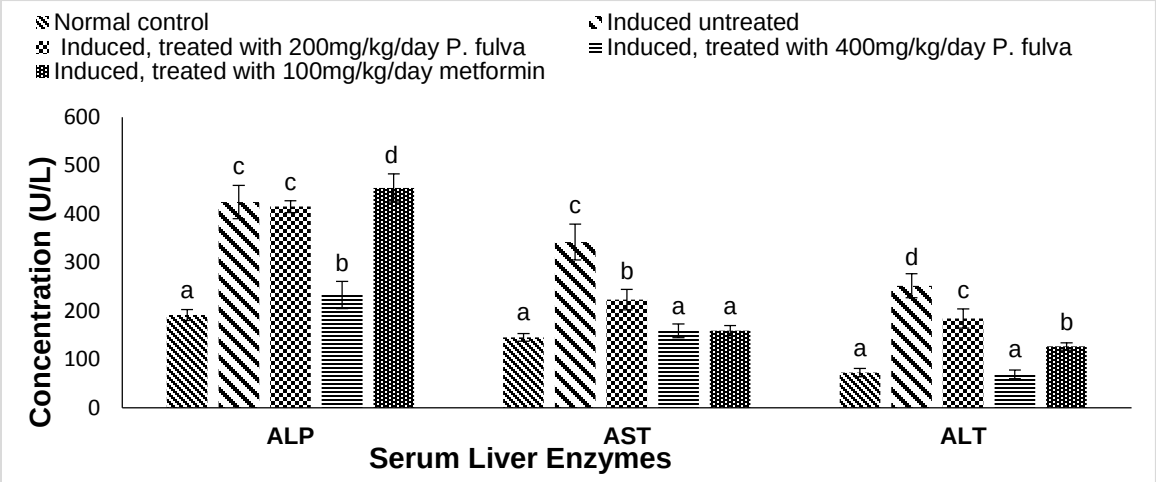


Figure 4. 2: The effect of *P. fulva* bark extract on serum liver enzymes

Bars are expressed as mean ± Standard Error of Mean (SEM); n= 5. Mean values with different letters in bar graphs show statistically significant differences ($p < 0.05$, Student t-test). ALP – Alkaline phosphatase; AST – Aspartate aminotransferase; ALT – Alanine aminotransferase

4.6 Effect of *P. fulva* bark extract on Liver Function Serum Proteins Indices

As shown in Figure 4.3, there was no significant differences in total proteins (TP) between the normal control rats (57.0 g/L) and the diabetic induced untreated rats (59.8 g/L). Significant increase in TP levels (64.2g/L) was recorded in the diabetic induced rats treated with 400 mg/kg bwt/day of *P. fulva* compared to diabetic induced untreated rats with TP levels of 59.8g/L. There was no statistical difference on TP levels (60.1g/L) of diabetic induced rats treated with 200mg/kg bwt/day of *P. fulva* when compared to diabetic induced untreated rats (59.8g/L). Also, TP levels of diabetic induced untreated group was not significantly different from diabetic induced rats treated with 100mg/kg bwt/day metformin (60.2g/L). On the analysis of albumin (ALB), comparison between the normal control rats and the diabetic induced untreated rats had no significant

difference. Results further showed that the induced rats treated with 400mg/kg bwt/day of *P. fulva* extract recorded significant higher ALB levels (37.4 g/L) than the induced untreated rats (29.9 g/L). For globulins, we found insignificant differences among treated groups although the diabetic induced untreated group had higher globulin levels (30g/L) compared to the normal control group (23.16g/L).

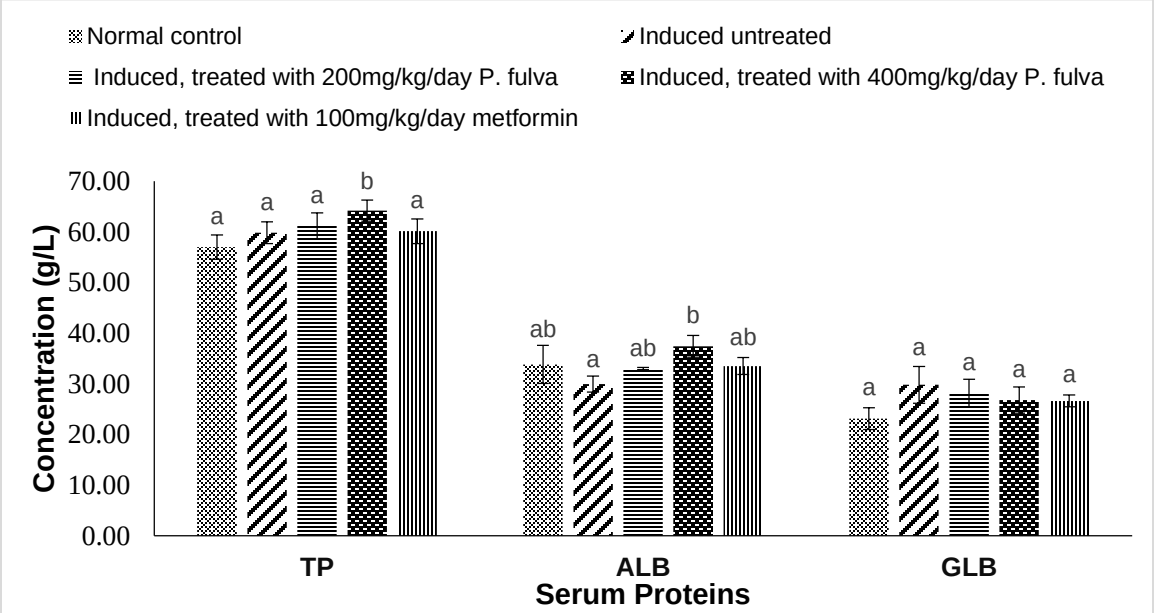


Figure 4. 3: The effect of *P. fulva* bark extract on serum proteins

Bars expressed as mean ± Standard Error of Mean (SEM); Mean values with different letters in bar graphs show statistically significant differences ($p < 0.05$, Student t-test) TP - Total Proteins; ALB – Albumin; GLB – Globulins

