

**EFFECTS OF CO-ADMINISTRATION OF SITAGLIPTIN AND ETHANOL  
LEAF EXTRACT OF *MORINGA OLEIFERA* LAM. IN ALLOXAN-INDUCED  
DIABETES MELLITUS AND ITS CHRONIC COMPLICATIONS IN RATS**

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## DECLARATION

I declare that the work in this thesis entitled ‘Effects of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* Lam. in Alloxan-Induced Diabetes Mellitus and its Chronic Complications in Rats’ has been carried out by me in the department of Pharmacology and Therapeutics. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

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## CERTIFICATION

This thesis entitled EFFECTS OF CO-ADMINISTRATION OF SITAGLIPTIN AND ETHANOL LEAF EXTRACT OF *MORINGA OLEIFERA* LAM. IN ALLOXAN-INDUCED DIABETES MELLITUS AND ITS CHRONIC COMPLICATIONS IN RATS by Comfort Omoigemetete OLURISHE meets the regulations governing the award of the degree of Doctor of Philosophy in Pharmacology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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## **DEDICATION**

Dedicated to the Lord God Almighty, my heavenly Father, my present help in all times of need and my tower of strength throughout the period of this research. To my biological father, Chief A. A. Alufohai for his exemplary life of humility, hardwork and great spirit of contentment.

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## **ABBREVIATIONS, DEFINITIONS, GLOSSARIES AND SYMBOLS**

ADA	American Diabetes Association
AACE	American Association of Clinical Endocrinologist
ADP	Adiponectin
ALT	Alkaline Transaminase
AST	Aspartate Transaminase
CAT	Catalase
CRPs	C Reactive Proteins
BNP	B-type Natriuretic Peptide
DPP-4	Dipeptidyl Peptidase-4
DCCT	Diabetes Control and Complications Trial
DC	Diabetic Control
DCM	Diabetic Cardiomyopathy
DM	Diabetes Mellitus
EASD	European Association for the Study of Diabetes
FDA	Food Drug Administration
FBG	Fasting Blood Glucose
GLP-1	Glucagon Like Polypeptide-1
2HPP	2 Hours Post Prandial
HbA1c	Glycosylated Haemoglobin
IDF	International Diabetes Federation
MDA	Malondialdehyde
MO	<i>Moringa oleifera</i> Treated Group
NC	Normal Control
NICE	National Institute of Clinical Excellence
PDN	Peripheral Diabetic Neuropathy

PPG	Post Prandial Glycaemia
PPC	Post Prandial Control
PPSM	Post prandial Combination Treated Group
ROS	Reactive Oxygen Specie
RBG	Random Blood Glucose
SIGN	Scottish Intercollegiate Guidelines Network
SM	Combination Treated Group
ST	Sitagliptin Treated Group
T2DM	Type 2 Diabetes Mellitus
TNF $\alpha$	Tissue Necrosis Factor $\alpha$
WHO	World Health Organization

## ABSTRACT

Incidence and prevalence of type 2 diabetes mellitus (T2DM), with its resultant complications continue to be on the increase. The outcome of management is highly dependent on efficacy, safety and suitability of medicines used concomitantly. An aspect of efficacy/safety that is of growing importance is that of drug-herb interactions. The co-administration of Sitagliptin, an antidiabetic agent and *Moringa oleifera*, a herb traditionally used in the management of T2DM is a real possibility in the diabetic population. This study investigated the effects of co-administration of Sitagliptin (50 mg/kg) and ethanol leaf extract of *M. oleifera* (300 mg/kg) on the onset, progression and amelioration of diabetic complications in alloxan-induced diabetic rats. Six groups of eight rats per group were used, with groups I and II as normal (NC) and diabetic controls (DC). Groups III to VI were diabetic rats treated with Sitagliptin (III), *M. oleifera* (IV), Sitagliptin and *M. oleifera* (SM) (V), in two phases of 28 days and 42 days respectively, with 2 weeks delayed treatment in a post prandial hyperglycaemic group (VI). Antihyperglycaemic efficacy of the drug and extract using fasting blood glucose (FBG), random blood glucose (RBG) and insulin (INS) levels were determined. Inflammatory, cardioactive and oxidative stress markers in addition to lipid profiles were investigated to ascertain effects on diabetic cardiomyopathy and macroangiopathy. Morphological and functional markers of the kidneys, eyes and pain perception using (hyperalgesic and allodynia) models were also investigated for the effects of the combination on microvascular complications. There was significant decrease in FBG ( $p<0.01$ ) from day 14 (56%) to day 42 (55%) and in RBG ( $p<0.01$ ) on day 42 (27%) compared to day 1 in rats treated with only Sitagliptin. In rats treated with only *M. oleifera*, significant decrease (45%) was observed in FBG ( $p<0.01$ ) on day 21 compared to day 1, thereafter, there was an increase in FBG from day 28 (29%) up to day 42 (65%) compared to day 21. The co-administration of the drug and extract, however showed significant decrease in FBG ( $p<0.01$ ) from day 14(60%) to day 28 (38%) compared to day 1, after which there was a steady increase of up to 57% on day 42 compared to day 28. There was also a significant decrease in RBG ( $p<0.001$ ) in SM group on day 42 (24%) compared to day 1. Body weights did not differ significantly in any of the groups for the entire period of the study. No significant difference was seen in mean serum levels of INS,

adiponectin (ADP) and C reactive proteins (CRP) comparing the combination group to DC in both phases. There was a significant decrease ( $p < 0.005$ ) in serum tissue necrosis factor alpha (TNF $\alpha$ ), and non significant decrease (21%) in mean serum levels of B type natriuretic peptide (BNP) in SM group, following 28 days administration, but with 11% increase after 42 days compared to DC. A significant increase ( $p < 0.05$ ), in serum levels of catalase (CAT) on day 28, and non significant increase on day 42, in SM group compared to the individual agents. Also, a non significant decrease in serum levels of malondialdehyde (MDA) in SM group after 28 days, compared to diabetic control, with no effect seen on day 42. There was a significant increase in triglyceride (TG) levels ( $p < 0.05$ ) compared to diabetic control, with no effect on high density lipoprotein (HDL), total cholesterol (TC) and low density lipoprotein (LDL) levels except decreases in TC and LDL in post prandial treated groups. No significant histopathological findings in sections of the aorta in the SM group compared to aortic vasculitis and thickening seen in DC. Sections of heart in SM group showed congested myofibrils (though no significant difference in relative heart weight). No significant difference were observed in serum urea, albumin and liver enzyme levels in SM group compared to DC and the individual agents, but significant increase in relative kidney weight ( $p < 0.001$ ) compared to NC. There was however, no significant amelioration of kidney necrosis compared to DC. There was a significant increase ( $p < 0.05$ ) in pain threshold (thermal and mechanical) in the 5<sup>th</sup> week in SM group, compared to week 2, but with a further decrease in the 6<sup>th</sup> week. Sections of the foot pad of rat treated with SM showed moderate intraepidermal nerve fibre density. Evidence of mild lenticular opacity was observed, with no significant effect in pathologic lesions in the retina. *M. oleifera* decreased antihyperglycaemic effect of Sitagliptin with slight delay in the onset and progression, without ameliorating chronic complications in diabetic rats. However prolonged administration showed negative effects on biomarkers of diabetic cardiomyopathy and nephropathy. Caution should therefore be exercised on chronic and indiscriminate use of *M. oleifera* alongside Sitagliptin.



## **CHAPTER ONE**

### **1.0 INTRODUCTION**

#### **1.1 General Background**

Diabetes is rapidly emerging as a global health problem which threatens to reach pandemic levels by 2030 (WHO, 2008a). It is growing at an exponential rate due to dietary habits, sedentary life style and increasing obesity in Western countries. However, in developing countries it has been ascribed to changes in lifestyle and urbanisation (a scenario of increased food quantity and reduced quality) which is a shift from the relatively healthy traditional lifestyle (Godfrey and Julien, 2005; Sierra, 2009). Epidemiologists predict that 70% of Africans will live in cities by 2025, invariably increasing the number of Africans at risk for diabetes mellitus (Levitt, 2008). In the year 2014, the world prevalence of diabetes was 8.3% (382 million adults) with a possible 46% undiagnosed, and is predicted to increase by 53% (592 million adults) by 2035 (IDF, 2014). In addition, as at the same year 2014, about 21.5 million (5.1%) people in Africa were estimated to be living with diabetes and this is projected to increase to 41.5 million (i.e. an increase of 93%) by 2035 with about 80% of cases undiagnosed (IDF, 2014).

Over 7 million people, between the ages of 20 to 79 years in sub-saharan Africa as at 2003 were living with diabetes giving a prevalence of 2.4%, with Nigeria accounting for about 1.2 million (IDF, 2003). However, in 2014 the population of Nigerians with diabetes mellitus had risen to 3.7 million with prevalence of 4.6% and deaths estimated as greater than 105,090. This obvious rise in prevalence of diabetes is most noticeable in developing countries with a 93% predicted increase by 2035 as compared to 32.5% increase in Europe (IDF, 2014). This implies an increasingly large

financial toll in the future, particularly on older adults in developed countries and on working-age adults in developing countries (ADA, 2008).

The combination of the rising prevalence of diabetes and the high rate of its long-term complications in Africans, will lead to a drastic increase in the burden of diabetes on health systems of African countries. About 4.9 million people in the world died of diabetes in 2013 with one person dying every 7 seconds. The complications of diabetes, being a result of chronic hyperglycemia is associated with long-term damage and dysfunction of small and large blood vessels resulting in microvascular (specific) and macrovascular (non-specific) complications (Stratton *et al.*, 2000). The macrovascular complications are coronary artery disease, cerebrovascular diseases, peripheral arterial disease, which can lead to ulcers, gangrene/amputation and stroke. Microvascular complications however include effects on arterioles, capillaries and venules which manifests as diabetic nephropathy, neuropathy and retinopathy. These complications can be delayed or prevented with intense and continuous glycaemic control accomplished by appropriate glucose monitoring, drug therapy, and ongoing disease state management. Two landmark studies in the 1990s, the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), showed that intensive control of hyperglycemia can reduce the occurrence or progression of retinopathy, nephropathy and neuropathy in both type 1 and type 2 diabetics (Shamoon, 1993; Turner, 1998).

However, diabetes co-exists with several diseases which include hypertension, obesity, dyslipidaemia, various inflammatory disorders and other cardiovascular abnormalities. Importantly, are co-morbid diseases that are pro-atherogenic in nature



and they include obesity, hypertension and dyslipidaemia (metabolic syndrome) as they also contribute significantly to the pathology of the vascular tree arising from atherosclerosis and vascular-related inflammation. The involvement of this other co-morbid states (risk factors) makes lowering the risk for developing macrovascular complications in diabetic patients complex and involves more than lowering glucose levels. The ultimate goal is prevention, aggressive management and treatment of all risk factors present to reduce development and progression of diabetic complications.

The management of type 2 diabetes mellitus (T2DM) has evolved over the years, with scientists and clinicians aiming for more potent, effective and less toxic medicinal compounds to treat hyperglycaemia. The conventional antidiabetic agents are saddled with quite a number of untoward side effects including hypoglycaemia and gastrointestinal tract disturbances. In addition, the complex nature of the disease, coupled with its apparent co-morbidities requires the use of antidiabetic medications not likely to interact with medications used in management of these co-morbid states and its own diverse complications. The employment of a therapeutic agent likely to control blood pressure, lower cholesterol levels and correct the metabolic abnormalities associated with T2DM in addition to maintaining adequate glycaemic control will be of great benefit. However, so far, the available therapeutic agents are unable to do this efficiently while preventing the evolution and progression of T2DM complications. (Hoerger *et al.*, 2008; ACCORD, 2008).

The pathogenesis of type 2 diabetes mellitus has been known to be due to defects in insulin secretion and insulin action. As such, the conventional antidiabetic agents targeted these two defects by acting as either secretagogues or insulin sensitizers.

However, it is now known that abnormalities in other hormones also contribute to the development of hyperglycemia. One of such hormones is the incretin hormone, which led to the development of the incretin concept. This concept is an important advancement which has come to the fore in diabetes research following the observation that enteral nutrition acted as a more potent stimulus to insulin release compared to intravenous glucose challenge (Creutzfeldt, 1979; Nauck, 1986). The incretin system, which contributes significantly to the insulin response in healthy individuals, is impaired in individuals with diabetes, offering a target for the development of a new class of antidiabetic agents. These agents include the incretin-mimetics (glucagon-like polypeptide-1 [GLP-1] receptor agonists and dipeptidyl peptidase-4 [DPP-4] inhibitors) (Davidson, 2009).

Sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor that prevents the breakdown of glucagon like polypeptide-1. In October 2006, Sitagliptin became the first DPP-4 inhibitor to gain Food Drug Administration (FDA) approval for the treatment of type 2 diabetes mellitus. Many gastrointestinal hormones, neuropeptides, vasoactive peptides, cardioactive peptides, cytokines, and chemokines are substrates for DPP-4 (Mentlein, 1999; De Meester *et al.*, 2003) and the complete spectrum of the DPP-4 substrate is still not known. This suggests that the therapeutic use of DPP-4 inhibitors may be more complex than just enhancing the action of endogenous GLP-1 (Ferreire *et al.*, 2010). More so, the increased levels of inactivated GLP-1 in the system, with receptors in various major organs in the body have also been of great concern. In addition, due to the widespread expression of DPP-4 on many cell types, including lymphocytes, there is considerable interest in the long term safety profile of DPP-4 inhibitors.

Alternatively, plants are also a potential source of antidiabetic drugs and are widely used in traditional medicine to prevent and treat diabetes. Several medicinal plants have been investigated for their beneficial use in different types of diabetes and many phytoconstituents have been isolated from such plants. Currently, ethnobotanical information indicates that more than 800 plants are used as traditional medicines for the treatment of diabetes due to their effectiveness, less side effects and relatively low cost (Jung *et al.*, 2006). They can improve glucose metabolism and the overall condition of individuals with diabetes not only by hypoglycemic effects but also by improving lipid metabolism, cardiovascular and capillary functions.

*Moringa oleifera* Lam. (Moringaceae), is an angiosperm, native of the Indian sub-continent and also widely cultivated in Africa, where its various parts are used as food, water purifier and medicine. Jarald *et al.* (2008) recorded *Moringa oleifera* as one of the herbs used as antidiabetic agent in Africa and one of the constituents of antidiabetic formulations available in the market. Studies have also revealed its antidiabetic properties in streptozotocin and alloxan induced diabetes in Wistar rats (Ndong *et al.*, 2007; Jaiswal *et al.*, 2009; Tende *et al.*, 2011). The plant is also documented to have antihypertensive (Faizi *et al.*, 1994, Edwards *et al.*, 2007), diuretic, anti-inflammatory, cholesterol-lowering activities and hepatoprotective effects (Ghasi *et al.*, 2000; Sulaiman *et al.*, 2008; Mahajan and Mehta, 2009) all of which are desirous in diabetic patients. It is also being used locally as an adjunct to low-calorie and high protein diets.

## 1.2 Statement of Research Problem

According to WHO (2014), an estimated 4.9 million people had died from consequences of high blood sugar. It also stated that more than 80% of diabetes deaths occur in low- and middle-income countries. T2DM is reported to account for well over 90% of diabetes in sub-Saharan Africa (Hall *et al.*, 2011) with 50% of deaths being associated with cardiovascular complications with projections that this figure would double between 2005 and 2035 (WHO, 2014). The IDF in 2014, also recorded mortality due to diabetes mellitus in Africa as 480,000 patients with greater than 105,000 in Nigeria. Predictably, this continent will be the least able to cope economically with this disease burden.

Cardiovascular and renal complications are responsible for most cases of mortality, especially in the presence of other co-morbid states while morbidity results from microvascular complications like neuropathy and retinopathy (Kings *et al.*, 2005; WHO, 2011). Populations in Africa have a much higher incidence of these microvascular complications than macrovascular (Oguejiofor *et al.*, 2014). There is also evidence of an increased risk of coronary and peripheral artery disease by 2 to 4 fold in diabetic patients, while the risk of stroke is increased 10 fold in individuals younger than 55 years (Newman *et al.*, 1993; You *et al.*, 1997). The increased burden of cardiovascular disease in sub-Saharan Africa, is worsened when it co-exists with diabetes mellitus (Akinboboye *et al.*, 2003). About 70-90% of diabetic patients in Nigeria have mild to severe forms of neuropathy (Oguejiofor, 2009; Olurishe *et al.*, 2012) while prevalence of diabetic retinopathy in Nigeria is 15-36% (Chinenye *et al.*, 2008; Omolase, 2010; Lawan and Mohammed, 2012). Most patients with DM in Nigeria have suboptimal glycemic control, are hypertensives and have chronic

complications. Diabetes mellitus remains one of the commonest reasons for admission in Tertiary Hospitals in Nigeria with diabetic foot ulceration (DFU) being the commonest indications for admission and notoriously responsible for prolonged hospital stay, morbidity and mortality (Oguejiofor *et al.*, 2014).