4.7 Effect of *P. fulva* Bark Extract on Lipid Peroxidation in Liver Tissues

MDA levels as an index of lipid peroxidation in liver tissues were analyzed and the results summarized in Figure 4.4. There was a significant increase of 57.2% in lipid peroxidation in the STZ induced untreated diabetic rats when compared to the normal control. However, diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* had the

highest significant reduction in lipid peroxidation (a decrease of 78.6%) followed by diabetic induced rats treated with 200mg/kg bwt/day *P. fulva* bark extract (a decrease of 62.31%) when compared to the diabetic induced untreated group. Additionally, significant reduction in lipid peroxidation was recorded in the 100mg/kg/ bwt/day metformin treated group (a decrease of 49.70%) when compared to the diabetic induced untreated rats.

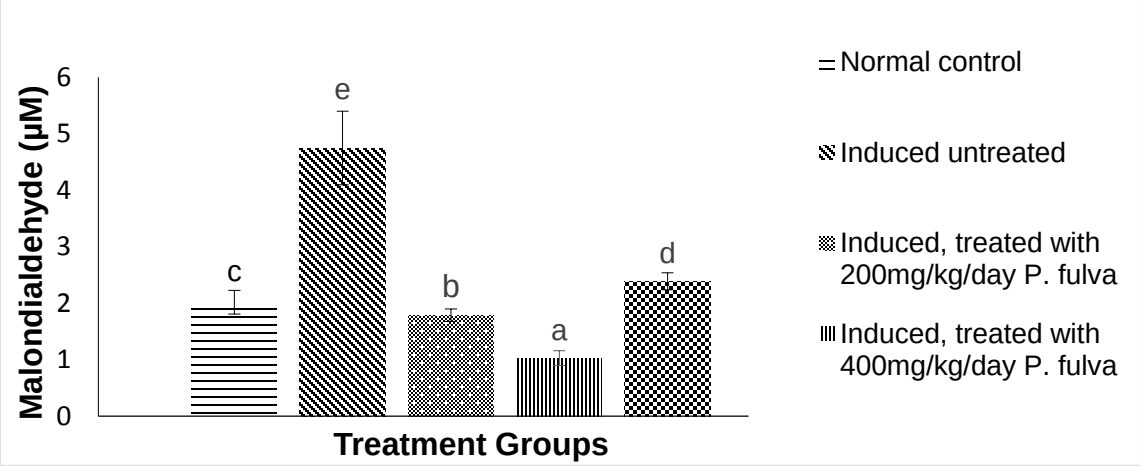


Figure 4. 4: Effect of *P. fulva* bark extract on lipid peroxidation in liver

Bars are expressed as mean ± Standard Error of Mean (SEM); n=5 Mean values with different letters in bar graphs show statistically significant differences ($p < 0.05$, Student t-test). MDA-Malondialdehyde.

4.8 Liver histopathology

Further effects of *P. fulva* treatment on liver tissues were assessed by histopathological examinations. Light microscope (Model CX21FS1) was used to examine the processed liver tissues and comparison of the normal control, the diabetic induced untreated, diabetic induced rats treated with crude bark extract of *P. fulva* (200mg/kg bwt/day and

400mg/kg bwt/day) and metformin (100mg/kg bwt/day) and histological changes were noted. Representative light microscopy photomicrographs of liver sections were selected from each of the experimental five animal groups after examination.

4.8.1 Liver morphology of normal control rats

Hematoxilin and eosin staining of liver tissue for the normal control group of rats showed no pathological changes with normal hepatocyte morphology and organized hepatic cell with no inflammation, fatty degeneration and proliferation (Figure 4.5).

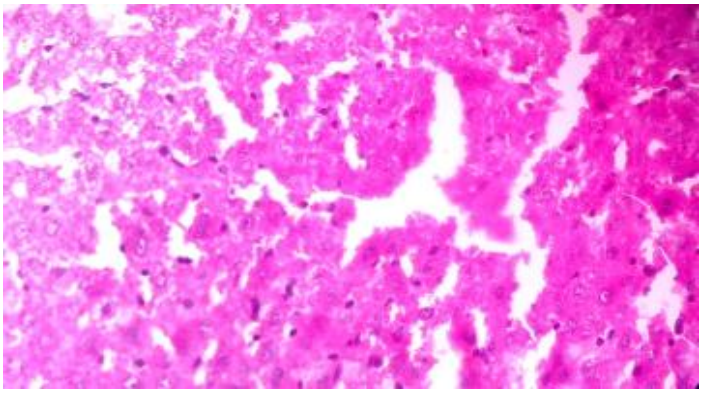
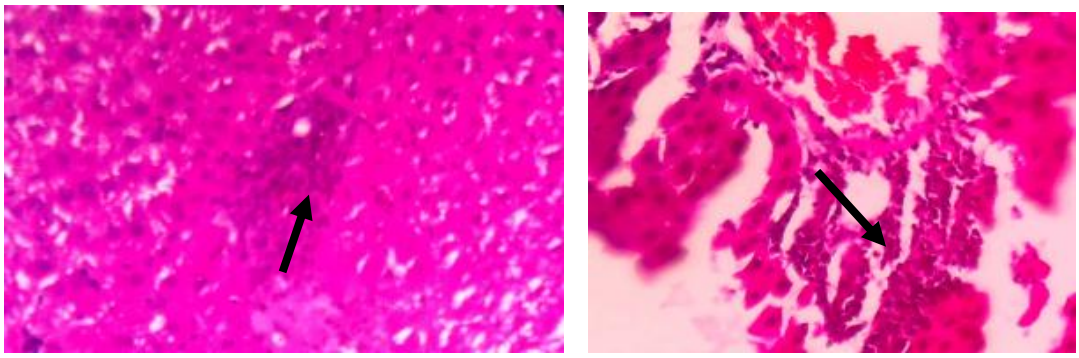


Figure 4. 5: Photomicrograph of liver tissue sections showing normal hepatic architecture (X40)

4.8.2 Liver morphology of STZ induced diabetic untreated rats

Hematoxilin and eosin staining of liver tissue for diabetic induced untreated group of rats upon examination showed pathological changes in hepatic morphology with mild periportal chronic inflammation (Figure 4.6).