Vascular pathology due to hyperglycaemia is responsible for most macrovascular complications in diabetes due to atherosclerosis as a result of lipid adhesion, thrombosis and of recent the involvement of inflammatory processes (Monero and Fuster, 2004). Atherosclerosis accounts for virtually 80% of all deaths among diabetic patients (Aronson and Rayfield, 2002) and the concept of the involvement of inflammation in atherosclerosis has spurred the discovery and adoption of inflammatory biomarkers for cardiovascular risk prediction (Packard and Libby, 2008). Hyperglycemia has been shown to also enhance oxidative stress, increase nuclear factor- $\kappa$ B, Tissue necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin 6 (IL-6), adhesion molecules; impair Nitric oxide (NO)-mediated vasodilatation; and has a procoagulant effect (Vincent *et al.*, 2004; Dandona *et al.*, 2007). Broadly speaking, the concept has arisen that an interplay between inflammatory and metabolic abnormalities leads to tissue damage in diabetes. In addition, previous studies have shown that inflammation, and more specifically inflammatory cytokines (Chemokines), are determinant in the development of microvascular diabetic complications, including neuropathy, retinopathy and nephropathy (Navarro and Mora, 2005; Mora and Navarro, 2006). Markers of well-accepted pathophysiologic pathways have been important in predicting development of diabetes and its complications as well as providing targets for therapy.

Research and clinical practice have demonstrated the benefits of Sitagliptin. Animal studies have also shown its likely benefits in lipid metabolism, inhibition of inflammatory responses of atherosclerotic plaque and beta-cell function preservation (Ferreire *et al.*, 2010; Matsubara *et al.*, 2012). These effects of Sitagliptin are largely due to enhanced effects of the increased concentration of available GLP-1 at its various receptors (Arakawa *et al.*, 2010). However, in clinical settings Sitagliptin, due to its action on various inflammatory and homeostatic chemokines, cytokines and neuropeptides have exhibited drug-disease interactions in patients with infections and various inflammatory disorders (Baraniuk and Jamieson, 2010) and rheumatoid arthritis (Yokota and Igaki, 2012). This calls for legitimate apprehension especially as inflammation plays a key role in the pathogenesis of vascular complications in diabetes.

Drug-drug interactions have also occurred with concomitant use of DPP-4 inhibitors with statins and angiotensin converting enzyme (ACE) inhibitors resulting in lowered blood pressure, increased risk of angioedema and limiting the use of ACE inhibitors in the management of nephropathy in diabetic patients on Sitagliptin (Grouzmann *et al.*, 2009; Marney *et al.*, 2010). Meta analyses, control trials and animal studies have demonstrated a tendency for increased risk of cardiac and vascular disorders from the use of DPP-4 inhibitors (Grieve *et al.*, 2009, GooBen and Graber, 2012). The renal effects of sitagliptin seen in clinical practice and diabetic rats calls for caution and closer examination in hypertensive diabetic patients due to augmentation of the renovascular effect of angiotensin II (Jackson and Mi, 2008). This is of great concern as this effect even persisted in rats with diabetic nephropathy and metabolic syndrome (Tofovic *et al.*, 2010). Other side effects commonly associated with sitagliptin include

asthenia, muscle paralysis, osteoarthritis and pain at extremities (Januvia monograph, 2007). These are obviously not comfortable developments for patients already with an increased risk of cardiovascular events, neuropathy and nephropathy.

Sitagliptin and other DPP-4 inhibitors have also been reported to cause upper respiratory tract infections and worsening of symptoms in allergic rhinitis and asthma patients (Baraniuk and Jamieson, 2010).

However, medicinal plants like *Moringa oleifera* are being used as nutraceuticals and herbs alongside with sitagliptin in the management of T2DM. As a food plant, it is considered relatively safe as it is likely to contain synergistic and/or side effect neutralizing combinations of activities (Gilani and Rahman, 2005). It is however a wrong concept to declare that medicinal plants are non toxic and without risks to human health since they are natural and have been tested worldwide through centuries. Safety issues related to herbal drugs continue to be ignored by the public, neglected by manufacturers and under-researched by professionals. An aspect of safety that is of growing importance is that of herb-drug interactions. *Moringa oleifera* like other herbs is a mixture of more than one pharmacologically active ingredient obviously increasing the likelihood of interactions taking place when used concomitantly with orthodox medicines. The likelihood of drug-herb interactions is theoretically higher than drug-drug interactions because of this fact of multiple chemical entities (Izo, 2005). Since there is an increase in consumer awareness and acceptance of *Moringa oleifera* for the management of diabetes, there is an increase in

the possibility of drug-herb interactions from the concomitant use of self-prescribed *Moringa oleifera* and prescribed sitagliptin in patients.

In the light of the aforementioned associated adverse effect of Sitagliptin, and the vascular beneficial effect of *Moringa oleifera* the possibility of an interaction between sitagliptin and *Moringa oleifera* exists when used concomitantly to either ameliorate or exacerbate complications in diabetic patients.

### **1.3 Justification**

Incidence and prevalence of T2DM with its resultant complications continue to be on the increase. The presence of metabolic syndrome coexisting with T2DM further worsens the development and progression of complications. This is because hyperglycaemia (leading to endothelial changes in blood vessels) and lipotoxicity, both hallmarks of T2DM, contribute immensely to atherosclerosis in vascular pathology.

Sitagliptin has been introduced into Nigeria as an antidiabetic agent, meanwhile meta analysis, had shown the association of cardiovascular events and asthenia with the use of DPP-4 inhibitors (Gooben and Gaber, 2012). However, the actual relationship between DPP-4 inhibition and actual cardiovascular outcomes remains unknown (Jose and Inzuchi, 2012). Research, information and awareness on the nutritional and diverse pharmacological benefits of *Moringa oleifera* is also beginning to gain much ground (Mbikay, 2012). There is scientific evidence in animals of its antihypertensive, diuretic, anti-inflammatory and cholesterol lowering activities increasing its acceptability and possible use amongst diabetic patients. *Moringa*



*oleifera* is also rich in multiple medicinally active chemicals, although it is possible that in the presence of other chemically active agents its effects could be enhanced or neutralized. Significant reductions in blood glucose levels have also been seen with the use of *Moringa oleifera* in humans, either given alone or in patients already on sulphonyl urea medications (Kumari, 2010; Ghiridhari *et al.*, 2011).

This evidence shows a possible augmentation of the pharmacological activities of orthodox antidiabetic medications by *Moringa oleifera*. Coupling this benefit, with possible neutralizing of side effects of sitagliptin will be an important advancement in medical research. The development will not only be cost effective but will go a long way in improving patient's quality of life while reducing morbidity and mortality. However, the contrary is a high possibility as has been reported with other herbs e.g. *Hypericum perforatum* (St. John's wort), *Opuntia ficus-indica* (Prickly pear cactus) and *Ginkgo biloba* when used with antidiabetic agents in various studies became detrimental with life threatening situations (Hruska *et al.*, 2005; Bush *et al.*, 2007). This is in addition to documented conflicting reports with respect to the effect of *Moringa oleifera* in hyperglycaemia induced neuropathy, nephropathy and retinopathy in various animal models (Manaheji *et al.*, 2011; Gupta *et al.*, 2013; Oyagbemi *et al.*, 2013).

Based on the aforementioned, the concomitant use of Sitagliptin and *Moringa oleifera* is a high possibility in the diabetic population. The concomitant use of Sitagliptin and *Moringa oleifera* in addition to a possible synergistic antidiabetic effect, might have potential favorable or otherwise cardiovascular/renal implications and lipid

metabolism. These effects will be of great benefit as possible risk of development and progression of complication in T2DM will likely be reduced.

The outcome of management of diabetes, other co-morbid states and its resultant complication is dependent on efficacy, safety and suitability of medicines used concomitantly. However these factors could be largely compromised due to drug-drug, drug-herb or drug-food interactions. One of the goals, of the WHO traditional medicine strategy for the next decade (2014-2035) is to promote the safe and effective use of traditional medicine by regulating, researching and integrating traditional medicine products, practitioners and practice into health systems where appropriate (WHO, 2013). As such, it is imperative that possible interaction between Sitagliptin and *Moringa oleifera* be investigated for long term effects on onset and progression of chronic complications in T2DM. Consequently the evaluation of a series of candidate biomarkers reflecting inflammation, oxidative stress, and thrombosis in addition to conventional lipids and glycaemic levels in animals will translate to potential clinical tools for predicting and improving vascular complication in patients on the drug and herb.

#### **1.4 Aim and Specific Objectives**

The aim of this research, was to investigate the effects of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on alloxan-induced diabetes mellitus and its chronic complications in rats.

The specific objectives were:

To study the effects of co-administration of Sitagliptin and *Moringa oleifera* on:

- I. Glycaemic control parameters [Fasting Blood Glucose (FBG), Post prandial Blood Glucose (PPBG), Insulin Levels], body weight and relative organ weight in alloxan-induced diabetic rats.
- II. Progression and possible amelioration of diabetic cardiomyopathy (DCM) using appropriate biomarkers [antiinflammatory and proinflammatory cytokines i.e. Tissue Necrosis Factor  $\alpha$  (TNF $\alpha$ ), C reactive proteins (CRP), Adiponectin (ADP)] and a cardioactive peptide [B type natriuretic peptide (BNP)] and heart histology in alloxan-induced diabetic rats.
- III. Biomarkers of diabetic macroangiopathy, i.e. oxidative stress markers [Catalase (CAT), Malondialdehyde (MDA)], serum lipid profiles and aorta histology in alloxan-induced diabetic rats.
- IV. Progression and possible amelioration of diabetic nephropathy using renal function biomarkers and morphology (serum electrolytes, urea, albumin and renal trophism and histology) in alloxan-induced nephropathy in diabetic rats.
- V. Onset, progression and possible amelioration of neuropathic pain (Hyperalgesia and Alodynia) and histology of skin of the hind paw in alloxan-induced neuropathy in diabetic rats.
- VI. Development and progression of retinopathy (Lens opacity and retinal cells/vessel damage) in alloxan-induced diabetic rats.

### **1.6 Null Hypothesis**

The co-administration of Sitagliptin and *Moringa oleifera* leaf extract does not delay the onset and progression of chronic complications in alloxan-induced diabetic rats.

## **CHAPTER TWO**

### **2.0 LITERATURE REVIEW**

Diabetes mellitus as a chronic medical disorder will soon surpass the ravaging of the infectious diseases like HIV/AIDS and malaria epidemic in few years to come. The prevalence in the African continent is to be doubled in the next twenty years, with greater effect being seen amongst the working class population. Africa also has the highest number of undiagnosed diabetes, with Nigeria leading the top ten African countries with this disease (IDF, 2014). The International Diabetic Federation in collaboration with the World health organization has put in place various measures to curb this global pandemic. However, mortality and morbidity from various diabetic complications and its contributions to poor prognosis of other co-morbid diseases, calls for re-evaluation of the various pharmacological and non pharmacological measures that have been put in place.

#### **2.1 Diabetes Mellitus**

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Fowler, 2008).

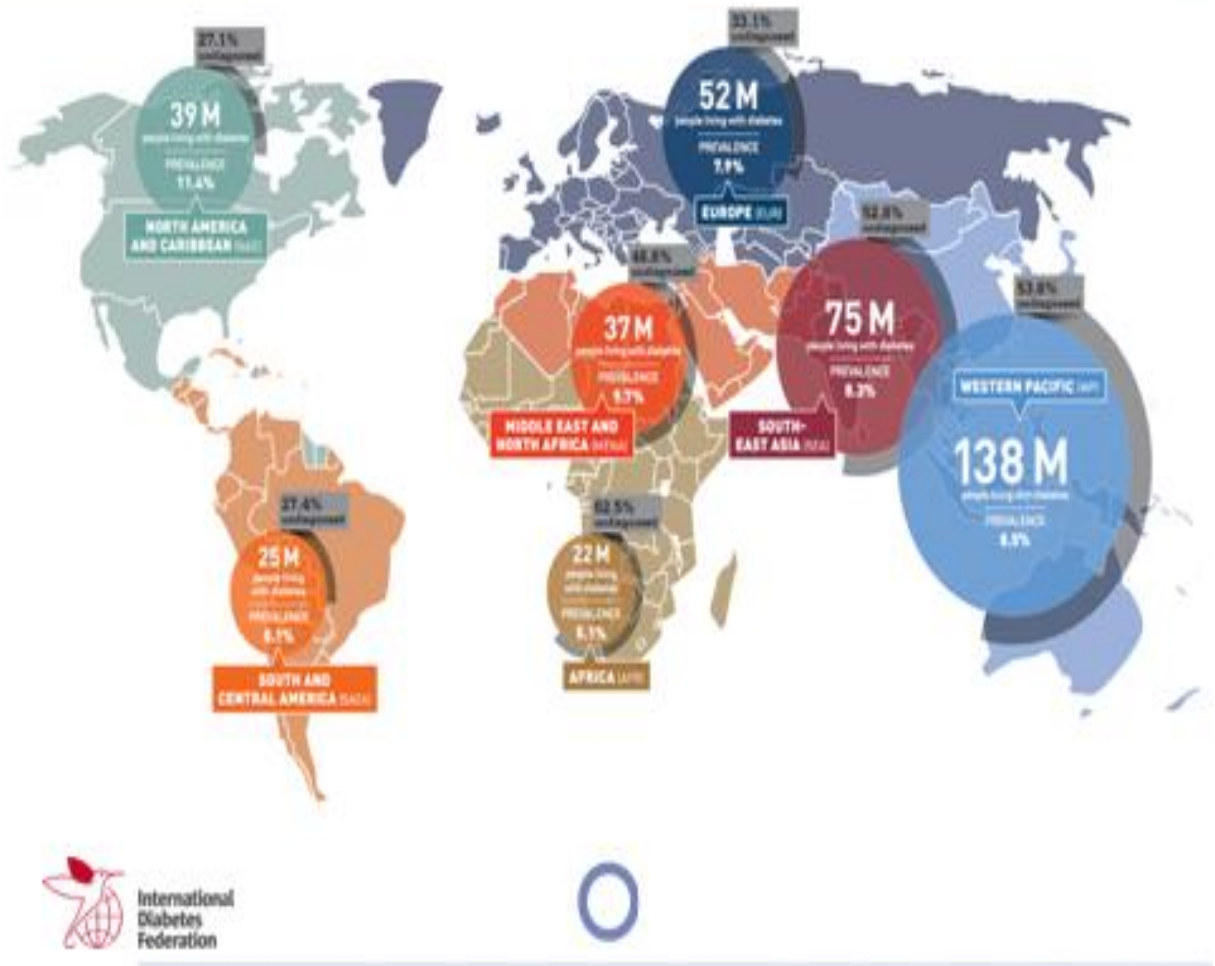
Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the  $\beta$ -cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is

deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome (Votey and Peters 2008).

### **2.1.1 Incidence and prevalence of diabetes mellitus**

Estimates from 2014 by the International Diabetes Federation suggest that the number of adults with diabetes mellitus in the world will expand by 53%, from 386.7 million in 2014 to 591.9 million in 2035 (IDF, 2014). The projected growth for Africa is 93%, from 21.5 million in 2014 to 41.5 million in 2035. This proportion is almost double the predicted global increase. The report also highlighted the paucity of data from Africa though a steady increase in number of literature over the past years. Mortality attributable to diabetes in sub-Saharan Africa is estimated, in 2014, at 8.3% of total mortality, a staggering 76.4% of those deaths occurred in people under the age of 60. In a review, (Mbanya and Sobngwi, 2003) populations of African origin had the highest prevalence of microvascular complications of diabetes mellitus. The IDF in 2014, also estimated the number of adults living with diabetes mellitus in Nigeria as 3.75 million and deaths attributable to diabetic complications as greater than 105, 000 adults.

# Global diabetes prevalence



**Plate I: Global Diabetes Prevalence, 2013**

Source: International Diabetic Federation ATLAS 2014

Map 3.1 Prevalence\* (%) estimates of diabetes (20-79 years), 2013

AFRICA REGION

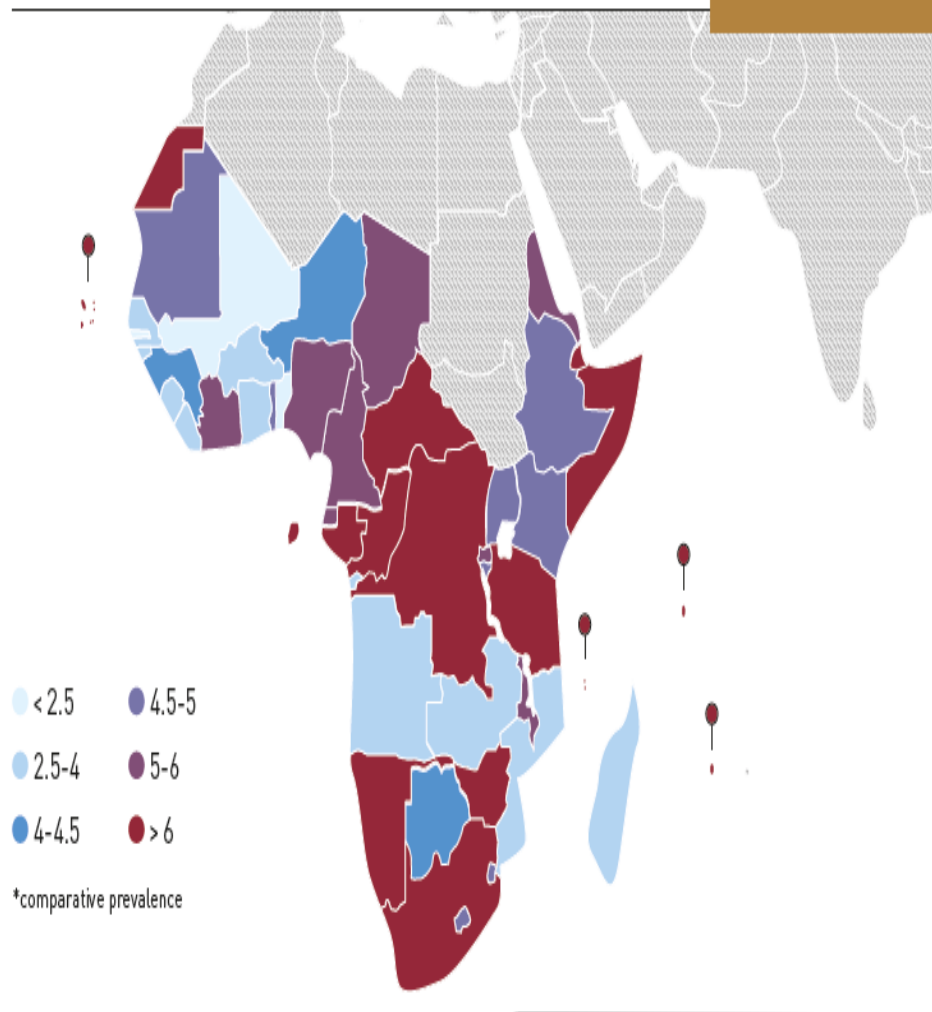


Plate II: Prevalence (%) Estimates of Diabetes (20-79 years) 2013

Source: International Diabetic Federation ATLAS 2014

### **2.1.2 Aetiology and pathogenesis of type 2 diabetes mellitus**

The aetiology of T2DM is multifactorial, with genetic background, physical inactivity and environmental factors to varying extent as critical components. The development of type 2 diabetes is clearly associated with a family history of diabetes. The significantly higher concordance rate between monozygotic twins than between dizygotic twins suggests the considerable involvement of genetic factors. The pathogenesis has been assumed to involve genetic abnormality in the molecules related to the regulatory system of glucose metabolism (Kaku, 2010). The effect of advanced age, obesity, alcohol drinking, smoking, are also independent risk factors of pathogenesis of T2DM. Obesity (particularly visceral fat obesity) due to a lack of exercise induces insulin resistance, and is closely associated with the rapid increase in the number of middle- and high-aged patients. Urbanization with respect to changes in dietary energy sources, particularly the increase in consumption of fatty meals and simple sugars and the decrease in starch and dietary fibre intake, contributes to obesity and cause deterioration of glucose tolerance (Levitt, 2008).

Any increase in blood glucose level is the net result of glucose influx exceeding glucose outflow from the plasma compartment. Insulin is also the major signal for conversion of glucose to glycogen for internal storage in liver and skeletal muscle cells. Fasting hyperglycaemia is a consequence of increased hepatic glucose production, while postprandial hyperglycaemia i.e. further glucose excursions result from the combination of insufficient insulin production and release to handle this glucose output and defective insulin stimulation of glucose uptake in target tissues, mainly skeletal muscle.



The pathophysiology of type 2 diabetes mellitus include a dysfunction of the pancreatic  $\beta$ -cells, a defect in insulin-mediated glucose uptake in muscle otherwise referred to as insulin resistance, a disruption of secretory function of adipocytes, and an impaired insulin action in liver. However current theories include that of altered glucose release and disposal, altered glucagon secretion, rapid gastric emptying, impaired satiety and impaired incretin system. Anti-hyperglycaemic agents are directed at one or more of the pathophysiological defects of type 2 diabetes, or modify physiological processes relating to appetite or to nutrient absorption or excretion.

#### 2.1.2.1 *beta-Cell dysfunction*

This is a key and basic feature of type 2 diabetes characterized by an initial impairment in the first phase of insulin secretion during glucose stimulation and this actually precedes the onset of glucose intolerance in type 2 diabetes (Ward *et al.*, 1986). In the early stage of the disease, insulin production is normal or increased, but disproportionately low for the degree of insulin sensitivity, which is already reduced. The ability of the pancreatic beta cell to release adequate hormone in this stage to compensate with rising blood glucose, is profoundly compromised and this functional islet incompetence is the main quantitative determinant of hyperglycaemia (Ferrannini *et al.*, 2005). Glucose transport in  $\beta$ -cells of type 2 diabetes patients is also greatly reduced and impairment in this first phase of insulin secretion may serve as a marker of risk for type 2 diabetes mellitus in family members of individuals with type 2 diabetes mellitus (Warram *et al.*, 1990; Vaag *et al.*, 1995). The delay in instant insulin response is followed by a secondary phase of insulin hypersecretion and release due to either an inherited or acquired defect within the  $\beta$ -cell or as a compensatory response to peripheral insulin resistance. Over a prolonged period of time, insulin

secretion gradually declines accompanied by a decline in  $\beta$ -cell mass with eventual  $\beta$ -cell failure

#### 2.1.2.2 *Insulin resistance (IR)*

IR has also been considered to play an integral role in the pathogenesis of T2DM and it refers to suppressed or delayed responses to insulin. In most patients with type 2 diabetes, especially the obese, insulin resistance in target tissues (liver, muscle, adipose tissue, myocardium) is a prominent feature. It appears to result from a complex interaction between abdominal (visceral) fat and the immune system that results in a state of chronic inflammation. Insulin resistance is generally ‘post-receptor’, which refers to a problem with the cells that respond to insulin rather than a problem with insulin production (Lin and Sun, 2010). An increase in intra-abdominal adipose tissue or fat cell (adipocytes) is associated with insulin resistance even in the absence of diabetes. In recent years, it has become clear that adipocytes are metabolically active, contains numerous macrophages and secretes a family of cytokines referred to as adipokines. One of these adipokines, is the pro-inflammatory cytokine referred to as tissue necrosis factor alpha (TNF- $\alpha$ ,) which exacerbates insulin resistance by desensitizing insulin receptors to the effect of insulin (Lin and Sun, 2010).

The presence of elevated levels of free fatty acids is a common feature in obesity, which further promotes insulin resistance (Aronne and Isoldi, 2007). Additionally, insulin resistance is found in hypertension, hyperlipidemia and ischemic heart disease, conditions commonly found in association with diabetes mellitus.

### 2.1.2.3 *Increase in hepatic gluconeogenesis*

Another important pathophysiologic pathway in type 2 diabetes mellitus is increase in hepatic gluconeogenesis and increase in hepatic insulin resistance which heralds the evolution of impaired glucose tolerance (IGT). Hepatic insulin resistance is characterized by a marked decrease in glucokinase activity and a catalytic increased conversion of substrates to glucose despite the presence of insulin. Thus, the liver in type 2 diabetes is programmed to both overproduce and underutilize glucose. Moreover, an increased delivery of fatty acids to the liver favours oxidation, which also contributes to increased gluconeogenesis, and the overabundance of lipids promoting hepatosteatosis. In addition, in type 2 diabetes, pancreatic alpha cells hypersecrete glucagon, further promoting hepatic glucose production (Nauck, 2011).

### 2.1.2.4 *Impaired incretin system*

It is now known that abnormalities in other hormones also contribute to the development of hyperglycemia. Gastrointestinal tract also secretes hormones that play an integral role in glucose homeostasis, one of such hormones is the incretin hormone, which led to the development of the incretin concept. The incretin effect, as this is termed, may be responsible for 50% to 70% of the total insulin secreted following oral glucose intake (Baggio and Drucker, 2008). The incretin system, which contributes significantly to the insulin response in healthy individuals, is impaired in individuals with diabetes. Glucose-dependent insulinotropic polypeptide (GIP) and glucagon like polypeptide - 1 (GLP-1) are the two most important incretin hormones secreted in response to food ingestion. A decreased secretion of GLP-1 after mixed meals is observed in most studies and the sensitivity of the pancreatic islets to the actions of the incretin hormones are also decreased in T2DM (Holst *et al.*, 2008).

Circulating levels of GLP-1 are low in the fasting state and rise quickly after meal, these circulating levels also decrease rapidly (half-life, less than 2 minutes) because of inactivation by the proteolytic enzyme dipeptidyl peptidase -4 (DPP-4) enzyme (Pratley and Gilbert, 2008). The discovery of the incretin system in the pathogenesis of T2DM offered the opportunity for the development of the new class of anti diabetic agents referred to as the incretin mimetics. These include the GLP-1 agonist and the DPP-4 inhibitors.

### **2.1.3. Diagnosis and classification of diabetes mellitus**

Diagnosis for diabetes according to WHO (2006), revised from the WHO (1999) “*Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia*” is based on fasting plasma glucose  $\geq 7.0$  mmol/l (126 mg/dl) or 2 hours post plasma glucose  $\geq 11.1$  mmol/l (200 mg/dl) after ingestion of 75 g oral glucose load. In addition, American Diabetes Association (ADA) and American Association of Clinical Endocrinologists (AACE) criteria provide for diagnosis of diabetes based on the presence of diabetes/hyperglycemic symptoms (polyuria, polydipsia, unexplained weight loss) and a casual prandial glucose level of  $\geq 200$  mg/dL.

In 2009, the World Health Organization further revised the criteria for diagnosing diabetes and added glycosylated haemoglobin (HbA1c) as additional diagnostic parameter while maintaining the parameters already stated in 2006. HbA1c reflects average plasma glucose over the previous 8 to 12 weeks. It can be performed at any time of the day and does not require any special preparation such as fasting. These properties have made it the preferred test for assessing glycaemic control in people with diabetes. More recently, there has been substantial interest in using it as a

diagnostic test for diabetes and as a screening test for persons at high risk of diabetes. HbA1c value greater or equal to 6.5% is diagnostic of diabetes. Nonetheless, it is important to note, that while HbA1c values have been accepted as biomarkers for glycaemic control, they represent an average measure of glycaemic exposure over time. This accounts for the reasons why individuals who have identical HbA1c values may have experienced widely varying blood glucose ranges

Fasting glucose levels are a function of endogenous, mostly hepatic, glucose production, and elevated concentrations reflect some combination of insulin deficiency, glucagon excess, and hepatic insulin resistance (Dunning and Gerich, 2007). However, prandial glycaemic levels measured after consumption of a standardized meal or a fixed quantity of liquid glucose provide information as to how the glucose homeostatic system can respond to a challenge and give insights into the adequacy of insulin secretion and the degree of insulin sensitivity. It is now evident that a large number of patients with type 2 diabetes experience significant Post prandial glycaemic (PPG) excursions, even in the context of good control assessed by HbA1c and FBG measurements (Bonora *et al.*, 2001)

During postprandial hyperglycemia, hyperglycemic spikes induce endothelial dysfunction, inflammatory reactions and oxidative stress, which may lead to progression of atherosclerosis and occurrence of cardiovascular events (Node and Inoue, 2009). There is evidence that postprandial hyperglycemia, but not fasting hyperglycemia, independently predicts the occurrence of cardiovascular event (Cavalot *et al.*, 2006). At the early stages of type 2 diabetes, even when fasting glucose and HbA1c are within normal ranges, postprandial hyperglycemia causes

macrovascular complications and increase risk of CVD (Ceriello *et al.*, 2004; Cavalot *et al.*, 2006). A multiple regression analysis also revealed that postmeal hyperglycaemia independently correlated with the incidence of diabetic retinopathy and neuropathy. Additionally, post-prandial hyperglycaemia was also associated, although not independently, with the incidence of diabetic nephropathy (Shiraiwa *et al.*, 2005). Evidence suggests that improving PPG control is useful for achieving therapeutic targets, and it has been hypothesized that minimizing wide swings in blood glucose may reduce the damaging vascular effects of hyperglycemia (Ceriello *et al.*, 2008). This evidence of hyperglycemic spikes being relevant to the pathophysiology of diabetes complications has received much attention which was sufficient to influence guidelines from key professional bodies, including the World Health Organization, the American Diabetes Association and the American College of Endocrinology (Ceriello, 2005).

#### 2.1.3.1. *Classification of diabetes mellitus*

Assigning a type of diabetes to an individual often depends on the circumstances present at the time of diagnosis, and many diabetic individuals do not easily fit into a single class (ADA, 2009). The revised classification (WHO, 2006) encompasses both clinical stages and aetiological types of Diabetes Mellitus and other categories of hyperglycaemia as suggested by Kuzuya and Matsuda (1997).

Clinical staging of diabetes mellitus: The clinical staging reflects that diabetes progresses through several clinical stages during its natural history and this is regardless of its aetiology. As such, individuals who have, or who are developing diabetes mellitus are categorized by stage according to the presenting clinical

characteristics even in the absence of information concerning the underlying aetiology. The clinical stages include:

Diabetes Mellitus, which is subdivided into (1) *Insulin requiring for survival*, representing the formal class of Insulin Dependent Diabetes Mellitus (IDDM).

(2a) *Insulin requiring for control*, i.e. endogenous insulin is still being produced but is not sufficient to achieve normoglycaemia without added exogenous insulin. (2b) *Not insulin requiring*, i.e. normoglycaemia can be achieved by non pharmacological methods, or oral antidiabetic Agents (OAA). 2a and 2b represents the formal class of non insulin dependent diabetes mellitus (NIDDM).

Impaired Glucose Regulation *i.e.* impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG). These terms refer to a metabolic stage intermediate between diabetes and normoglycaemia *i.e.* individuals whose glucose levels, although not meeting criteria for diabetes, are nevertheless too high to be considered normal. These groups of individuals are considered to be prediabetic. Impaired glucose tolerance is defined as oral glucose tolerance test (OGTT) 2-h value between 140-199 mg/dl (7.8-11.1 mmol/l). IGT is considered to be a risk category for cardiovascular disease. Impaired fasting glycaemia is defined as fasting venous plasma glucose value between 100-125 mg/dl (5.6-6.9 mmol/l). It is fasting glucose concentration lower than those required to diagnose diabetes but higher than the normal reference range. IFG is considered to be a risk category for subsequent development of diabetes mellitus. It is recommended that people with IFG should have an OGTT done to exclude diabetes.

Normoglycaemia, which refers to individuals with fasting plasma glucose values less than 6.1mmol/L

Classification based on the etiology of the disease (*ADA, 2009; WHO, 2006*): The aetiological classification is based on the fact that the processes that lead to diabetes may be identifiable at any stage of the development of the disease. Thus the presence of islet cell antibodies for example in a normoglycaemic individual makes it likely that the person has Type 1 autoimmune process.

Type 1 diabetes mellitus (T1DM) also referred to as juvenile onset or insulin dependent diabetes mellitus, accounts for only 5-10% of those with diabetes and is characterized by auto immune destruction of pancreatic beta-cell in which "insulin is required for survival" to prevent the development of ketoacidosis, coma and death. Several immunological markers, reflecting ongoing immune activity and possible beta-cell damage have been identified. These include islet cell cytoplasmic antibodies (ICA), insulin autoantibodies (IAA) and antibodies to glutamate decarboxylase (anti-GAD) (*Winter et al., 2002*). In some subjects with this clinical form of diabetes, particularly non-caucasians, no evidence of an autoimmune disorder is demonstrable and these are classified as "type 1 idiopathic (*WHO, 1999*). In this form of diabetes, the rate of  $\beta$ -cell destruction is quite variable, in infants and children it is rapid and slow in adults. At the time of diagnosis of the disease, children and adolescents in particular may present with ketoacidosis as the first manifestation. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. In some cases, particularly in adults, there may be residual  $\beta$ -cell function sufficient to prevent ketoacidosis for



many years; such individuals eventually become dependent on insulin for survival and are at risk for ketoacidosis. As the disease progresses to its latter stage, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide (ADA, 2009). Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life.

Type 2 diabetes mellitus (T2DM) also referred to as adult onset or non insulin dependent diabetes mellitus is the most common form of diabetes occurring in 90-95% of patients. This form of diabetes frequently goes undiagnosed for many years because the hyperglycemia develops gradually and at the early stage, is often not severe enough for the patient to notice any of the classic symptoms of diabetes. It is a heterogeneous group of disorders characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production. It is preceded by a period of abnormal glucose homeostasis classified as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). Resistance to the action of insulin takes the form of a decrease in the ability of the skeletal muscle both to store glucose (due to a reduction in the activities of the enzyme glycogen synthetase) and to metabolize glucose (due to a reduction in pyruvate dehydrogenase activity) (Patel *et al.*, 2008). There is also an increase in hepatic glucose output (HGO) due to inhibition of glycolysis and an increase in gluconeogenesis leading to chronic hyperglycaemia. Type 2 diabetes is becoming more prevalent and the risk of developing this form of diabetes increases with age, obesity, and lack of physical activity (Levitt, 2008). It occurs frequently in women with prior gestational diabetes mellitus (GDM), in individuals with hypertension or dyslipidemia, and its frequency varies in different racial/ethnic subgroups. It is often associated with a strong genetic predisposition.

Gestational hyperglycemia/diabetes: Gestational hyperglycaemia is carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy. It does not exclude the possibility that the glucose intolerance may antedate pregnancy but has been previously unrecognized. Women who become pregnant and who are known to have diabetes mellitus which antedates pregnancy do not have gestational diabetes but have "diabetes mellitus and pregnancy" and are treated accordingly before, during, and after the pregnancy (WHO, 1999).

Other specific types of diabetes are categorized according to specific causes. It includes genetic defects in beta-cell function and insulin action, endocrinopathies, drug or chemical induced, infections and gestational induced diabetes mellitus. These specific types are currently less common causes of diabetes mellitus and also include fibrocalculous pancreatopathy, a form of diabetes which was formerly classified as malnutrition-related diabetes mellitus.