(a) **(b)**

Figure 4. 6: Photomicrographs of liver tissue sections of diabetic induced untreated rats

The black arrow indicate mild periportal chronic inflammation for both a & b at X40.

4.8.3 Liver Morphology of STZ induced diabetic rats treated with 200mg/kg bwt/day of *P. fulva*

Hematoxilin and eosin staining of liver tissue for the induced diabetic rats treated with 200mg/kg/day *P. fulva* showed improved hepatic architecture with very mild bile duct proliferation with no inflammation (Figure 4.7).

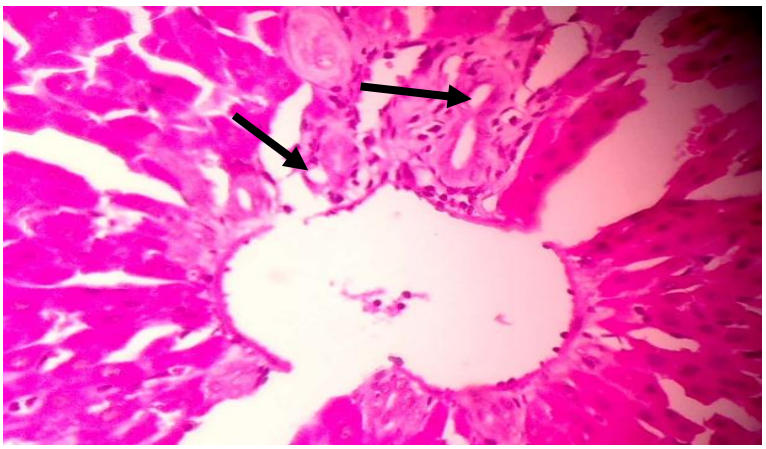


Figure 4. 7: Photomicrograph of the liver tissue sections of diabetic induced rats treated with 200mg/kg bwt/day *of P. fulva*

The arrows indicate mild bile duct proliferation with no inflammation at X40.

4.8.4 Liver Morphology of STZ induced diabetic rats treated with 400mg/kg bwt/day of *P. fulva*

Hematoxilin and eosin staining of liver tissue for the diabetic induced rats treated with 400mg/kg bwt/day of *P.fulva* showed normal hepatic architecture with prominent nucleoli and no inflammation (Figure 4.8).

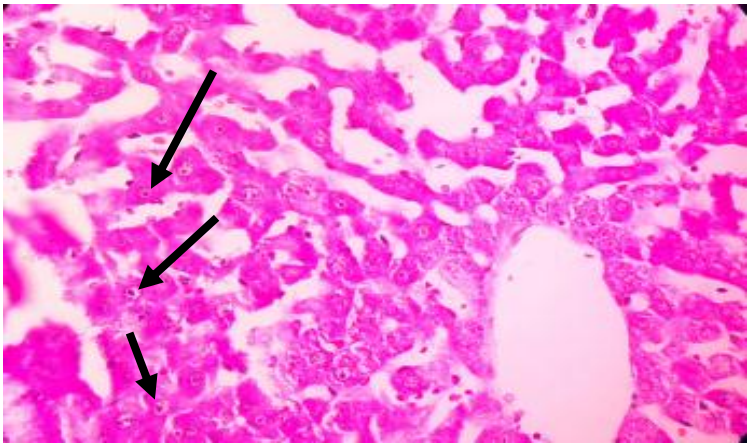


Figure 4. 8: Photomicrograph of liver tissue sections of diabetic induced rats treated with 400mg/kg bwt/day of *P. fulva*

The arrows indicate prominent nucleoli with no inflammation at X40.

4.8.5 Liver morphology of STZ induced diabetic rats treated with 100mg/kg bwt/day of metformin

Hematoxilin and eosin staining of liver tissue for the diabetic induced group of rats treated with 100mg/kg bwt/day of metformin showed normal hepatocytes morphology with prominent nucleoli and no inflammation (Figure 4.9).

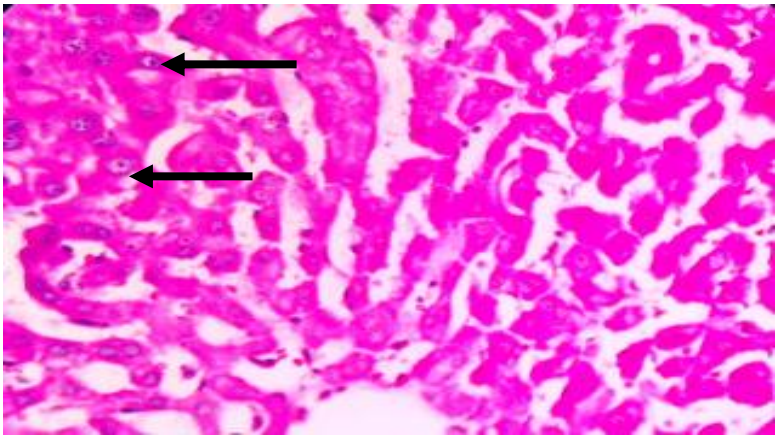


Figure 4. 9: Photomicrograph of liver tissue sections of diabetic induced rats treated with 100mg/kg bwt /day of metformin

The arrows indicate prominent nucleoli with no inflammation at X40

CHAPTER FIVE

DISCUSSION

Medicinal plants contain different phytochemicals with biological activity that can be of valuable therapeutic index. The phytochemicals present in medicinal plants have been found to possess various activities which may help against chronic diseases (Kumar *et al*., 2009). In this study, *P. fulva* ethanolic bark extract yielded a crude extract which upon analysis of general phytochemicals showed the presence of tannins, anthraquinones, terpenoids, saponins, flavonoids and steroids. However, alkaloids were not detected in the crude extract. According to Yadav & Agarwala, (2011), these phytochemical constituents are known to exhibit medicinal as well as physiological activities.