## **2.2 Complications of Diabetes Mellitus**

The United Kingdom prospective diabetic study (UKPDS) showed that around 50% of T2DM patients already had indication of diabetes related tissue damage such as retinopathy, heart disease or microalbuminuria at the time of diagnosis (Turner *et al.*, 1998). The end organ morbidities associated with diabetes places a huge tax on healthcare resources and diminish patients' quality of life with diabetic nephropathy being the complication with greatest socioeconomic impact (Rodbard *et al.*, 2007). Both population- and hospital based studies provide evidence for an increasing burden of cardiovascular disease in sub-Saharan Africa, with diabetes mellitus as a major

contributor (Muna, 1993, Akinboboye *et al.*, 2003). Prevention and treatment of complications are considered to be most important for general care of diabetic patients. The basic causes of complications include tissue metabolism disorders caused by chronic hyperglycemia, which results in damage to many organs.

### **2.2.1 Acute and chronic complications**

Acute complications of diabetes mellitus include hypoglycaemia mainly due to effects of oral antidiabetic agents and hyperglycaemia due to improperly managed T2DM. Hyperglycemia can exacerbate a number of problems, including cardiac, neurologic, and infectious complications and generally improves with treatment of the hyperglycemia (Clement *et al.*, 2004). Acute hyperglycaemic complications also manifests as ketoacidosis in type 1 diabetes mellitus and non-ketotic hyperosmolar coma in T2DM and rarely, lactic acidosis. Infection in acute complications is a clinical condition that is not specific to but can easily become complicated in diabetic states because diabetic patients have reduced immune function (Powers, 2006). Infections associated with either increased frequency or severity among individuals with diabetes include mucormycosis, cystitis, complicated urinary tract infections (e.g. pyelonephritis and intra renal abscesses). Others include pneumonia, lower-extremity soft tissue infection, polymicrobial gangrene, emphysematous cholecystitis and malignant otitis externa (Joshi *et al.*, 1999).

Chronic complications are divided into microvascular diseases that are specific to and common in diabetes and macrovascular diseases that are not specific but frequent and thus important for a prognosis.

### 2.2.1.1. *Macrovascular complications*

Long-term exposure of macrovessels to high-concentration of glucose and low density lipoproteins leads to the development of macrovascular complications in diabetes and strongly increases the risk of cardiovascular, cerebrovascular and peripheral arterial diseases. Aggressive management of blood glucose only does not substantially improve most macrovascular complications especially cardiovascular risk factors commonly present in T2DM. Implying that management requires more than optimizing glycaemic control. Most antidiabetic medications show a neutral, and in some cases even harmful effect on some cardiovascular risk factors and these risk factors are already present at increased levels in individuals at high risk for T2DM contributing to their increased cardiovascular risk (Hu *et al.*, 2002; D'Agostino *et al.*, 2004).

Diabetic cardiomyopathy (DCM): It is defined as structural and functional changes in the myocardium, independent of hypertension, coronary artery disease (CAD) or any other known cardiac diseases, and are caused by metabolic and cellular abnormalities induced by diabetes mellitus ultimately resulting in heart failure (HF) (Fang *et al.*, 2004). The condition is associated with important clinical consequences, such as increased susceptibility to hypertension-mediated damage, an increased mortality rate after acute myocardial infarction, and progression to symptomatic heart failure (Factor *et al.*, 1980; Bell, 2003). DCM is mainly characterized by left ventricular dysfunction and cardiomyocyte hypertrophy. There are a number of mechanisms by which hyperglycemia can contribute to the development and progression of diabetic cardiomyopathy. It increases the levels of free fatty acids and growth factors and

causes abnormalities in substrate supply and utilization, calcium homeostasis, and lipid metabolism.

The diastolic dysfunction seen in diabetic cardiomyopathy is postulated to be the result of an initial myocellular hypertrophy and myocardial fibrosis. Evidence from laboratory investigation shows that compromised cardiac efficiency in diabetes is a result of increased fatty acid utilization due to insulin insensitivity, which leads to an increased in production of reactive oxygen specie (ROS) (Boudina and Abel, 2005). The increase in oxidative stress decreases nitric oxide levels thereby worsening endothelial function, and induce myocardial injury and cardiomyocytes apoptosis through stimulation of inflammatory mediators. Cardiac inflammation, characterized by increased levels of proinflammatory cytokines, plays an important role in the pathophysiology of diabetic cardiomyopathy. Studies reveal a strong correlation between biomarkers of chronic inflammation (fibrinogen and C-reactive protein [CRP]), oxidative stress and left ventricular hypertrophy/diastolic dysfunction in patients with type 2 diabetes independent of traditional cardiovascular risk factors (Palmieri *et al.*, 2003; Dokken, 2008). In addition to generalized oxidation resulting in cell dysfunction, necrosis or apoptosis, ROS also induce specific post translational modifications that alter the function of important cellular proteins and signaling pathways in the heart (Figtree *et al.*, 2012).

The disease course consists of a hidden subclinical period, during which cellular structural insults and abnormalities lead initially to diastolic dysfunction, later to systolic dysfunction, and eventually to heart failure. The important contributors to the onset and progression of diabetic cardiomyopathy include left ventricular

hypertrophy, metabolic abnormalities, extracellular matrix changes, small vessel disease, cardiac autonomic neuropathy, hyperinsulinemia, insulin resistance, oxidative stress, and apoptosis (Voulgari *et al.*, 2010; Boudina and Abel, 2005). Overexpression of human CRP in DCM exacerbates left ventricular dysfunction and remodeling possibly through enhancement of inflammatory processes, renin-angiotensin system, and oxidative stress (Mano *et al.*, 2011).

The B-type Natriuretic peptide (BNP), a peptide hormone is also upregulated in DCM. It is released from the cardiac ventricles in response to physiological (pressure and volume overload) and pathological stimuli and is among the most relevant molecular markers of left ventricular hypertrophy (Vanderheyden, 2004). Left ventricle hypertrophy is the main characteristic of DCM (Nunes *et al.*, 2013) and its role in diagnosis has been endorsed by National Institute for Clinical Excellence (NICE) and the recent Scottish Intercollegiate Guidelines Network (SIGN) for the management of heart failure. Cardiac overexpression of the proinflammatory cytokine TNF- $\alpha$  has also been associated with cardiac hypertrophy and fibrosis, as well as with left ventricular dysfunction (Sun *et al.*, 2004).

Various studies reveal the ability of DPP4 inhibitors to reduce infarct size in myocardial infarction and also increase tendency of regeneration after reperfusion in genetically induced and other forms of myocardial injury due to up regulation of BNP and Stromal derived factor (SDF) (Saxena *et al.*, 2008; Ye *et al.*, 2010). These cardioactive peptides are both substrates of DPP4 and are involve in angiogenesis and vasodilation and also increase in circulating GLP-1 hormone. However DPP-4 inhibition causes increase in heart rate due to its central syphathomimetic effect

(Gomez *et al.*, 2011) and consequently increase in blood pressure and cardiac arrhythmia. Evidence has also shown the positive effect of the metabolically inactive metabolite GLP (9-39) on cardiovascular system suggesting a kind of haemodynamism which will be obstructed with DPP4 inhibitors (Nikolaidis *et al.*, 2005).

In summary, diabetic cardiomyopathy demonstrates multiple mechanisms by which diabetes affects the cardiovascular system. Microvascular disease, including endothelial dysfunction and decreased NO bioavailability is also of importance in DCM.

#### *2.2.1.2 Microvascular complications*

Microvessels are the smallest functional unit of the cardiovascular system and consist of arterioles, capillaries, and venules. These vessels differ significantly from macrovessels with respect to their architecture and cellular components. Microvessels have specific roles of regulating blood pressure and offering nutrient delivery while larger vessels basically provide blood to organs. The microcirculation also has regulatory systems such as vasomotion, permeability, and myogenic responses that can adapt flow to local metabolic needs (Sheetz and King, 2002). The incidence of microvascular complications positively correlates with extent and duration of hyperglycaemia. Hyperglycaemia-induced microvascular complications are especially evident in insulin-insensitive cells that are unable to regulate glucose handling. They include capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons and Schwann cells in peripheral nerves. The impact of improved glucose control in preventing or limiting progression of microvascular

complications strongly implicates hyperglycaemia in these complications (DCCT, 1993). Actually the current fasting plasma glucose parameters used to diagnose diabetes is derived largely from diabetes specific microvascular complication data, especially retinopathy (WHO, 2006).

Pathological changes in the diabetic microvasculature alter organ perfusion and this is particularly evident in organs that are heavily dependent on their microvasculature supply, namely the retina, kidneys, and peripheral nervous system. The clinical problem associated with these changes (retinopathy, nephropathy, and neuropathy) is responsible for the huge burden of morbidity in T2DM. Microvascular disease also contributes to peripheral vascular disease, reduced myocardium vascularization, and poor wound healing. Alteration of microvessel function is a consequence of structural modification resulting in thickening of the capillary basement membrane, including arterioles in the glomeruli, retina, myocardium, skin, and muscle, resulting in the classic diabetic microangiopathy (Orasanu and Plutzky 2009). Inflammation, more specifically inflammatory cytokines (Chemokines), also play a role in the development of microvascular diabetic complications (Navarro and Mora, 2005; Mora and Navarro, 2006).

Diabetic nephropathy (DN): DN is a progressive development of renal insufficiency caused by angiopathy of capillaries in the kidney glomeruli in the setting of hyperglycaemia. Diabetic nephropathy occurs in approximately one-third of all people with diabetes and is the leading cause of end stage renal disease (ESRD) and renal failure in developed and developing countries (WHO, 2008a). The underlying pathological changes involve thickening of basement membrane, atrophy, mesangial



expansion and interstitial fibrosis. This initially results in glomerular hyperfiltration and subsequently progressive loss of renal function (Williams and Pickup, 2004). Persistent albuminuria, increased levels of creatinine and blood urea nitrogen (BUN) are clinically significant biomarkers. Mechanisms of albuminuria involve abnormalities of the glomerular endothelial barrier, causing excessive filtration, as well as reduction of renal tubular cell albumin degradation and reabsorption.

The cardinal lesion of diabetic nephropathy resides in renal glomeruli and is called diabetic glomerulosclerosis. Hyperglycemia is responsible for the development and progression of diabetic nephropathy through metabolic derangements, including increased oxidative stress, renal polyol formation, activation of protein kinase C (PKC) as well as such hemodynamic factors as systemic hypertension and increased intraglomerular pressure (Kikkawa *et al.*, 2003). Studies also reveal that inflammatory cytokines (TNF $\alpha$ , interleukins) exert important diversity of actions implicated in diabetic nephropathy, from development, to progression and finally to late stages of renal failure (Navarro and Mora, 2008).

Management of diabetic nephropathy employs the use of angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs). Low dose ACEi is recommended for all diabetic patients as renoprotectives irrespective of blood pressure status. However, the concurrent use of DPP-4 inhibitors and ACEi in diabetic patients has been associated with an increase risk of angioedema (Byrd *et al.*, 2008; Grouzmann *et al.*, 2009).

Diabetic peripheral neuropathy (DPN): DPN is a spectrum of various neurological disorders associated with diabetes characterised by the presence of symptoms and/or signs of peripheral nerve dysfunction in diabetes, after the exclusion of other causes. It is a precursor for foot ulcers, and other nerve problems (King *et al.*, 2005). It accounts for hospitalization more frequently than other complications of diabetes and also is the most frequent cause of non-traumatic amputation. It could be distal symmetrical polyneuropathy (DSPN) or asymmetrical (involving cranial nerves, thoracic or limb nerves, mononeuropathies i.e. involving elbow and wrist and are of acute onset). Peripheral neuropathy in diabetes may manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. Physical examination reveals sensory loss to light touch, vibration, and temperature. Diabetic amyotrophy, a manifestation of diabetic mononeuropathy is characterized by severe pain and muscle weakness and atrophy, usually in large thigh muscles and attributed to immunological changes.

DSPN accounts for about 75% of diabetic neuropathy (Bransal *et al.*, 2006) and is further classified into large fibre and small fibre neuropathy. Large fibre neuropathy is characterised by painless paresthesia with impairment of vibration, joint position, touch and pressure sensations, and loss of ankle reflex. In advanced stage, sensory ataxia may occur. Large fibre neuropathy results in slowing of nerve conduction, impairment of quality of life, and of daily activities. Small fibre neuropathy on the other hand is associated with pain, burning, and impairment of pain and temperature sensations, which are also characteristics of autonomic neuropathy (Bransal *et al.*, 2006). Sensory impairment occurs in glove and stocking distribution and motor signs are not prominent. Nerve conduction studies are usually normal but quantitative

sensory and autonomic tests are abnormal. Small fibre neuropathy results in morbidity and mortality.

Autonomic neuropathy is usually associated with DSPN; but diabetic autonomic neuropathy does not occur without sensory motor neuropathy. It is characterized by stimulus-independent persistent pain or abnormal sensory perception of pain, such as allodynia (a painful response to a normally innocuous stimulus e.,g. light touch, pressure or mild temperature changes) and hyperalgesia (exaggerated pain sensations as a result of exposure to a mildly noxious stimulus) (Ueda and Rashid, 2003). DPN pain is typically worse at night and can be described as burning, pins and needles, shooting, aching, jabbing, sharp, cramping, tingling and cold. Management of neuropathic pain involves the use of analgesics, non-steroidal anti-inflammatory drugs, antidepressants, and anticonvulsants.

Diabetic retinopathy (DR): This is hyperglycaemia-mediated damage within the retinal microvasculature. It is characterized by microaneurysms (saccular pouches due to capillary distension), presence of small haemorrhages in the middle layers of the retina and capillary basement membrane. Also present is neovascularization (formation of new blood vessels on the surface of the retina) with eventual progression to diabetic macular oedema (Vithian, 2010). Glycosylation of retinal proteins and the abnormalities in retinal microvasculature eventually leads to blindness. While hyperglycaemic increases in sorbitol formation and glycated lysine residues in lens protein is responsible for the development of cataract. Diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years (King *et al.*, 2005). Zheng *et al.* (2012) estimated that the number of

patients with vision-threatening diabetic retinopathy (VTDR) will increase from 37.3 million in 2010 to 56.3 million in 2030, if prompt action is not taken.

High levels of inflammatory cytokines are implicated in the pathogenesis of diabetic retinopathy as seen in peripheral blood and vitreous/aqueous humour of patients with proliferative diabetic retinopathy (Lee *et al.*, 2008; Rangasamy *et al.*, 2012). Methods of diagnosing DR, includes: ophthalmoscopy, fluorescence angiography and fundus photography; while definition is based on clinical and photographic grading. Management involves strict glycaemic control and adequate blood pressure regulation.

### **2.2.2 Pathophysiology of diabetic vascular complications**

Vascular complications from diabetes are pathologic responses to hyperglycemia which are manifest in the vascular cells that directly encounter elevated blood glucose levels. In the microvasculature, these cells include endothelial cells (Ecs) and vascular smooth muscle cells (VSMCs), in addition to Ecs, are contractile cells of the retina vessels (pericytes) and the glomerular vessels (podocytes). Additionally, neovascularization arising from the vasa vasorum promotes atherosclerotic plaque progression and contribute to plaque rupture (Orasanu and Plutzky, 2009). Atherosclerosis is a progressive disease characterized by the response of the vessel wall to chronic multifactorial injury, which leads ultimately to the formation of atheromatous or fibrous plaques. Endothelial dysfunction is thought to be the initial stage of atherosclerosis. In addition to endothelial dysfunction, smooth muscle cell dysfunction, metabolic abnormalities of the vessel wall including inflammation, oxidative stress and breakdown of neurohormonal balance occur in the early stage of

the atherosclerosis process. Accelerated atherosclerosis and the increased risk of thrombotic vascular events in diabetes mellitus result from chronic inflammation, dyslipidemia, endothelial dysfunction, platelet hyper-reactivity, impaired fibrinolytic balance and abnormal blood flow.

#### 2.2.2.1 *Pro and anti-inflammatory mediators*

The concept of inflammation as a pathogenic principle in atherosclerosis came to the fore following observations that despite the important role of cholesterol in atherosclerosis, many individuals who experience myocardial infarction have cholesterol concentrations at or below the National Cholesterol Education Program thresholds. Inflammation plays a key and central role in the pathophysiology of atherosclerosis, starting from initiation, through progression, and ultimately the thrombotic complications of atherosclerosis. An early feature of inflammation is the release of chemokines such as monocyte chemo-attractant protein (MCP)-1 and macrophage migration inhibition factor (MIF) from stressed tissues. Chemokines (chemotactic cytokines) are small heparin molecules that direct or influence the movement of leucocytes to the site of inflammation or injury. They consist of inflammatory chemokines or immune chemokines. Inflammatory chemokines cause chronic low-grade inflammation and immune chemokines cause activation of the innate immune system, both are closely involved in the pathogenesis of diabetes and its microvascular complications (Navarro and Mora, 2008). The presence of these chemokines have been clearly demonstrated in vascular endothelium and adipose tissue (Christiansen *et al.*, 2005). They increase expression of interstitial and vascular cellular adhesion molecules (ICAM-1, VCAM-1), and E-selectin and attract monocytes and immunocytes that gain access to the inflammatory site. In addition,

they undergo chemokine-induced proliferation and proinflammatory gene activation producing the well established inflammatory cytokines like TNF alpha, interleukins 1, 6 and 8 (IL-1, IL-6, IL-18) (Ehnes *et al.*, 2008). In addition, other relevant TNF-alpha effects have been reported, such as induction of apoptosis, necrotic cell death (Boyle *et al.*, 2003) and induction of reactive oxygen species. Summarily, the chemokines induced proliferation of macrophages which contributes to the generation of altered vasoreactivity and a procoagulant state (Goldberg, 2009). Additionally, they stimulate the production of acute-phase reactants such as C-reactive protein (CRP), an important contributor in endothelial dysfunction and atherosclerosis (Packard and Libby, 2008).