Flavonoids have the ability to reduce free radicals formation as well as scavenge free radicals. They therefore exhibit antioxidant activity. The hydroxyl groups in flavonoids are responsible for the free radical scavenging activity. Flavonoids and terpenoids have also been known to have protective effects of β – cells in diabetic rats (Kumkrai *et al*., 2015). Studies indicate that saponins have antihyperglycemic properties (Prabhakar & Doble, 2011) as well as treatment of cancer and inflamed or ulcerated tissues. Tannins have strong anti-platelet and antihypercholesterolemic effects by reducing cholesterol absorption in animal studies (Baskaran *et al*., 2015). Saponins are involved in complexation with cholesterol to form pores in cell membrane bilayers and may be used as anti-cholesterol agents or cholesterol lowering agents (Usunobun & Ngozi, 2016). Saponins have also shown cholesterol-lowering effects by reducing LDL and total cholesterol concentrations without altering high-density lipoprotein cholesterol levels in hypercholesterolemia-induced rats and were also found to reduce the risk of

atherosclerosis in rats (Baskaran *et al*., 2015). Findings obtained in the present study are in agreement with those reported by Njateng *et al*. (2013) where the presence of saponins, tannins, alkaloids, anthraquinones and phenol was recorded in crude extract of *P. fulva* ethyl acetate fraction in antidermatophytic study. In another study, researchers also characterized dichloromethane-methanol (1:1 v/v) stem bark extract of *P. fulva* resulting in the isolation of 10 known compounds and one new saponin (3-*O*-[*α*-L-rhamnopyranosyl (1–2)-*α*-L-arabinopyranosyl]-28-*O*-[*α*-L-4-*O*-acetyl-rhamnopyranosyl (1–4)-*β*-D-glucopyranosyl-(1–6)-*β*-D-glucopyranosyl]-hederagenin) and investigated their antifungal properties (Njateng *et al*., 2015)

STZ-induced hyperglycemia in animal models has been described as a useful experimental tool to study the activity of hypoglycemic agents (Kurup & Mini, 2017). In the present study, STZ induced hyperglycemia in rats and this is postulated to be through STZ selectively destroying the pancreatic insulin secreting β– cells, leaving less active β-cells resulting in a diabetic state. STZ is a potent alkylating agent that exerts its effect on the pancreatic β-cells via glucose transporter-GLUT2 and cause induction of methylation of DNA resulting in damage (Irudayaraj *et al*., 2017). Damaged DNA in turn activates the poly (ADP-Ribose) polymerase, leading to NAD^+ depletion in pancreatic β-cells. STZ also acts as a nitric oxide donor in pancreatic islets and enhances O_2^- radical fragmentation in islet cells. As a result, pancreatic β-cell apoptosis and necrosis ensued leading to decrease in pro-insulin synthesis and therefore fasting blood glucose was elevated significantly in diabetic rats leading to hyperglycemia as a result of pancreatic β-cell damage by STZ or few remaining β- cells were less active in insulin production (Kumar *et al*., 2014b).

In this study, maximum percent fall of fasting blood sugar (FBS) in diabetic rats was found at the dose of 400 mg/Kg bwt/day (74.2%) as compared to 200 mg/Kg bwt (65.6%) of *P. fulva* on the last day of treatment period. These results was comparable with the standard drug, metformin at 100mg/kg bwt/day which reduced fasting blood glucose level by 74.3% after 21 days. The difference between the two extract doses, 400 and 200 mg/kg bwt of *P. fulva* extract, might be attributed to the former containing higher concentration of the phytochemicals responsible for more fall of fasting blood glucose than the latter. The biochemical mechanism of anti-hyperglycemic actions of *P. fulva* bark extract might be due to an insulin mimetic effect on muscle and adipose tissues by either stimulating glucose uptake and metabolism (Daisy *et al*., 2010), by inhibiting hepatic gluconeogenesis (Andrade-Cetto & Vázquez, 2010) and glycogenolysis (Rawi *et al.*, 2011), by stimulation or regeneration process of remnant β - cells.

The antioxidant potential of *P. fulva* extract could have potentially reduced the oxidative stress induced by STZ in beta cells leading to increased pancreatic secretion of insulin from the few existing pancreatic β-cells from extract treated rats and/ or inhibition activity against α-glucosidase enzymes in small intestine which convert disaccharides into monosaccharaides for the sake of absorption (Shinde *et al*., 2008). Similar findings were reported by Sengupta *et al.* (2011). The researchers reported a decrease in blood glucose level after administration of leaf extract of *Psidium guajava* (250 and 400 mg/kg) to STZ-induced diabetic mice with increase in induction time (Sengupta *et al*., 2011). In addition, fasting blood glucose lowering effect of *A. remota* was similar and *Ajuga iva* were found to have hypoglycemic effect in streptozotocin induced diabetic rats (El Hilaly & Lyoussi, 2002).