CRP is not a mere marker of inflammation, but an active pathogenic substance (Mano *et al.*, 2011). In comparison to LDL cholesterol, CRP has been found to be a stronger and reliable predictor of incident cardiovascular accident (CVA) as it adds prognostic information at all levels of calculated Framingham risk for developing CVA and at all levels of the metabolic syndrome. CRP, with the advent of high-sensitivity assays, has emerged as one of the most powerful independent predictors of cardiovascular disease (Verma *et al.*, 2004) and currently the best validated inflammatory biomarker. Adiponectin is another adipokine (a cytokine produced by the adipose tissue), possessing insulin sensitizing, antiatherogenic, and antiinflammatory properties (Okamoto *et al.*, 2006). It modulates the differentiation of preadipocytes and favors the formation of mature adipocytes. This adipokine also functions as an endocrine factor, influencing whole-body metabolism via effects on target organs. It exerts multiple biologic effects essential to cardiovascular homeostasis, including increasing insulin sensitivity, reducing visceral adipose mass, reducing plasma triglycerides, and

increasing high-density lipoprotein (HDL) cholesterol (Matsuzawa, 2006). Adiponectin alters the concentrations and activity of enzymes responsible for the catabolism of triglyceride-rich lipoproteins and high density lipoproteins, such as lipoprotein lipase and hepatic lipase. Adiponectin suppresses the attachment of monocytes to endothelial cells which is a fundamental step in experimental vascular damage as well as an early event in the atherosclerotic process. It thus influences atherosclerosis by affecting the balance of atherogenic and antiatherogenic lipoproteins in plasma. Adiponectin also directly affects the function of endothelial cells, reducing VCAM-1 expression, and macrophages, decreasing the expression of scavenger receptors and the production of tumor necrosis factor (Okamoto *et al.*, 2006). Obese adult patients with type 2 diabetes mellitus, dyslipidemia, and cardiovascular disease have reduced adiponectin concentrations. Elevated levels of CRP and fibrinogen and reduced level of adiponectin can be used for early diagnosis of T2DM and can predict the incidence of diabetic complications (Swellam *et al.*, 2009).

#### 2.2.2.2 Dyslipidaemia

Dyslipidaemia is evident as low concentration of high density lipoproteins (HDL) and high concentrations of low density lipoproteins (LDL) also contributes significantly to the development of arteriosclerosis. The HDLs are responsible for the removal of free cholesterol from the blood. Low plasma levels of HDL are associated with increased cardiovascular risk. Low levels of HDL are frequently seen in patients with insulin resistance, metabolic syndrome and overt diabetes mellitus. Badimon *et al.* (1990), elegantly demonstrated the antiatherogenic properties of HDL, reducing the number of fatty streaks and inducing disease regression. Apolipoprotein B and

modified LDL retained in the arterial intima recruits monocyte-derived macrophages, which take up lipoproteins and differentiate into foam cells. Cytokines and chemokines released from macrophage foam cells and other immune cells recruit additional immune cells further accelerating the process of atherosclerosis (Rask-Madsen and King, 2013). Lipids are targets of oxidation because of their molecular structure of abundance of reactive double bonds. Oxidized low-density lipoprotein (oxLDL) and other biologically active moieties are localized in the lipid core of the atheroma, and these modified lipids induce the expression of adhesion molecules. OxLDL levels are higher in patients with CVD and coronary artery disease (Holvoet *et al.*, 1998; Meisinger *et al.*, 2005) and increasing OxLDL levels correlate with increasing severity of disease. One of the mechanisms of protection by HDL against the atherosclerotic process is by decreasing lipoprotein oxidation and generation of OxLDL.

Two of the most well studied markers of lipid peroxidation are isoprostanes (IsoPs) and malondialdehyde (MDA). MDA is generated *in vivo* via peroxidation of polyunsaturated fatty acids. MDA interacts with proteins and is itself potentially atherogenic. Lipid peroxidation is the formation of lipid peroxides via enzymatic and/or non-enzymatic mechanisms. ROS resulting from hyperglycaemia are thought to contribute to the initiation of lipid peroxidation. Once formed, lipid peroxides undergo a series of complex reactions, ultimately binding chemically to proteins and yielding advanced lipoxidation end products (ALEs) (Esterbauer *et al.*, 1992). Effects of oxidized LDL and ALE-containing LDL are proatherogenic and include, increased smooth muscle cell proliferation, increased apoptosis in endothelial cells, induction of macrophage-derived foam cell, decreased nitric oxide bioavailability, pro-



inflammatory effects, pro-clotting effects and inhibition of antioxidant enzymes (Jenkins *et al.*, 2004)

#### 2.2.2.3 *Endothelial dysfunction*

This is believed to be an important link between the postprandial state, atherosclerosis and cardiovascular diseases. Endothelial dysfunction is a state of imbalance between relative contribution of endothelium-derived relaxing and contracting factors. It refers to a condition in which the endothelium loses its physiological properties i.e. the tendency to promote vasodilation, fibrinolysis, and anti-aggregation. Abnormalities in endothelial and vascular smooth muscle cell function, as well as an increase in tendency to cause thrombosis, contribute to atherosclerosis and its complications. Endothelial cells are the single layer of the inner surface of all blood vessels, and provide a metabolically active interface between blood and tissue that modulates blood flow, nutrient delivery, coagulation and thrombosis (Cines *et al.*, 1998). It synthesizes important bioactive substances, including nitric oxide and other reactive oxygen species, prostaglandins, endothelin, and angiotensin II, that regulate blood vessel function and structure. In diabetes mellitus, hyperglycemia induces endothelial dysfunctions and hypercoagulation which accelerates the process of atherothrombotic complications (Maiti and Agrawal, 2007). There are four hypotheses explaining the mechanisms of hyperglycemia-induced endothelial dysfunction and diabetic complications. They include (1) Increased polyol pathway. (2) Increased advanced glycation end products (AGEs) formation leading to modification of low density lipoproteins (AGE-LDL) and AGE-modified peptides which contributes to tissue injury by reattaching to susceptible target proteins both within and outside the vasculature, thereby accelerating vascular pathology in

diabetic patients (Makita *et al.*, 1996). (3) Activation of protein kinase C (PKC) isoforms which causes upregulation of VEGF through activation of NADPH and increase in reactive oxygen species (Thallas-Bonke *et al.*, 2008). (4) Increased hexosamine pathway flux (Brownlee, 2001) which is a source of oxidative stress and studies show that glucosamine, which is an intermediate metabolite in this process, also brings about oxidative stress (Kaneto *et al.*, 2007). All of these mechanisms are independently associated with overproduction of superoxide anion by the mitochondrial electron transport chain leading to the production of ROS.

Increased superoxide production is the central and major mediator of diabetes tissue damage also causing direct inactivation of two antiatherosclerotic enzymes, nitric oxide synthetase (eNOS) and prostacyclin synthase. The effect of this hyperglycemia-induced formation of reactive oxygen species (ROS) is that of endothelial dysfunction, decrease in the bioavailability of nitric oxide, increase in the synthesis of vasoconstrictor prostanoids and endothelin-1 and promoting atherosclerotic plaque formation (Ceriello and Motz, 2004). The key endothelium-derived relaxing factor is nitric oxide (NO), and it plays a pivotal role in the regulation of vascular tone and vasomotor function. Apart from its vasodilatory effect, NO also protects vessels from injury, inflammation and thrombosis. The bioavailability of NO reflects a balance between its production via (eNOS) and its degradation, particularly by oxygen-derived free radicals. Oxidized LDL also reduces intracellular concentration of NO and causes endothelium activation (Cominacini *et al.*, 2001). The metabolic derangements known to occur in diabetes, including hyperglycemia, excess free fatty acid liberation, and insulin resistance, cause

abnormalities in endothelial cell function by interfering with the synthesis or degradation of NO (Creager *et al.*, 2003).

#### 2.2.2.4 Platelet hyperactivity

In addition to potentiating platelet function, diabetes augments blood coagulability, making it more likely that atherosclerotic plaque rupture or erosion will result in thrombotic occlusion of the artery. Patients with type 2 diabetes have impaired fibrinolytic capacity because of elevated levels of plasminogen activator inhibitor type 1 (Carr, 2001). Diabetes increases the expression of tissue factor, a potent procoagulant, and plasma coagulation factors such as factor VII (Dandona *et al.*, 2007) and decreases levels of endogenous anticoagulants such as antithrombin III and protein C.

Various biomarkers have been identified and studied that are specific for the accepted and highlighted pathophysiologic pathways implicated in the vascular complications of type 2 diabetes mellitus. The evaluation of these series of candidate biomarkers reflecting inflammation, oxidative stress, endothelial activation and thrombosis in addition to conventional lipids and glycaemic levels acts as potential clinical tools for predicting the development of diabetes and its complications as well as providing therapeutic objectives.

### **2.3. Management of Type 2 Diabetes Mellitus**

Lifestyle modifications and/or pharmacotherapy remain the mainstay in management of T2DM as these delays or prevent the progression from prediabetes to overt diabetes (Nathan *et al.*, 2007). Diet and exercise (Physical activities) can improve PPG and

HbA1c levels and are recommended as first-line therapeutic approaches for patients with type 2 diabetes. While lifestyle interventions, including appropriately prescribed physical activity and medical nutrition therapy have been of great benefit in patients, most patients with type 2 diabetes will also require pharmacotherapy (Blonde, 2010).

The current management approach for T2DM continues to employ the conventional drugs that focus on  $\beta$ -cell failure and/or insulin resistance. However newer agents that target other defects (e.g. incretin deficiency/resistance) are now increasingly incorporated especially in post prandial hyperglycaemia. The effect of therapies on associated comorbidities (eg, dyslipidemia, hypertension, obesity, hypercoagulability) has become an additional therapeutic focus for a more effective control of vascular complications and improved prognosis.

### **2.3.1 Algorithm for management of type 2 diabetes mellitus**

A number of medical organizations have developed guidelines and algorithms for the treatment of patients with type 2 diabetes. Most recent recommendations are derived from evidence-based information and expert opinion. They include the 2007 American Association of Clinical Endocrinologists/American College of Endocrinology (AACE/ACE), The 2009 American Diabetes Association/ European Association for the Study of Diabetes (ADA/EASD). Guidelines for treating patients with type 2 diabetes emphasize the need for individualized treatment targets to facilitate attaining and maintaining glycemic goals while minimizing the potential for adverse effects.

According to a consensus algorithm (released by the ADA/EASD) on initiation and adjustment of therapy for T2DM, the choice of specific antihyperglycemic agents is based on several considerations: their effectiveness in lowering glucose levels, extraglycemic effects that may reduce long-term complications, safety profiles, ease of use, and expense. In regard to reducing long-term complications, the consensus statement refrains from recommending one class of glucose-lowering agents (or one combination of medications) over others, since the beneficial effects of therapy on long-term complications appear to be derived from the level of glycemic control achieved, rather than from any other attributes of a particular drug.

The goal of therapy is to maintain a HbA1c level of less than 7%. This goal was selected because of the practicality and potential for reduction in complications. The guidelines states that a HbA1c of 7% or higher should be “a call to action to initiate or change therapy with the goal of achieving a level as close to the non diabetic range as possible. The ADA in the algorithm recognized that lifestyle changes alone are often ineffective for long-term control of blood glucose because of failure to lose weight, the high rate of weight regain, and progression of the disease. Consequently, it is recommended that metformin be started at the time of diagnosis, along with lifestyle modification. If glucose control is not achieved with lifestyle modification in addition to the maximal dose of metformin that can be tolerated by the patient, a second medication should be added. Second-line medications include sulfonylureas, a thiazolidinedione, or insulin. The decision of which agent to use should depend on the degree of necessary HbA1c lowering. In patients with HbA1c levels greater than 8.5% or those who are symptomatic, insulin should be considered (Nathan *et al.*, 2009). If at the end of three months, HbA1c value is greater than 7%, a third agent which is

either a sulfonylurea or a thiazolidinedione is added and insulin therapy intensified for patients on insulin. In the use of triple combinations the essential consideration is obviously to use agents with complementary mechanisms of action. Increasing the number of drugs heightens the potential for side effects and drug–drug interactions, raises costs with a possible negative impact on patient adherence. Data from the UK Prospective Diabetes Study suggest that 53% of patients will require insulin after 6 years following diagnosis and 75% of patients will need multiple treatments after 9 years (Turner *et al.*, 1999; Wright *et al.*, 2002).

However, the development of new classes of glucose-lowering medications to supplement the traditional therapies (insulin, sulfonylureas, biguanides) has certainly broadened the number of available treatments and possible combinations. Conversely, it has also increased the uncertainty that accompanies the selection of appropriate therapeutic regimens for the diverse population of patients with diabetes (Mazzola, 2012). The development of new antidiabetic agents, such as insulin analogs and incretin-based therapies, has led to treatment strategies that enable many patients with T2DM to achieve target HbA1c levels less than or equal to 7.0% (Lebovitz, 2011). The National Institute for Health and Clinical Excellence (NICE) clinical guideline for type 2 diabetes suggests adding a DPP-4 inhibitor instead of a sulfonylurea as second line treatment to first line metformin if there is a considerable risk for hypoglycaemia or if a sulfonylurea is contraindicated or not tolerated (NICE, 2009). In addition, the AACE/ACE recently developed ‘road maps’ for managing patients with T2DM. They stated that in patients with T2DM who are naïve to therapy, DPP-4 inhibitors are among the recommended first options when the initial HbA1c is 6.0% to 7.0% and as a combination therapy component when HbA1c

reaches 7.0% to 9.0%. In patients who have already received monotherapy for 2 to 3 months and whose HbA1c is 6.5% to 8.5%, treatment options include combination therapy with a DPP-4 inhibitor and metformin or a thiazolidinedione (Jellinger *et al.*, 2007).

### **2.3.2 Pharmacotherapy of type 2 diabetes mellitus**

Pharmacological agents that have been employed include (1) The secretagogues (sulphonyl ureas and meglitinides) (2) Biguanides, (3) Thiazolidinediones, (4) Alpha glucosidase inhibitors, (5) Amylin analogues (6) Incretin mimetics.

#### *2.3.2.1 The secretagogues*

They act by increasing the secretion of insulin from the  $\beta$ -cells in the pancreas, with resultant increase in plasma insulin concentrations which suppresses hepatic glucose production and facilitates glucose uptake by the muscles (Best *et al.*, 1992). The major side effect associated with the secretagogues is the potential for hypoglycemia and weight gain, though with lower incidence in patients on meglitinides. Most patients receiving monotherapy with secretagogues eventually require a second agent from another class because the production of insulin by  $\beta$ -cells eventually declines and stimulating the  $\beta$ -cells no longer improves insulin secretion.

#### *2.3.2.2 The biguanides*

They act by suppressing basal hepatic glucose production and enhancing insulin uptake by the muscle. The fasting blood glucose level will begin to decrease within 3–5 days after initiating therapy, but the full effect takes 1–2 weeks. Biguanides also reduce plasma triglycerides and low density lipoprotein cholesterol levels. Metformin

is the only approved agent in this class and the only oral antidiabetic drug that can reduce both microvascular and macrovascular complications when used as monotherapy for the treatment of type 2 diabetes. Metformin is usually well tolerated, especially if the dose is gradually titrated to the effective dose. Metformin as monotherapy is not associated with hypoglycaemia unless in combination with the secretagogues. In the absence of contraindication, it is the drug of choice in obese T2DM patients since it is weight neutral or produces moderate weight loss. It is contraindicated in patients with clinical conditions predisposing to hypoxemia, conditions such as respiratory failure, acute myocardial infarction, acute congestive heart failure, acute or chronic metabolic acidosis with renal disease or dysfunction, that requires pharmacological treatment, and acute or chronic metabolic acidosis (Wyne *et al.*, 2003). The most important and potentially life-threatening adverse effect associated with its use is lactic acidosis.

#### 2.3.2.4 Thiazolidinediones (TZDs)

They rejuvenate  $\beta$ -cell activity and act to improve sensitivity to insulin. The fasting blood glucose levels begin to decrease within 5-7 days, but the maximum potential of a given dose is not reached for 3-4 weeks. Liver toxicity occurs in some patients, necessitating monthly monitoring of liver function. The only agent in this class of drugs presently in use (pioglitazone) has recently been associated with a possible increased risk of bladder cancer (Lewis *et al.*, 2011). Recognised side effects of TZDs include weight gain, fluid retention leading to oedema and/or heart failure in predisposed individuals and increased risk of bone fractures (Kahn *et al.*, 2006).



#### 2.3.2.5 *Alpha glucosidase inhibitors*

They inhibit the action of alpha glucosidase, an enzyme that metabolizes complex carbohydrates into simple sugars. This reduces postprandial glucose levels by delaying the digestion of carbohydrates and absorption of glucose. Although it does not reverse any pathophysiological defects, slowing the rate of absorption of glucose is thought to give the  $\beta$ -cells more time to secrete the necessary insulin. Acarbose and miglitol are the alpha-glucosidase inhibitors in use, they are not as effective as other available antidiabetic agents and expected reduction in HbA1c is approximately 0.5% to 0.8%. The major advantage of these agents is a lack of effect on weight while disadvantages include the high incidence of gastrointestinal adverse effects, especially gas and bloating. The effects can be so severe and lead to discontinuation in up to about 45% of patients. These agents are contraindicated in patients with intestinal or bowel disease, and intestinal obstruction.