Findings from this study also recorded a decrease in body weights of untreated diabetic rats. This body loss could be due to increased muscle wasting (Ravi *et al*., 2004). Moreover, loss of body weight in diabetic rats is due to dehydration, loss of carbohydrates and the excessive break down of tissue proteins and fat (Kurup & Mini, 2017). However, *P. fulva* bark ethanol extract treatment in this study, slightly improved the body weight in the treatment groups as compared to diabetic untreated rats. The ability of the extract to protect massive body weight loss seems to be due to its ability to reduce hyperglycemia which was an indication of proper glucose utilization (Pari & Satheesh, 2004), its protective effect in muscle wasting and controlling protein turn over and/or improvement in diabetes mellitus associated disorders (Oyedemi *et al*., 2012). Results of the study further revealed that the diabetic treated groups recorded slight increase in body weight gain (158.50 – 144.58 g) and (161.20 – 148.82 g) for the rats treated with 200 and 400mg/kg bwt/day respectively as compared to the diabetic untreated group (160.88 – 142.27 g), which could support that *P. fulva* bark extract could be a potential strong candidate for treatment of diabetes mellitus over synthetic drugs which are mostly known to cause body weight gain in diabetes mellitus treatment (Prabhakar & Doble, 2011). Past studies have shown that STZ diabetic induced rats have their body weights lowered (King, 2012). Similar studies show that phytoconstituents from plants have been known to increase the body weights of animal models as a result of administration of STZ induction (Zhang & Tan, 2000).

Diabetes mellitus is related to lipid metabolic abnormality characterized mainly by high CHOL-T, high TGs, high LDL-C and low HDL-C. An elevation in serum lipid levels may lead to a higher risk for cardiovascular disease in some cases. In this study, CHOL -

T was significantly elevated in the normal control croup when compared to the diabetic untreated group. However, there was a significant drop in Chol-T levels of the diabetic group treated with 200mg/kg bwt/day as compared to diabetic untreated group (Figure 4.1). Decrease in cholesterol levels could be due to insulin releasing effect of *P. fulva* bark extract sensitizing activity from regenarated beta cells of the pancrease as insulin has been proved to inhibit the activity of sensitive lipases in the adipose tissue and suppresses the release of lipids (Patel *et al*., 2009). Decline in cholesterol levels has been known to decrease blood glucose levels (Kumar *et al*., 2014b) which could be the reason for the hypoglycemic activity of *P fulva*. It has also been reported that flavonoids, that we also found present in *P. fulva* ethanolic extract, prevent the oxidation of LDL-C and lowers the blood levels of cholesterol and triglycerides hence the reduction in the risk for the development of atherosclerosis. Saponins detected in *P. fulva* ethanol bark extract is reported to have beneficial effects on blood cholesterol levels by binding with bile salt and cholesterol in the intestinal tract. This causes a reduction of blood cholesterol by preventing its re-absorption. (Oyewole & Akingbala, 2011). Moreover, saponins are known to elicit serum cholesterol lowering activity by causing resin-like action leading to reduction in the enterohepatic circulation of bile acids. In the process, conversion of cholesterol to bile acid is enhanced in the liver resulting in concomitant hypocholesterolemia (Adeneye & Olagunju, 2009). The hypocholesterolemic effect noted in our study may also be due to decreased intestinal absorption or decreased cholesterol biosynthesis (Ahmed *et al*., 2014). In addition, other researchers have suggested that some phytoconstituents of plant extract may act as inhibitors for some enzymes such as

hydroxy methyl glutaryl CoA reductase, which participates in cholesterol biosynthesis (Baskaran *et al*., 2015).

This study also showed that the highest HDL-C levels was in the diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* extract when compared to the diabetic untreated rats. This was in line with other studies which reported high levels of HDL-C that were beneficial because it mediates the transfer of excess cholesterol from the peripheral cells to the liver for its catabolism. Increased serum HDL-C levels may prove beneficial in lipid disorders (Ochani & D'Mello, 2009). Other studies by Ahmed *et al*. (2014) showed that stem bark extract of *Albizzia lebbck benth* treatment of STZ diabetic rats showed elevated levels of HDL-C and reduction of CHOL-T, LDL-C, VLDL-C and TGs in a dose dependent manner. Similarly, findings by (Rouhi *et al*., 2017) showed that polyphenols of plants increase the activity of serum HDL associated paraoxonase 1, which can in turn hydrolyze lipid peroxides in oxidized – LDL-C and convert them to a less atherogenic LDL-C; thus causing more reduction in oxidized - LDL-C content.

On the other hand, our study showed that STZ induced untreated diabetic rats had higher LDL-C levels as compared to the normal control rats. This could be due to the STZ induced diabetes in untreated rats that possibly had increased level of glucose-6-phosphatase, which in turn enhance the production of fats from the carbohydrates resulting in increased deposition of fats in the liver and kidney. In STZ induced untreated diabetic rats, the collapse in glucose metabolism alter the capacity of liver in synthesizing glycogen, decline the level of hexokinase and lead to decrease in the use of energy Kumar *et al*. (2014b). A significant decline in LDL-C was noted after treatment with 200mg/kg bwt/day, 400mg/kg bwt/day of ethanolic bark extract of *P. fulva* and 100mg/kg

bwt/day of metformin. The decrease in LDL-C could have been due to improved level of hexokinase in the liver that take part in the conversion of glucose into glucose-6-phosphatase and utilization of the glucose into energy (Kumar *et al*., 2014b).

In this study, hypertriglyceridemia was noted in STZ induced untreated diabetic rats versus the normal control rats (Figure 4.2). A significant (*P>0.05*) drop in the TGs level after treatment with ethanolilc bark extract of *P. fulva* at a dose of 200 and 400mg/kg/day and 100mg/kg/day of metformin was also noted. According to Kumar *et al*. (2014b), STZ induced diabetes mostly involve abnormal lipid metabolism which is a metabolic disorder in diabetes complications. In diabetic state, hypertriglyceridemia is one of the common factors involved in development of atherosclerosis and coronary heart disease. The level of the triglyceride increased in STZ induced diabetic rats possibly due to decreased level of insulin, leading to inactivation of lipoprotein lipase that hydrolyzes TGs into glycerol and fatty acids. The reduction in TGs in the treated groups could be due to inhibition of fatty acid synthesis (Baskaran *et al*., 2015). Findings from the present study are similar to those reported by other researchers for instance, phytochemical constituent of *A. remota* extract, from other Ajuga species have been reported to show significant reduction of lipid profile parameters in diabetic rat (Fan *et al*., 2011; Mamadalieva *et al*., 2013).

Lipid peroxidation is an autocatalytic free radical mediated destructive process whereby poly-unsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides including conjugated dienes and MDA (Tangvarasittichai, 2015). In this study, the results showed that STZ-induced diabetic rats were susceptible to lipid peroxidation as observed with the elevation of MDA in the diabetic untreated group versus the normal control (Figure 4.7). This lipid peroxidation could have resulted from

the rise in generation of ROS such as superoxide (O_2^-) and hydroxyl (OH^-) radical by STZ. Also, the disruption of hepatic antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), and Glucose peroxidase (GPx) could contribute the abundance of ROS in cells. Additionally, prolonged exposure to hyperglycemia increases the generation of free radicals and reduces capacities of the antioxidant defense system which leads to oxidative stress (Lee, 2006). SOD mop up superoxide radicals by converting them to hydrogen peroxide (H_2O_2) and oxygen while both CAT and GPx are involved in the elimination of H_2O_2 (Kazeem *et al*., 2013).

Treatment with *P. fulva* crude stem bark extract at doses of 200 and 400mg/kg bwt/day led to lipid peroxidation being drastically reduced compared to the STZ diabetes induced untreated rats. It was also observed that both dosages of 200 and 400mg/kg bwt/day of *P. fulva* had higher lipid peroxidation lowering effect compared to metformin at a dose of 100mg/kg bwt/day as the standard drug. The *P. fulva* crude bark extract antioxidant potential could be explained in part because our phytochemical analysis showed the presence of flavonoids, tannins and saponins amongst others. Flavonoids and tannins are phenolic compounds that are a major group of compounds that act as primary antioxidants (Ayoola *et al*., 2008). Flavonoids have been reported to prevent the oxidation of low-density lipoprotein, lowers cholesterol and triglycerides levels. The mechanisms of antioxidant action of medicinal plants which have flavonoids include the suppression of reactive oxygen species (ROS) formation either by inhibition of ROS-generating enzymes; the chelation of trace elements that are involved in free radical generation; or by the induction of antioxidant defenses. The effectiveness of flavonoids is due to their chemical structure. The non-sugar part of saponins also has antioxidant

activity which may help to reduce risk of heart diseases (Oyewole & Akingbala, 2011). In addition, there is increasing evidence that polyphenols protect cellular constituents against oxidative damage and, therefore, limit the risk of chronic diseases associated with oxidative stress (González-Ponce *et al*., 2016). The antioxidant potential exhibited by *P. fulva* ethanolic bark extract may therefore be beneficial in correcting hyperglycemia and preventing diabetic complications due to free radicals and lipid peroxidation (Patel *et al*., 2009). The beneficial effect of hypoglycemia exhibited could mainly be due to metformin-like action in glucose reduction output owing to inhibition of liver gluconeogenesis and to a lesser extent, increased insulin-mediated glucose uptake in the skeletal muscle and slight delay in the absorption process of glucose (Pernicova & Korbonits, 2014).

The effect of *P. fulva* ethanolic crude bark extract on AST, ALT and ALP was evaluated in serum samples of rats in this study. Diabetes mellitus is frequently associated with the elevated activities of liver toxicity marker enzymes like ALT, AST and ALP in serum, which might be mostly due to the out flow of these enzymes from the liver cytosol into the bloodstream (Kurup & Mini, 2017). Moreover, diabetes mellitus and liver disease are commonly associated. The liver maintains glucoregulation, carbohydrate homeostasis and insulin deterioration through MAPK signaling as potent regulators of insulin signal transduction, glucose and lipid metabolism (Wang *et al*., 2018) but diabetes leads to declined hepatic insulin sensitivity which is responsible to augment the estimation of liver function test like serum ALT, AST, ALP, and proteins (Chaudh7ary *et al*., 2016). Estimating the activities of these enzymes can make assessment of liver function (Rathi *et al*., 2015). This is because serum transaminases (AST and ALT) levels are most widely

used as a measure of hepatic injury, due to their ease of measurement with high degree of sensitivity and their usefulness in detection of early damage of hepatic tissue (Ray *et al*., 2006).

Results on the effect of the *P. fulva* extract on the serum AST, ALT and ALP in Figure 4.4 showed a significant increase in these serum enzymes ($P< 0.05$) in diabetic untreated rats when compared to the normal control rats. Specifically, administration of *P. fulva* and metformin reduced the AST and ALT levels in diabetic condition and the effect was superior for *P. fulva* at a dose of 400mg/kg bwt/day attributable to higher concentration of phytochemicals. This could imply that both metformin at a dose of 100mg/kg bwt/day and *P. fulva* at a dose of 400mg/day bwt/kg have hepatoprotective effect. The increase in the activities of serum AST and ALT in diabetic untreated rats versus mormal rats could be due to the leakage of these enzymes from the liver cytosol into the blood stream which is a pointer to the hepatotoxic effect of STZ which causes necrosis of liver tissues (Kazeem *et al*., 2013). Moreover, hyperglycemia causes over production of free radicals thereby creating oxidative stress leading to cellular injury as also supported from our liver lipid peroxidation results in the diabetic rats (Figure 4.4). Increased lipolysis during diabetes increases free fatty acids levels in tissues that causes the generation of free radicals (Balasubashini *et al*., 2004). On the other hand, treatment of diabetic rats with *P. fulva* ethanolic bark extract of 400mg/kg bwt/day showed a significant decrease in serum ALP levels. ALP levels is related to functioning of hepatocytes and its increase in serum could have been due to increased synthesis in the presence of increased biliary pressure (Hegde & Joshi, 2009). In addition, ALP has been reported to be involved in the transport of metabolites across the cell membranes, protein synthesis, secretory activities and

glycogen metabolism (Sharma *et al*., 1996). Therefore, the gradual rise in the serum ALP levels in the diabetic untreated group when compared to the normal control in the present study may be due to disturbance in the secretory activity of cell or the transport of metabolites or may be due to altered synthesis of certain enzymes as in other hepatotoxic conditions. Overally, the decrease in the levels of ALT, AST and ALP enzymes might be due to hepatoprotective effect and normalization capability of impaired liver metabolism in treated diabetic rats (Sharma *et al*., 2011).