#### 2.3.2.6 *Incretin mimetics*

The GLP-1 agonist and the DPP-4 Inhibitors are referred to as incretin mimetics. Metabolic control is markedly improved by administration of exogenous GLP-1, but it is almost immediately degraded by the enzyme DPP IV and therefore, has little clinical value. Activation of the GLP-1 receptor with exogenous GLP-1 receptor agonist (Exenatide from the Gila monster saliva) which is resistant to the effect of DPP4 enzyme has produced significant lowering of both fasting and postprandial glucose levels. This agent which is already licenced and is being used, is plagued by the fact that in addition to its being available only as injectable, is associated with additional side effects (Holst *et al* ., 2008). The effects of GLP-1 agonists tend to be greater, probably because they produce enhanced pharmacologic effects of GLP-1

compared to effects seen with increased physiologic levels with the DPP-4 inhibitors. Another difference is that unlike the DPP-4 inhibitors, the GLP-1 agonists also slow gastric emptying and promotes satiety (Campbell *et al.*, 2010). In addition to insulin secretion, GLP-1 also reduces glucagon concentrations, delays gastric emptying and increases CNS-mediated satiety leading to reduced food intake (Nauck *et al.*, 2002). GLP-1 may also play a role in the proliferation of beta cells and the decrease in beta-cell apoptosis by inducing the transcriptional activation of the insulin gene and insulin biosynthesis (Drucker, 2003; Farilla *et al.*, 2003). It also improves myocardial function (Girard, 2008), increases insulin sensitivity and nutrient uptake in skeletal muscle and adipose tissue, and exerts neuroprotective effects (Baggio and Drucker, 2008). These changes lead to improved glycemic control and a reduction of free fatty acids, which, in turn, may result in attenuation of both glucotoxicity and lipotoxicity in patients (Drucker, 2003).

Orally active inhibitors of DPP IV have now been developed and have been shown to enhance endogenous levels of GLP-1, resulting in improved glucose tolerance, lasting improvement of HbA1C and improved beta-cell function. In general the DPP IV inhibitors are weight neutral, and well tolerated. DPP-4 is a 766 amino acid transmembrane glycoprotein, also known as adenosine deaminase complexing protein 2 or CD26. It is expressed on the surface of several cell types, including monocytes and lymphocytes and not only acts as a proteolytic enzyme, but as a T-cell activator (Fadini and Avogaro, 2011). It is a serine aminopeptidase enzyme which inactivates GLP-1, GIP and other proteins *in vivo* via dipeptide cleavage of the N-terminal amino acid. The cleavage yields the inactive metabolite GLP (9-39).



**Plate III: Structure of GLP-1 and Site of Proteolytic Inactivation by DPP-4 Enzyme**

Source: Drucker and Nauck (2006)

Notably, DPP-4 substrates are extensive and include several proline or alanine containing peptides, such as growth factors, chemokines, neuropeptides and vasoactive peptides. Inhibition of the DPP-4 enzyme also modulates the activities of several cardioactive factors, like the B- type Natriuretic peptide (BNP), neuropeptide Y and stromal cell derived factor-1 (SDF-1) (Drucker, 2007). Due to its enzymatic effect on a wide range of substrates, DPP-4 has the potential to mediate a wide range of pleiotropic effects (both positive and negative), that is independent of GLP-1.

The DPP-4 inhibitors comprise a diverse group of compounds, which can be broadly divided into those that mimic the dipeptide structure of DPP-4 substrates and those which are non-peptidomimetic (Deacon, 2011). They are competitive reversible inhibitors, which display high affinity for DPP-4. They are orally active and well tolerated, after once- or twice-daily dosing they effectively inhibit DPP-4 and lead to a postprandial elevation of endogenous GLP-1 concentrations 2-3 times above normal physiological levels (Mest, 2006; Ahren, 2008). They also have the advantage of higher stability and bioavailability when compared with the GLP-1 receptor agonists.

In patients who do not achieve the glycaemic targets with metformin alone, DPP-4 inhibitors can lower HbA<sub>1c</sub>, in a similar way to sulfonylureas or pioglitazone (Karagiannis, *et al.*, 2012). Nevertheless there is still uncertainty about their long term safety. In recognition of growing evidence regarding the importance of PPG control, clinical practice guidelines from the ADA and AACE have been revised to address PPG control and to include recommendations for the use of the relatively new categories of incretin-based therapies (Jellinger *et al.*, 2007; Nathan *et al.*, 2009).

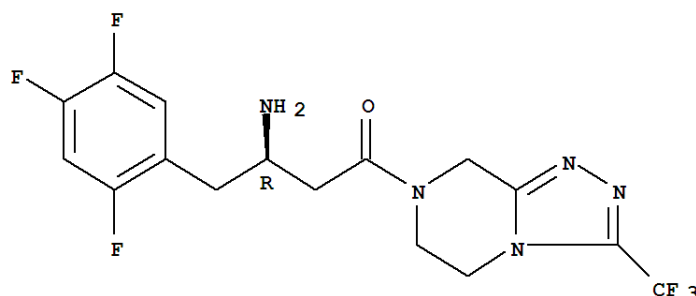
In clinical trials, the most commonly reported adverse effects associated with DPP-4 inhibitor therapy included nasopharyngitis, upper respiratory tract infection, urinary tract infection and headache. Urticarial dermatological reactions and angioedema have also been reported with pancreatitis occurring rarely (Jose and Inzucchi, 2012). A tendency for increased risk of cardiac and vascular disorders and asthenia was detected when comparing certain DPP-4 inhibitors treatments to placebo, but statistical significance was marginal (Gooben and Graber, 2012).

**Sitagliptin:** In October 2006, sitagliptin became the first DPP-IV inhibitor to gain United States Food and Drug Administration (FDA) approval for the treatment of type 2 diabetes. Sitagliptin is currently licensed by the European Union (EU) and FDA as a monotherapy or in combination with metformin, sulfonylureas or TZDs. It is also approved for use in triple combination therapy with a sulfonylurea and metformin. In clinical studies, sitagliptin improved the glycemic parameters (HbA<sub>1c</sub>, FBG, PPG) in patients with type 2 diabetes in doses of 100 mg and 200 mg given once daily in a 24-week study (Gallwitz, 2007). Doses of 50–200 mg of sitagliptin administered once daily led to a  $\geq 80\%$  inhibition of DPP-4 enzyme over 24 hours. As a result, the

concentrations of biologically active, intact GLP-1 are increased 2-3 fold in the postprandial state. Pre clinical studies show preservation of beta-cell function and beta-cell mass (Mu *et al.*, 2006). Applying this to humans show sitagliptin could also have the potential to be useful in pre-diabetic stages and early stages of type 2 diabetes in retarding or preventing the disease progression. Sitagliptin is weight neutral and does not increase the incidence of hypoglycemic episodes in patients.

Sitagliptin is rapidly absorbed (peak concentration at 1-4 hours) following oral administration and has a high oral bioavailability ( $F = 0.87$ ). Clinical trials to date have reported no correlation between changes in the pharmacokinetic parameters of sitagliptin and age, sex, race, or body mass index. The average volume of distribution ( $V_d$ ) at steady state is 1981 after a single dose of sitagliptin. Sitagliptin is moderately bound to plasma proteins (bound fraction = 38%) (Januvia monograph, 2007). Sitagliptin undergoes limited metabolism to produce six metabolites in trace amounts each accounting for <1% to 7% of sitagliptin-related material in plasma and the primary enzyme responsible is CYP3A4 with a lesser contribution from CYP2C8. Three of these metabolites (M1, M2 and M5) are active, but do not contribute to the pharmacodynamic profile of sitagliptin because of their very low concentration in plasma and low affinity for DPP-4 (Vincent *et al.*, 2007). The primary route of elimination and excretion of sitagliptin is via the kidneys (75% of an oral dose is found in the urine as unchanged drug); the elimination half-time is 12-14 h (Herman *et al.*, 2006). As such, dosage adjustments are required for patients with moderate to severe renal impairment. Sitagliptin is available commercially in 25 mg, 50 mg, and 100 mg tablets. A dose of 50 mg/day is recommended in patients with a creatinine clearance ( $CrCl$ )  $\geq 30$  to  $< 50$  ml/min and 25 mg/day in patients with a  $CrCl$

less than 30 ml/min or in patients with end-stage renal disease requiring dialysis (White, 2008). Sitagliptin is contraindicated in patients with type 1 diabetes and is not intended for use in the treatment of diabetic ketoacidosis.



**Figure 2.1: Chemical Structure of Sitagliptin**

Source: Deacon, 2011

Chemical name : (2R)-4-Oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine

Other DPP-4 inhibitors that have been approved for treatment of patients with T2DM either as monotherapy or in combination with metformin, sulfonylurea, or a thiazolidinedione include Saxagliptin and linagliptin (5-10 mg daily), Vildagliptin (50-100 mg daily) (Davidson *et al.*, 2008). Studies reveal that Saxagliptin and Vildagliptin also lowered glycaemic parameters (HBA1c, FBG and PPG) significantly in diabetic patients (Rosenstock *et al.*, 2009; Kikuchi *et al.*, 2009).

### 2.3.3 Herbal remedies

The World Health Organization (WHO, 1996) defined herbal medicine as finished labeled medicinal products that contain as active ingredients aerial or underground parts of plants or other plant materials or combinations thereof whether in the crude

state or as plant preparations. The WHO also estimates that about 80% of the populations in African and Asian countries rely on traditional medicine for their primary healthcare, recognizing it as ‘an accessible, affordable and culturally acceptable form of healthcare trusted by large numbers of people’ (WHO, 2008b). Herbs, which are a form of traditional medicine, may contain other constituents in addition to the active ingredients. Herbal medicines remain part of the history of the people despite the fact that orthodox medicines which came with civilization, is now the main stay in the treatment of disease states especially in modern medical practice. The development of herbal medicine and its incorporation into clinical practice, have been associated with a number of issues these includes the presence of a number of active ingredients with different pharmacological profiles, lack of quality control, lack of government regulations regarding safety and efficacy, insufficient clinical trials, and inadequate information on the adverse effects and drug-herbal interactions (Philp, 2004).

Herbal and orthodox medicines are sometimes used interchangeably and concomitantly by patients. There is an increase in reliance on the use of medicinal plants in the industrialized societies, which has been traced to the extraction and development of several drugs and chemotherapeutic agents from plants as well as from traditionally used herbal remedies (Jarald *et al.*, 2008). However, the use of herbal remedies for management of health is more prominent in the developing countries. In the African setting, herbs are generally employed to remedy disrupted physiological processes in order to restore homoeostasis rather than for appropriate treatment of the disease process (Osemene *et al.*, 2011). Various studies have shown

a high prevalence of herb use in Nigeria, with Onyiapat *et al.* (2011) recording an 84.7%, prevalence in patients and 66.4% by Oreagba *et al.* (2011) in the southwest.

Plants are a potential source of antidiabetic agents that are widely used in traditional medicine (TM) to prevent and treat diabetes. Ethnobotanical information indicates that more than 800 plants are used in TM for treatment of DM (Grover *et al.*, 2002; Jung *et al.*, 2006). This is due to evidence of their ability to improve glucose and lipid metabolism and enhance cardiovascular and capillary function. The incidence of herb use in diabetic patients is about 1.6 times more than in persons without diabetes (Egede *et al.*, 2002). Yeh *et al.* (2002) in another study, also reported that 35% of respondents with diabetes used herbs to treat their condition.

#### 2.3.3.1 Herbal remedies evaluated/validated for diabetes mellitus

A number of herbs have shown antidiabetic activities when evaluated using available experimental techniques and some of these herbs, include, *Brassica juncea* (Cruciferae) seed extract which showed antihyperglycaemic activities at doses of 250, 300 and 400 mg/kg (Thirumalai *et al.*, 2011). The methanolic leaf extract of *Catharanthus roseus* (Apocynaceae) reduced blood glucose more significantly than glibenclamide and metformin (Ohadoma and Michael, 2011). The aqueous leaf extract of *Cassia auriculata* (Caesalpiniaceae) also showed antihyperglycaemic effect in diabetic rats (Gupta *et al.*, 2011).

A number of edible vegetables and fruits in have also shown antihyperglycaemic activities in Wistar rats. Two common examples include, aqueous leaf extract of *Vernonia amygdalina* (bitter leaf), with significant reduction in blood glucose in



alloxan induced diabetic rats (Akah *et al.*, 2004). The rhizome of Ginger (*Zingiber officinale*) also showed antihyperglycaemic effects in rats (Jafri *et al.*, 2011). Another scientifically validated antidiabetic dietary plant is *Moringa oleifera*.

#### 2.3.3.2 *Moringa oleifera*

*Moringa oleifera* Lam. (*M. oleifera*) also known as *Moringa pterygosperma* Gaertn is an angiosperm plant and member of the Moringaceae family. It is a native of the Indian subcontinent, where its various parts have been utilized throughout history as food and medicine. It is now cultivated in all tropical and sub-tropical regions of the world with evidence of nutritional, prophylactic, and therapeutic effects. Dietary consumption of various part of the plant is been promoted as a strategy of personal health preservation and self-medication in various diseases. *Moringa oleifera* is an edible plant and a wide variety of nutritional and medicinal virtues have been attributed to its roots, bark, leaves, flowers, fruits, and seeds (Anwar *et al.*, 2007; Kumar *et al.*, 2010). It is recommended as a remedy for malnutrition and a vast range of ailments and is variably referred to as Miracle Tree, Tree of Life, Mother's Best Friend, God's Gift to Man and Saviour of the Poor.

*Moringa oleifera* is well distributed across ecological zones in Nigeria. It is called by the Hausa tribe in Nigeria as *Zogale* and *Bagagruwa maka*, while the Fulanis refer to it as *Gawara*, *Rini maka* and *Kanimarate*. The Yorubas refer to it as *Ewe Igbale*, *Ewe ile* and *Adagba maloye*, while the Igbos call it *Ogwe oyibo* and *Odudu oyibo* and Nupes, *Chigban Wawa*. In addition to being well adapted to the varied climatic condition in the nation, it has found wide acceptance, recognition and usefulness among various ethnicities (Popoola and Obembe, 2013). The seeds and leaves are

mostly utilized locally as food, medicine and water purifier, while the stem is mainly used as fodder, fence and firewood. It has been used for both prophylactic and therapeutic treatments in Nigeria. The plant has also been listed as an identified and selected herb with ethnomedicinal information in two agricultural research institutes in Nigeria and ranked as 8 out of 34 most used herbs in the treatment of diabetes in North West, Nigeria (Etuk and Mohammed, 2009). Ethnomedicinal uses documented across five agro-ecological zones in Nigeria include, fever, eye and ear infections, hypertension, diabetes, human immune deficiency virus infection, respiratory tract infections, male impotence, skin diseases/ infection and as an immune booster (Stevens *et al.*, 2013)

*Moringa oleifera* is also described as the most nutrient rich plant on this planet and the leaves can be eaten either fresh or cooked (Mehta and Agrawal, 2008). It is a potent natural source of protein and among the most widely cultivated species of the monogeneric family. *Moringa oleifera* is used as an antidiabetic agent in Africa and one of the constituents of antidiabetic formulations available in the market (Jarald *et al.*, 2008). Animal studies using streptozotocin and alloxan induced diabetes in Wistar rats and rabbits, reveals its antidiabetic properties (Ndong *et al.*, 2007; Jaiswal *et al.*, 2009; Manohar *et al.*, 2012). Various animal and human studies also show antihypertensive and antilipidaemic potentials and properties (Faizi *et al.*, 1994, Ghasi *et al.*, 2000; Edwards *et al.*, 2007).

Phytochemical analyses have shown that its leaves are particularly rich in potassium, calcium, phosphorous, iron, vitamins A and D, essential amino acids, as well as such known antioxidants such as  $\beta$ -carotene, vitamin C, and flavonoids (Anwar *et al.*,

2007; Amaglo *et al.*, 2010). Other phytochemicals isolated from *Moringa oleifera* include, zeatin, quercetin,  $\beta$ -sitosterol, caffeoylquinic acid phenolics and kaempferol. These phytochemicals are responsible for the various pharmacological activities of the herb. However, three structural classes of phytochemicals are of major medicinal interest and they are the glucosinolates, flavonoids, and phenolic acids (Amaglo *et al.*, 2010; Kasolo *et al.*, 2010; Coppin *et al.*, 2013). Enzymatic hydrolysis of the glucosinolate is responsible for its antihypertensive properties (Faizi *et al.*, 1994), while other flavanoids/phenolic compounds are responsible for its antilipidaemic, antioxidant and antiinflammatory effects (Bour *et al.*, 2005; Zhang *et al.*, 2011). Antihyperglycaemic activity of *Moringa oleifera* has been attributed to moriginine an alkaloid and quercetin a flavanoid (Bour *et al.*, 2005; Rivera *et al.*, 2012).

Research has shown that quercetin lowers fasting and postprandial blood glucose levels in diabetic animals without any alteration in serum insulin level and like acarbose, it also inhibits the activity of  $\alpha$  glucosidase enzyme. *In vitro* activity demonstrates that quercetin also suppresses DPP-4 activity, implying a probability to simulate an increase in GLP-1 levels *in vivo* (Chang *et al.*, 2013).