The present findings are similar to those reported by various researchers (Hegde & Joshi, 2009). The researchers reported significant reduction in liver enzymes of diabetic rats treated with crude extracts of plants. The studies are also in agreement that the mechanism of the hepatoprotective effect of the plant extract is related to the ability of the plant to inhibit lipid peroxidation in the liver. Findings by Ramadan *et al*. (2013) also showed that increase in the activities of serum AST, ALT, and ALP indicated that diabetes may result due to liver dysfunction and acute effects on hepatocytes. Other studies have reported that increased activity of ALT in the blood of diabetic patients might be due to hepatic dysfunction involving changes in the integrity of cell membranes and tissue injury caused by the oxidative stress of which oxidative stress is also supported by our results as shown in Figure 4.4 on lipid peroxidation in the liver (Oliveira *et al*., 2016).

Apart from the analysis of serum enzymes indicative of liver function, we also analyzed the serum proteins (TP, ALB and GLB) in this study since the liver is the major source of most serum proteins. For instance, albumin is a protein synthesized by the liver and is a useful indicator of hepatic function (Thapa & Walia, 2007). A significant increase of total

protein and albumin levels coupled with insignificant decrease in globulins in diabetic induced rats treated with 400mg/kg bwt/day *P. fulva* when compared to the diabetic untreated rats was recorded in this study. Decrease in total protein and albumin with increased globulin levels indicate that diabetes may result to liver dysfunction and acute hepatocytes infection (Ramadan *et al*., 2013). Alongside with liver marker enzymes, albumin has been known to be a significant biomarker for liver function (Sobeh *et al*., 2017). *P. fulva* extract could have trapped the oxygen related free radicals and therefore hinder their interaction with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidation processes leading to MDA generation. *P. fulva* ethanolic bark extract could also have increased the hepatic content of GSH and blood glutathione since higher content of glutathione in blood and liver would lead to a better tissue protection against oxidative stress hence reduction in hepatotoxic effect of hyperglycemia and STZ effects. This may have consequently improved liver damage caused by STZ-induced diabetes.

Our studies on histopathology of the liver tissues was necessary to evaluate the effect of treatment of *P. fulva* bark extract on diabetic rats. Our results from liver histolopathology after 21 days for normal control rats (Figure 4.5) showed no pathological changes with normal hepatocyte morphology and organized hepatic cell with no inflammation, fatty degeneration and proliferation indicating that the cell architecture was normal. In contrast, the diabetic induced untreated rats resulted in liver pathological changes such as mild periportal chronic inflammation (Figure 4.6). STZ induced diabetes leads to liver damage and chronic hyperglycemia in diabetic rats exhibit high oxidative stress (Figure 4.4) resulting in depleted activity of the antioxidant defense mechanism, promoting the

generation of free radicals as result of lack of insulin. The pathological changes in diabetic untreated rats could have been due to injury to the liver hepatocytes resulting from these effects of STZ induction and chronic hyperglycemia. STZ-induced diabetic rats could have disrupted the activities of hepatic antioxidant enzymes accompanied with an increase in the susceptibility to lipid peroxidation. Moreover, antioxidant defense mechanisms (decrease in the activity of antioxidant enzymes) could have been altered due to hyperglycemia, promoting free radical generation. Additionally, liver biomarker indices were also significantly increased in liver due to altered permeability of the hepatocellular membrane as a result of damage (Figure 4.2). Furthermore, dyslipidemia (high Chol- T, LDL- C and TGs with decreased HDL- C) could have led to the pathological changes in diabetic induced untreated rat liver. However, administration of 200mg/kg/day of *P. fulva* led to the reduction of liver pathological changes (Figure 4.7) to a small extent with very mild bile duct proliferation with no inflammation and treatment with a higher concentration of 400mg/kg/day of *P. fulva* and metformin at a dose of 100mg/kg/day restored the hepatocytes architecture with prominent nucleoli and no inflammation (Figure 4.8 and 4.9). Therefore, the reversal of the pathological changes induced by STZ induction and chronic hyperglycemia by the treatment *P. fulva* could suggest the hepatoprotective effect (because it is rich in phytochemicals) with the antioxidant potential and can be a good candidate for the treatment of complications arising from diabetes. The antioxidant action of *P. fulva* against reactive oxygen species (ROS) formation could be through inhibition of ROS-generating enzymes.

The findings are in agreement with research done by (Venkatesh *et al*., 2010) who reported diabetic untreated rats showing pathological changes but the architecture of the

liver in diabetic rats treated with butanol extract of *Helicteres isora* roots was similar to that of normal liver, indicating that the degenerative changes initiated by diabetes were completely reversed by butanol extract administration in alloxan induced diabetic rats. Other findings on liver histopathology in normal control rats showed normal liver architecture with prominent nucleus and nucleolus but STZ induced untreated diabetic rats showed several pathological changes (Saeed *et al*., 2008). However, treatment with extract fractions of *Cephalotaxus sinensis* tolbutamide (standard drug) showed protection against STZ- induced changes in the liver (Saeed *et al*., 2008). Other similar histopathological evaluation of the liver in rats also revealed *Hibiscus sabdariffa* extract reduced the incidence of liver lesions, including hepatocyte swelling and necrosis induced by STZ (Adeyemi *et al*., 2014).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The qualitative phytochemicals analysis of *P. fulva* ethanolic bark extracts from this study showed the presence of tannins, terpenoids, saponins, flavonoids, anthraquinones and steroids with absence of alkaloids. This study also showed that administration of ethanolic extract of *P. fulva* bark to STZ diabetic induced rats reported a significant reduction in fasting blood glucose level and a slight increase in body weights.