□ sitosterol is the active principle responsible for its antilipidaemic effect as it helps in reducing cholesterol by inhibiting intestinal cholesterol absorption. (Frawley, 2009). It has structural similarity with cholesterol and is preferentially absorbed taking the place of dietary and biliary cholesterol in micelles produced from the intestinal lumen. Chlorogenic acid and moriginine also reduce serum total cholesterol (Cho *et al.*, 2010).

The content in *Moringa oleifera* leaves varies to some extent with the geographic and climatic conditions under which the plant is grown, as well as with the processing methods for the collected leaves. Studies in two different locations in Abuja reveals variation in macro and micro elemental composition in leaves of the plant (Anjorin *et al.*, 2010) and from Ghana, higher nutrient values were seen in *Moringa oleifera* leaf samples from the semi-deciduous forest zone compared to that from the Guinea savanna (Asante *et al.*, 2014). Worthy of note is the fact that, in spite of the widely held “belief” in the health benefits of *Moringa oleifera*, the international biomedical community has been rather indifferent in the medicinal potential of this plant (Mbikay, 2012).



**Plate IV: *Moringa oleifera* Tree in its Natural Habitat**



**Plate V: *Moringa oleifera* Leaves**

#### 2.3.4 Drug-herb interactions

Drug-herb interactions are said to occur when the pharmacokinetics and/or the pharmacodynamics of a drug are altered due to the presence of a herb. Herbal medicines have been associated with a variety of adverse herb-drug interactions and these interactions are theoretically more prevalent than drug-drug interactions, because synthetic drugs usually contain single chemical entities (Izo, 2005) and the concentrations of active ingredients vary widely from one formulation to another. Interactions also arise from inhibition or induction of the cytochrome P450 enzymes e.g. St John's wort has been found to induce the cytochrome P450 3A4 enzyme that may result in the increased clearance (and decreased effect) of a wide variety of medications (Markowitz *et al.*, 2003). Herbal medications are also involved in slowing elimination and excretion of orthodox medications e.g. the use of *Azadirachta indica* which increases the half life of Chloroquin phosphate tablets and increases the potential for toxicity (Nwafor *et al.*, 2003). These interactions have led to compromised therapeutic efficacies of orthodox medications and sometimes life threatening events.

Drug-herb interactions involving oral antidiabetic agents have been seen in numerous animals and human studies and also in clinical settings. Interactions have led to compromised glycaemic control as seen with use of *Ginkgo biloba*, where patients experienced significant worsening of glucose tolerance and increase in glucose level. The herb increased hepatic clearance of insulin and oral antidiabetic drugs leading to compromised glycaemic control and possibly to the development of macrovascular and microvascular complications. Synergistic and antagonisms interactions have also been seen in OAA-Herb interactions e.g. Ginger (*Zingiber officinale*) when co-

administered with glibenclamide in animals brought about a 25% reduction in random blood glucose than the administration of glibenclamide alone (7.9%) (Al-Omaria *et al.*, 2012). In addition drug toxicities leading to hypoglycaemic emergencies have also been seen e.g. is the use of prickly pear cactus (*Opuntia ficus-indica*) with oral antidiabetic drugs (Bush *et al.*, 2007). In other words, diabetic patients using both hypoglycemic medications and herbs that may improve glucose metabolism should be carefully monitored for hypoglycemia and may need to lower their dose of hypoglycemic medications (Bush *et al.*, 2007).

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Collection, identification and extraction of plant material

The branch of *Moringa oleifera* with the leaves and flowers were collected from Graceland, Zaria, Nigeria in January 2013. The plant was identified and authenticated by a taxonomist (Mr U. S. Gallah) in Department of Biological Sciences, Ahmadu Bello University Zaria and given a voucher specimen number (571). The leaves were then harvested, washed with distilled water, dried under shade until constant weight was obtained and then pulverized with mortar and pestle. Dried and pulverized leaves were weighed (500 g) and macerated in a percolator with 2 litres of 50% ethanol for 72 hours under room temperature. Thereafter, the extract was obtained on filtration using a filter paper. The resulting extract was dried using a rotavapour at 50-60°C to obtain a brownish greasy residue. The yield was determined using the formular shown below (25.6%) and the extract refrigerated at 2-8 °C.

$$\% \text{Yeild} = \frac{\text{Weight of extract}}{\text{Weight of pulverised leaves}} \times 100$$

##### 3.1.2 Drugs, chemicals, reagents, equipments and sundry consumables

- *Drug*: Sitagliptin was obtained from Merck Pharmaceuticals in Switzerland, Batch no: A000512; Expiry Date: 02/2016.
- *Chemicals*: Alloxan monohydrate was obtained from Sigma Aldrich St. Louis, U.S.A. Ethanol manufactured by Guanding Guanghua Chemical Factory Ltd. Shanhou Guanding China, (Lot: 20120519) used for extraction.



Methylated spirit from Mopson Pharmaceuticals and Picric acid from Hopkin and Williams, Essex, England.

Formaldehyde and chloroform from British Drug House, Poole, England.

- *Reagents:* Rat specific ELISA Kits used for determination of pro and anti inflammatory cytokines, insulin, B type Natriuretic peptide and oxidative stress markers and were obtained from WKEA Med Supplies Corp, Changohin, China. Randox kits used for determination of lipid profiles, serum albumin and liver function biomarkers, all obtained from SEPPIM S.A.S, in France.

- *Equipments:* Hettich Centrifuge, model (Universal 280).

Techne Dry Block Incubator, model (DB – 3A), United Kingdom.

Electrical Thermostatic Water Bath, model (DK – 600) by Search Instrument.

Audicom Ion Selective Electrolyte Analyser, Model number (AC 99 – 100)

Rayto Autoanalyser, model (Chemray 120), United States of America.

MRC spectrophotometer, model (V-IID), United States of America.

Biorad microplate reader, model (B 900), United States of America.

Rotavapour, United States of America.

- *Consumables:* Animal cages and animal feeds (Vital Feeds, Jos-Nigeria)

Test tubes, conical flasks, measuring cylinders and plain sample bottles

Digital balance, stop clock, scissors, cotton wool, gloves and antiseptics.

Normal saline (0.9% sodium chloride solution), 5% Dextrose and distilled water from Juhel Nigeria Limited.

Disposable syringes and needles (1ml, 2 ml, 5 ml and 10 ml), oral gavage needle (16G) and dissecting Kit.

Digital glucometer/glucose strips (Accucheck Active from Roche Ltd, India.)

### **3.1.3 Experimental animals and animal care**

Animals used for the study were obtained from the animal house facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The animal species selected and used for all the experiments were albino Wistar rats (*Rattus norvegicus*) of same age and both sexes (150-200 g). The rats were housed in aluminium cages and were fed with standard feed from Vital Feeds, Jos-Nigeria and water from municipal supply, both allowed *ad libitum* except otherwise required by specific experimental protocols such as fasting blood glucose determination. Wood shavings were used as beddings for the rats and this was changed frequently to prevent the rats from contacting any form of infection due to heavily soiled beddings containing ammonia and other waste faecal matter. Animal handling and care was according to recommended guidelines (CPCSEA, 1986).

### **3.1.4 Identification of animals**

Rats for the experiments were identified by a simple coded identification which was applied using picric acid solution with the aid of cotton buds. The coding method allows for animals to be numbered literally from 1-999 (see Appendix I). Records of experiment were taken using the code of each rat as its representation in its group. Where fading of the code occurred gradually with time the number codes were reapplied to prevent mis-identification (Ritschel, 1974).

## **3.2 Methods**

### **3.2.1 Induction of experimental diabetes**

Wistar rats of both sexes were weighed and fasted overnight but allowed free access to water, *ad libitum*. Their fasting blood glucose levels were determined on day 0

using the glucose oxidase method with the aid of a digital glucometer. This consisted of taking blood sample from the terminal (3 mm) of the tail of each rat (Tail tipping). Alloxan (freshly prepared in 0.9% normal saline solution) at a dose of 150 mg/kg was administered intraperitoneally to the rats with the aid of a 1 ml needle and syringe (Reddy *et al.*, 2006; Bhatti *et al.*, 2011). The rats were further denied food for a period of about 30 minutes after alloxan administration and 5% dextrose solution was offered to the rats via drinking bottles to prevent transient hypoglycaemia for the first 24 hours. After a period of one week, the blood glucose level in the rats was determined following an overnight fast to ascertain sustained hyperglycaemia. Rats with blood glucose levels greater than 150 mg/dl were considered diabetic and grouped for the experiment (Stanley and Venugopal, 2001).

### **3.2.2 Preparation of drug and extract**

Sitagliptin and 50% ethanol leaf extract of *Moringa oleifera* were prepared by dissolving in distilled water. Procedure for preparation and calculation are as shown in Appendix II.

### **3.2.3 Experimental design and animal groupings for pilot studies**

#### *3.2.3.1 Acute toxicity study of 50% ethanol leaf extract of Moringa oleifera (OECD, Limit test)*

Seven non-pregnant, nulliparous female rats (5 rats as test group and 2 as control) were used to determine the LD<sub>50</sub> of the 50% ethanol leaf extract using the OECD limit test according to the protocol described in OECD/OCDE 420 (OECD, 2001). The rats were weighed and fasted overnight prior to dosing and a sighting starting oral dose of 2000 mg/kg of *Moringa oleifera* leaf extract (Das and Kanodia, 2012) was

administered to the first rat and four others at the stated dose with food still withheld for another 3.5 hours. The animals were then observed at intervals of 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours and thereafter daily for 2 weeks respectively for changes in skin, fur, eyes, behavioural pattern and somatomotor activity. Time of onset of toxic reactions, length of recovery period and mortality were recorded from which the oral LD<sub>50</sub> was estimated. At the end of two weeks, the rats were weighed and euthanized. The heart, lungs, liver, kidney, spleen and pancreas were harvested, weighed and fixed in 10% buffered neutral formalin before processing for histopathology according to the protocol described by Tulpule and Ghaji (1987).

#### 3.2.3.2 *Efficacy and dose selection study for Moringa oleifera*

To determine the efficacy of *Moringa oleifera* leaf extract and thus a selection of appropriate dose for the study, two doses were chosen. The selection was based on optimal glycaemic control associated with these doses of *Moringa oleifera* in Wistar rats from previous studies in Zaria, Nigeria and globally (Jaiswal *et al.*, 2009, Tende *et al.*, 2011). Twelve rats were grouped into two groups of six rats as shown below:

Group I: 200 mg/kg of *M. oleifera* leaf extract

Group II: 300 mg/kg of *M. oleifera* leaf extract.

Diabetes was induced in the animals as described in the experimental protocol above. Rats in group I were treated with 200 mg/kg of leaf extract of *Moringa oleifera* orally, while rats in group II 300 mg/kg of leaf extract of *Moringa oleifera*. Blood glucose was determined at 2, 4, 8 hours and then weekly for 3 weeks as previously described. The values were recorded and the more effective dose with respect to better glycaemic control was used for the study.

### 3.2.3.3. Dose Determination for Sitagliptin monohydrate

The oral glucose tolerance test was used to determine the dose of Sitagliptin that was used in the study. Fifteen Wistar rats were grouped into three of five rats per group as shown below:

Group I: Normal control

Group II: 50 mg/kg Sitagliptin

Group III: 100 mg/kg Sitagliptin

The rats were weighed and fasted overnight but were however allowed free access to water *ad libitum*, and fasting blood glucose determined. Oral glucose tolerance test was carried out by administering 2 g/kg of glucose (freshly prepared in distilled water) orally. The blood glucose was then determined at time 0, 30, 60, and 120 minutes according to the method previously described (Pari and Saravanan, 2008). The selected doses were from previous studies (Agravat *et al.*, 2013). The Sitagliptin dose with better glycaemic control was determined and used for the study.

### 3.2.4 Experimental design and animal groupings for main study

The study was carried out in two phases, as such the protocol consisted of two different sets of animal groupings. Rats of both sexes were used. The drug and the extract were administered orally by gavage (16G Needle).

#### 3.2.4.1 Phase I Grouping

The first phase consisted of six groups of eight rats per group as shown below.

Group	Treatment
I	Non-diabetic rats on distilled water

II	Diabetic control on distilled water
III	Sitagliptin-treated diabetic rats (50mg/kg)
IV	<i>M. oleifera</i> -treated diabetic rats (300mg/kg)
V	Sitagliptin & <i>M. oleifera</i> -treated diabetic rats
VI	Ameliorative (Sitagliptin & <i>M. oleifera</i> -treated diabetic rats)

The first phase of the study was to investigate the effect of sitagliptin and the extract on the onset of chronic complications. Duration of treatment was 28 days. Wistar rats of both sexes in groups I and II represented the non diabetic control and diabetic control on distilled water respectively. Rats in group III and group IV were diabetic animals that received Sitagliptin and 50% ethanol leaf extract of *Moringa oleifera* as single agents respectively. Group V and VI rats represented diabetic rats that received a combination of Sitagliptin and the extract, however rats in group VI received the drug and extract combination 2 weeks after administration commenced in groups III, IV and V. This was for the purpose of determining the possibility of the drug-herb combination in amelioration of chronic diabetic complications that had set in during the first two weeks before drug treatment.

#### 3.2.4.2 Phase 2 grouping

The grouping for the second phase of the study is as shown below:

Group	Treatment
I	Non-diabetic animals on distilled water
II	Diabetic control on distilled water
III	Sitagliptin-treated animals
IV	<i>M. oleifera</i> -treated animals

- V Sitagliptin + *M. oleifera*-treated animals
- VI Ameliorative (Sitagliptin + *M. oleifera* treated animals)
- VII Postprandial Control

The second phase of the study was for determining the effect of Sitagliptin and the leaf extract of *Moringa oleifera* on possible delay in the progression of chronic complication of diabetes following further drug administration for another 2 weeks. Duration of treatment was for 42 days. Wistar rats of both sexes were grouped into seven groups of eight rats each. Wistar rats in groups I-VI represented the drug treated groups as described in phase 1 grouping above. However, in phase 2, a 7<sup>th</sup> group was added, and this, represented a post prandial control group, i.e. rats in group VI and VII had only high random blood glucose levels and not fasting hyperglycaemia at the end of seven days following alloxan administration. Rats in group VII acted as positive control for rats in group VI for the purpose of investigating the possible effect of the combination of Sitagliptin and 50% ethanol leaf extract of *Moringa oleifera* on post prandial hyperglycaemia-induced chronic complications in diabetes.

### **3.2.5 Experimental protocol**

Rats were grouped and treated as previously described. Rats in phase 2 were weighed weekly and FBG and RBG were also determined weekly. FBG was determined following an overnight fast of 12 hours and RBG determined during the day. At the end of the experiment, the rats were euthanised after light anaesthesia with chloroform, and blood sample taken from the jugular vein using careful aseptic technique into pre-labeled centrifuge test tubes. The blood was allowed to clot and then centrifuged at 1800 revolutions per minute for 10 minutes. The serum was then

aspirated carefully and transferred into plain sample bottles and stored at - 20°C in a freezer until subsequent use for assay of the parameters e.g. insulin, electrolytes and inflammatory biomarkers. The heart, kidney, pancreas, aorta, liver were removed and weighed using a Denver Instrument Digital Scale with 0.01 g sensitivity and the relative organ weight (ROW) was determined using the formula as shown below:

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat (g)}}$$

The eyes and foot pad of animals in phase 2 were also removed. The organs were thereafter fixed in 10% buffered neutral formalin (until they were taken to the histology laboratory for the preparation of the tissues) for histopathology studies.

### **3.2.6 Determination of pro and anti-inflammatory biomarkers**

The serum levels of the pro-inflammatory biomarkers (Tumour necrosis factor alpha and C-reactive protein) and anti-inflammatory biomarker (Adiponectin) were determined using rat-specific Quantikine Enzyme Linked Immunosorbent Assay (ELISA) kits.

#### *3.2.6.1 The principle of the ELISA technology*

The principle is that of an antigen-antibody reaction. The assay uses a stationary phase of microtiter plate which consists of wells pre-coated with a monoclonal antibody specific to that particular biomarker in rats, the biomarker under investigation being referred to, as the antigen. The mobile phase consists of the antibody linked to an enzyme (Streptavidin Horseradish peroxidase) and is referred to



as enzyme conjugate. The sample is added to the coated well followed by the enzyme conjugate to form a sandwich mixture of antibody- antigen- enzyme linked antibody. Substrate A (Hydrogen Peroxide) was added to generate reactive oxygen species (ROS) and water and substrate B (Tetramethyl benzidine), a chromogene and colourless compound was further introduced which becomes yellow in colour from oxidation by the ROS previously generated. The quantity of the antigen (biomarker) in the sample that the enzyme has conjugated is directly proportional to the ROS generated and invariably the oxidized coloured complex formed. The absorbance of the coloured complex was read off the microwell reader. The amount of the biomarker in the sample was determined by direct extrapolation from the standard regression curve generated from the absorbances of the serially diluted concentrations of the standard in the assay.

#### *3.2.6.2 Determination of Biomarkers using ELISA technology*

Step 1: Serial dilutions of the biomarker standard provided were prepared and 50 µl of each dilution introduced into the first 5 wells of the microtiter plate with the sixth well maintained as a blank.

Step 2: Ten µl of each test sample was pipetted into separate wells and diluted with 40µl of sample diluents to obtain 50µl and mixed gently.

Step 3: The plate was then covered with a thin plate cover and incubated for 30 minutes at 37°C.

Step 4: After incubation, the reaction mixture was discarded into a sink and each well rinsed using a wash solution provided. The rinsing was repeated 5 times and the plates were dried using filter papers until no trace of moisture was seen.