Treatment of ethanolic extract of *P. fulva* bark in STZ induced diabetic rats reported almost normalization of lipid profile parameters that is lowering of cholesterol total, low density lipoprotein cholesterol triglycerides, and an increase in high density lipoprotein cholesterol. Additionally, Serum levels of Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were significantly lowered in extract-treated diabetic rats while Total Proteins (TP) and Albumin (ALB) were significantly elevated in the 400mg/ kg bwt /day *P. fulva* extract treated rats when compared to untreated rats.

Malondialdehyde levels in liver tissues (as an index of lipid peroxidation) was significantly elevated in diabetic rats when compared to normal control while the *P. fulva* extract treated rats had significantly lowered levels when compared to diabetic untreated group.

Furthermore, our results on liver histopathology showed normal hepatocytes in the normal control group but the diabetic untreated group showed liver pathological changes

such as bile duct proliferation and mild periportal chronic inflammation. Treatment with *P. fulva* extract at a concentration of 400mg/kg bwt/day showed the reversal of the pathological changes in the diabetic rats i.e there was prominent nucleoli with no inflammation after the extract treatment.

Overall, we conclude that the phytochemical–rich extract of *P. fulva* crude bark extract showed hypoglycemic, hypolipidemic, and hepatoprotective effects in STZ diabetic induced rats. These findings have provided important insights into the potential of *P. fulva* bark extract for alternative medicine use in the management of diabetes mellitus.

6.2 Recommendations

- Results obtained in the study showed that the ethanolic crude extract of *P. fulva* bark was effective on STZ induced diabetic rats. Therefore, *P. fulva* bark may be used as an alternative herbal remedy for the treatment of diabetes and its resultant complications.
- More studies are needed to establish a solid foundation to this finding and understand the mechanism (s) of action of the crude extract. Further fractionation should be done to identify the active component(s) responsible for the anti-diabetic, antioxidant and hepatoprotective activity of *P. fulva* bark extract.
- In addition, other studies can be done on the other plant parts of the *P. fulva* and a comparative study done on the effectiveness of the plant extract found in various climatical conditions.
- Toxicological studies should be carried out on *P. fulva* bark extract and effective dose established for administration in diabetic patients and /or for disease prevention.

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APPENDICES

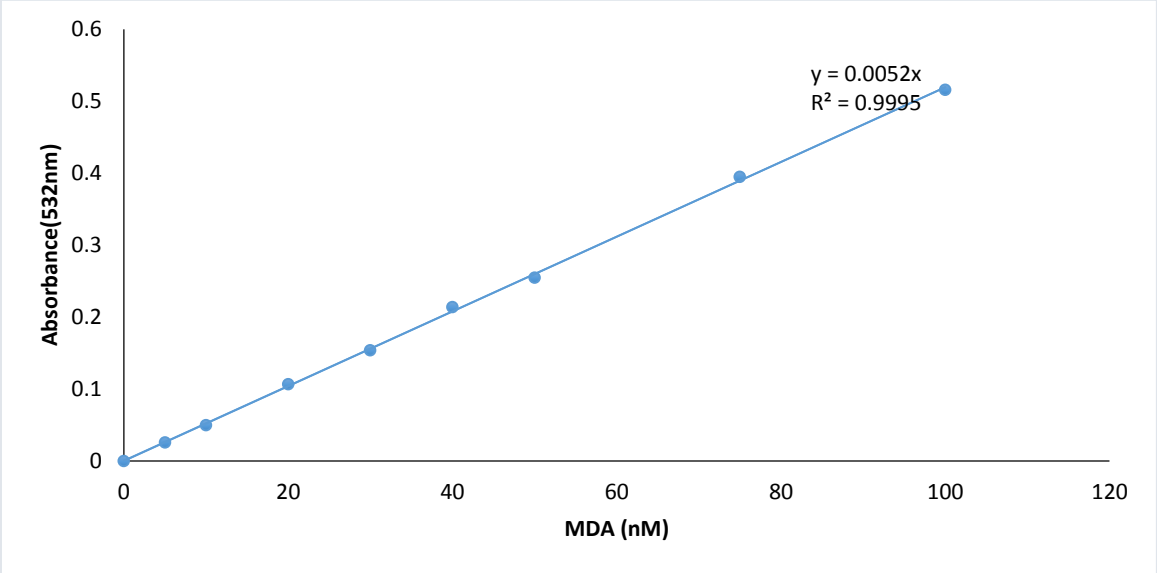
APPENDIX I: ANOVA Results on FBS

Source of Variation	F-Value	P-Value
Treatment	146.74	0.000**
Duration	171.89	0.001**
Treatment × Duration	18.93	0.000**

APPENDIX II: ANOVA Results on FBWTS

Source of Variation	F-Value	P-Value
Treatment	2.841	0.030**
Duration	4.417	0.006**
Treatment × Duration	1.499	0.142

APPENDIX III: Standard Calibration Curve for Malondialdehyde (MDA) Analysis



APPENDIX IV: Ethical Clearance Letter

OFFICE OF THE DIRECTOR OF GRADUATE STUDIES AND RESEARCH

UNIVERSITY OF EASTERN AFRICA, BARATON

P. O. Box 2500-30100, Eldoret, Kenya, East Africa

April 5, 2017

Koech Julius Kirwa
University of Eldoret
Department of Chemistry and Biochemistry

Dear Koech,

Re: ETHICS CLEARANCE FOR RESEARCH PROPOSAL (REC: UEAB/14/3/2017)

Your research proposal entitled ***"Hypoglycemic, Hypolipidemic and Hepatoprotective effects of Polyscias fulva Ethanolic Bark Extract in Diabetic Rats"*** was discussed by the Research Ethics Committee (REC) of the University and your request for ethics clearance was granted approval.

This approval is for one year effective April 5, 2017 until April 5, 2018. For any extension beyond this time period, you will need to apply to this committee one month prior to expiry date. Note that you will need a clearance from the study site before you start gathering your data.

We wish you success in your research.

Sincerely yours,

05 APR 2017

Dr. Jackie K. Obey
Chairperson, Research Ethics Committee

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