Step 5: Fifty  $\mu$ l of the enzyme conjugate was added to all wells except the blank well and the mixing process repeated.

Step 6: The plate was again covered and incubated as in step 3.

Step 7: Thereafter the reaction mixture was discarded and rinsed again as in step 4.

Step 8: Fifty  $\mu$ l of substrate (A) was pipetted and added to each well and step 3 was repeated but for 15 minutes.

Step 9: Fifty  $\mu$ l of substrate (B) was further added to each well to stop the reaction and step 3 repeated

Step 10: The absorbance of each well was then measured at 450 nm using a microplate reader and the concentration of the biomarker, calculated. The absorbance of each different concentration produced from serial dilution of the standard provided was entered into an excel spread sheet and a regression curve was plotted. The concentration of biomarker in each sample was extrapolated from the curve based on its absorbance value (Appendix III). The determination of each biomarker required the use of specific reagents and substrates (Appendix IV).

### **3.2.7 Determination of serum insulin levels**

The protocol, principle and procedure for determination of serum insulin levels was as described in 3.2.5 above using ELISA rat specific insulin kits and the concentration of insulin in each sample in units/litre (U/L) was calculated as shown in Appendix III.

### **3.2.8 Determination of cardioactive peptide (B-type natriuretic peptide)**

The protocol, principle and procedure for determination of serum levels of B-type natriuretic peptide was as described in 3.2.5 above using ELISA rat specific B-type

natriuretic peptide kits and the concentration of B-type natriuretic peptide in each sample in microgramme/litre (mcg/L) was calculated as shown in Appendix III.

### **3.2.9 Determination of oxidative stress markers**

The protocol, principle and procedure for determination of the oxidative stress markers which included Malondialdehyde and Catalase was as described in 3.2.5 above using ELISA rat specific kits and the concentration of the biomarkers in each sample in nmol/L for MDA and units/litre (U/L) for catalase was calculated as shown in Appendix III.

### **3.2.10 Determination of lipid profile**

The blood sample collected during phase 2 study was used for this analysis. The lipids investigated included triglycerides (TG), total cholesterol (TC), high density lipoproteins (HDL) and low density lipoprotein (LDL).

#### *3.2.10.1 Triglycerides*

Determination of triglyceride was done using an Auto analyzer (Rayto Chemray 120) with the aid of Randox kits. The principle is based on an enzymatic colourimetric end point, where triglyceride react with a combination of lipoprotein lipase, glycerol kinase and glycerol-3-phosphate oxidase to yield a coloured quinonemine compound dependent on the concentration of the triglyceride in the sample.

The procedure involved a withdrawal of 50 µl of each rats serum into separate customized sample fills of the auto analyser, and also 20 mls of the triglyceride reagent into the reagent compartment. The autoanalyser did the mixing of the reagent

and the test sample and also incubated for about 12 minutes. The result was produced by the autoanalyser as printed sheet of triglyceride concentration of each sample in millimoles/litre (mmol/l).

#### 3.2.10.2 *Total cholesterol*

Determination of total cholesterol was done using Randox kits. The principle is based on an enzymatic colourimetric end point, where cholesterol react with a combination of cholesterol esterase, cholesterol oxidase and peroxidase (cholesterol reagent) to yield a coloured quinonemine compound dependent on the concentration of the cholesterol in the sample.

The procedure was according to the method described by (Tinder, 1959). Ten  $\mu\text{l}$  of each sample and 10  $\mu\text{l}$  of the cholesterol standard was pipetted into separate test tubes. One ml of the reagent was then added and incubated for 10 minutes at 37°C. The absorbance of each test sample and the standard were read from a spectrophotometer at 540 nm. The concentration of cholesterol in each test sample was then calculated as shown in Appendix V.

#### 3.2.10.3 *High density lipoproteins (HDL) and low density lipoprotein (LDL)*

Determination of high density lipoproteins was done using Randox kits. Five hundred  $\mu\text{l}$  of precipitant was added to 200  $\mu\text{l}$  of each test sample previously withdrawn into separate test tubes and centrifuged at 1800 rpm for 10 seconds. Thereafter, 100  $\mu\text{l}$  of the supernatant and 100  $\mu\text{l}$  of the HDL standard (3 mmol/l) were withdrawn into separate test tubes respectively and 1,000  $\mu\text{l}$  of the cholesterol reagent added and left to stand for 10 minutes. The absorbance was then read with a spectrophotometer at a

wavelength of 500 nm. The concentration of HDL in each sample was calculated as shown below:

$$\text{Conc of HDL} = \frac{\text{Abs of HDL}}{\text{Abs of Std}} \times \text{Conc of Std}$$

Where

Conc = Concentration

Abs = Absorbance

Std = Standard

The concentration of LDL was derived using the triglyceride, cholesterol and high density lipoprotein values previously determined as shown below:

$$LDL = TC - \left( \frac{TG - HDL}{2.2} \right)$$

### **3.2.11 Determination of kidney function biomarkers (Serum Urea, Electrolytes and Albumin)**

Blood samples taken during phase 2 was used for this study.

#### *3.2.11.1 Serum urea*

The principle of determination of serum urea is based on the diacetyl monoxime method using thiosemicarbazide (Natelson *et al.*, 1951, Marsh *et al.*, 1965). The principle is based on the fact that when urea is heated in strongly acidic conditions with substances such as diacetyl containing two adjacent groups or monoxime, yellow condensation compounds are formed. The reaction is intensified by the presence of polyvalent ions such as ferric ions, such that a red coloured complex is formed which

is more linear with concentration than the yellow compound. The intensity of the red complex is measured colourimetrically and its intensity is proportional to the concentration of urea in the sample.

The procedure involved the dilution of 10  $\mu\text{l}$  of serum from each individual rat previously introduced into separate test tubes with 2 ml of distilled water and same repeated for 10  $\mu\text{l}$  of urea standard (3.25 mmol/L). A blank sample was also prepared using 2 ml of distilled water. Two millilitres of the working colour reagent (containing a mixture of diacetyl monoxime and thiosemicarboxide) was added into test, standard and blank sample tubes respectively and mixed gently. Another 2 ml of the already prepared mixed acid reagent (ferric chloride), concentrated sulphuric acid and phosphoric acid were further added into the mixture in each tube and mixed thoroughly. The tubes were then placed in water bath at 100 °C for 20 minutes, allowed to cool and the optical density was read at 520 nm with a spectrophotometer. The concentration of urea in each sample was calculated as shown in Appendix V.

#### 3.2.11.2 *Serum electrolytes*

Sodium ( $\text{Na}^+$ ), Potassium ( $\text{K}^+$ ), Chloride ( $\text{Cl}^-$ ) and bicarbonates ( $\text{HCO}_3^-$ ) were assayed using the ion selective electrode method (Potentiometry) with the aid of an Audicom (AC 9900). The audicom has an in-built precalibrated reference electrode which consists of a thin membrane across which only the intended ion can be transported. The transport of ions from a high concentration to a low one through a selective binding with some sites within the membrane creates a potential difference. It measures the flow of that particular ion by sensing the rate of flow of the ion in the sample. It then measures the concentration of that ion by relating its rate of flow to the

already existing calibrated value of the reference electrode. Three hundred  $\mu\text{l}$  of each test sample was introduced into customized sample fills and fed back into the audicom. The specific ions to be analysed were selected and the audicom ran the analysis based on the potentiometric principle explained above. The result was produced directly by the audicom in printed sheets in millimoles/litre (mmol/l).

#### 3.2.11.3 *Serum albumin*

The concentration of serum albumin was determined using an auto analyzer (Rayto Chemray 120) with the aid of Randox kits and the principle is based on a colourimetric reaction with the reagent (bromocresol green) in an acidic medium (pH of 4.20). The albumin in the sample binds with bromocresol green to form a green coloured complex and the intensity depends on the concentration of albumin in the test sample.

The procedure involved the withdrawal of 50  $\mu\text{l}$  of each sample into separate customized sample fills of the auto analyser, and also 20 ml of the albumin reagent into the reagent compartment. This was fed back into the auto analyser that did the mixing of the reagent (bromocresol green, 0.2 mmol/l and succinate buffer pH 4.20) and the test sample. The result was produced directly by the autoanalyser in printed sheet with albumin concentration of each sample in gram/litre (g/l).

#### **3.2.12 Determination of liver function biomarkers (ALT and AST)**

The concentrations of serum alanine transaminase and serum aspartate transaminase were determined using an auto analyzer (Rayto Chemray 120) with the aid of Randox kits and is based on the kinetics principle. AST reacts with the combined reagent (L-

Aspartate,  $\alpha$ -Ketoglutarate and malate dehydrogenase) to yield L-malate with the oxidation of NADH to NAD. The reaction was monitored by measurement of the decrease in absorbance of NADH. The rate of deduction in absorbance is proportional to AST activity in the sample. The same principle applies to ALT using the combined reagent (L-Alanine,  $\alpha$ -ketoglutarate and lactate dehydrogenase) (Reitman and Frankel, 1957). The procedure was as described in 3.2.10.3 and the result produced directly by the autoanalyser in printed sheet with AST and ALT concentrations of each sample in Units/litre (U/l).

### **3.2.13 Preparation of tissue for histopathology**

Tissues for histopathological examination were processed according to the protocol described by Tulpule and Ghaji (1987). It involved dehydration, clearing, impregnation and embedding. The tissues that had been previously fixed in 10% buffered neutral formalin solution were dehydrated with successive ascending concentrations of ethanol starting from 50% to 70%, 90% and finally absolute (100%) ethanol. Besides the 50% ethanol in which dehydration was left to continue overnight, the tissues were subjected to two changes of half an hour each before the 70% change. After the dehydration of the tissues was completed, the tissues were cleared in three changes of xylene lasting for one hour each. The tissues were then impregnated through two changes of molten paraffin wax for a period of one hour each. This was followed by embedding in molten paraffin wax and the tissues were then allowed to solidify. The blocked embedded tissue was then mounted on a wooden block and trimmed. Sections were cut to a thickness of about 6 microns using a rotary microtome. The resulting serial sections were allowed to float in a warm water bath and selected sections were placed on clean microscope slides. The tissues were further



dewaxed in two changes of xylene lasting for a period of three minutes each. Rehydration was then carried out through descending concentrations of ethanol (100-50%).

### **3.2.14 Staining of Tissues**

Two different staining procedures were used.

#### *3.2.14.1 Haematoxylin and Eosin staining (H and E)*

The staining procedure was used for all tissues except the rat footpad (where it acted as a counter stain) and involved dewaxing the tissues in xylene for two changes. Thereafter the tissues were passed through running water for a few minutes after which they were stained in Harris haematoxylin solution for ten minutes and then a further rinsing in tap water. The tissues were further differentiated using 1% ethanol and washed again in running tap water. This was followed by staining with 1% Eosin solution for one minute and then subsequent dehydration in graded concentrations of ethanol. The tissues were thereafter cleared in xylene and then mounted with cover slips using tricresylphosphate and xylene (DPX). Slides were allowed to dry overnight after which they were preserved in microscope slide box.

#### *3.2.14.2 Luxol fast blue staining (LFB)*

The principle of the luxol fast blue stain is that of an acid-base reaction which produces a salt. It stains particularly for myelin sheath in nerve fibres with the base of the lipoprotein in the myelin being replaced by the base of the dye (Klüver and Barrera, 1953). This staining procedure was used for the rat foot pad to investigate the intraepidermal nerve fibre density. The procedure involved staining the processed tissue in 0.1% LFB in 95% alcohol for 2 hours at 60°C. The tissue was then washed in

water and differentiated with saturated lithium carbonate in water and the washing procedure repeated. It was thereafter stained in Harris's haematoxylin for 5 minutes, washed in water and differentiated in acid alcohol with 3 to 6 dips. The washing process was repeated again in running water and rinsed in 95% alcohol. Eosin was used for the counter stain for 1 minute, making sure stain covers slides completely. The slides were then rinsed well in running water and dehydrated in 95% alcohol and 3 changes of absolute alcohol, 10 dips in each. The final process was clearing the slides in 3 changes of xylene, 10 dips each. The result appeared as myelin sheaths staining dark blue and nuclei staining blue-black on a pink background (Ralis, 1973).

### **3.2.15 Determination of onset and progression of neuropathic pain**

Experimental design, animal groupings and drug administration followed the protocol as described for phase 2 of the experiment, as the same groups of rats were followed throughout to the 42<sup>nd</sup> day of the study. The principle is that alloxan-induced diabetic rat, displays both functional and morphological changes of human peripheral diabetic neuropathy and is evidenced as slow nerve conduction velocity, thermal hyperalgesia and allodynia (Shaikh and Somani, 2010). The biomarkers of neuropathic pain i.e. hyperalgesia and allodynia were therefore assessed using response to both thermal and mechanical pain stimuli.

#### *3.2.15.1 Assessment of thermal hyperalgesia (Hot plate latency method)*

Thermal hyperalgesia was assessed using the hot plate latency method as initially described by Eddy and Leimbach (1953) and modified by Ibrinke *et al.* (2004). The test evaluates thermal pain reflexes due to footpad contact with a heated surface. The experiment was carried out once weekly for five weeks starting from the second week

following alloxan administration. During the experiment, each rat from a group was introduced into an open-ended cylindrical space with a floor consisting of a heated plate at a constant temperature of  $55 \pm 1^\circ\text{C}$  using a hot plate. Pain sensitivity was evaluated by the response latency (in seconds) for jumping off the hot plate using a stopwatch and a cut off time of 15 seconds was maintained to prevent damage to the tissues of the foot. Every rat from each group was used for the experiment and the mean latency value for withdrawal from the hot plate was calculated for each group.

#### 3.2.15.2 Assessment of mechanical hyperalgesia (Randal Sellito test)

Mechanical hyperalgesia was evaluated by the response to mechanical stimulus using the Randall-Sellito test with the aid of an analgometer (IITC 2500 Digital Paw Pressure Meter) as described by Santos-Noquiera *et al.* (2012). The instrument requires the application of increasing pressure by a foot pedal on the rats paw between a flat surface and a blunt pointer. The procedure involved depressing a pedal switch to start the mechanism which exerts a steadily increasing force (16 grams per second) on the medial portion of the dorsal surface of rats' right hind paw until the rat struggles and makes a stereotyped flinch response. The electronic measures the time interval between the start of the slide motion and the moment the slide is stopped by the operator and converts time into force. The force is measured on the scale calibrated in 10-gram steps, by a pointer riveted to the slide. The scale can be multiplied by 2 or 3, by placing one or two disc weights (70 g) on the slide.

The experiment was carried out at the end of every week for 5 weeks using each rat from the seven groups following the protocol as described above. Two discs were used as the initial weight for the experiment following no response from only one

disc. Two readings were taken for each rat following a 15 minutes interval and the mean values were further used to calculate the mean paw pressure required to elicit the flinching response for each group, (Appendix VI).

#### 3.2.15.3 Assessment of thermal allodynia (Tail immersion test)

Allodynia was assessed at the end of every week using the tail immersion (warm water) test as described by Anjaneyulu and Chopra (2004) at temperature of  $47 \pm 1$  °C with a cut off time of 15 seconds. The tails of the rats were immersed in warm water bath and the time in seconds at which every rat flicks its tail off the bath, was recorded. Two readings were taken for each rat at an interval of 15 minutes and the mean values were further used to calculate the mean latency for tail flick for each group.

#### 3.2.16 Determination of intraepidermal nerve fibre density

Following the histologic preparation, staining and microscopic examination of sections of the plantar surface of the rats' hind paw, intraepidermal nerve fiber density was determined according to the method described by Beiswenger *et al.* (2008). The method involved counting only the nerve fibres that can be seen crossing the dermal-epidermal border, from the dermis into the epidermis while excluding branches within the epidermis. The number of fibres counted represented the direct density of intra epidermal nerve fibre for the rat in its group.

#### 3.2.17 Determination of the onset and progression of retinopathy

Experimental design, animal groupings and drug administration also followed the protocol as described for phase 2 study. At the end of the 3<sup>rd</sup> and 6<sup>th</sup> week, the eyes of

the animals were examined externally using an ophthalmoscope for signs of opacity or cataract formation and this was graded according to the staging method described by Suryanarayana *et al.* (2005), (Appendix VII).

### **3.3 Data Analysis**

All data obtained were entered into the SPSS version 11 statistical package which was used for all statistical analysis. Single point variables were analysed using one way ANOVA followed by Levene's test of Homogeneity of variance and Games-Howell or Horchberg post hoc test, were used as appropriate. For parameters taken over time, split plot ANOVA followed by Bonferoni Adjustment was used. Results of the study are expressed as mean  $\pm$  SEM. P values of  $\leq 0.05$  were considered to be statistically significant.

### **3.4 Presentation of Data**

Data were presented as tables and charts as well as line graphs as applicable to the collected data. Histological observations were presented as photomicrographs.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Acute Toxicity Test

##### 4.1.1 Acute oral toxicity of 50% ethanol leaf extract of *Moringa oleifera*

Body weights of rats, at days 3, 7 and 14 did not differ significantly from the weights before dosing (Table 4.1). Physical observation of the skin, fur, eyes, changes in behavior and somatomotor activity within the first 4 hours, and daily for 14 days did not show any noticeable or significant changes in the rats. There was no death recorded throughout the 14 days study period and the oral LD<sub>50</sub> was estimated to be greater than 2,000 mg/kg.

##### 4.1.2 Effect of administration of 2,000 mg/kg (single dose) of *Moringa oleifera* leaf extract on histology of major organs in rats in acute toxicity studies

No significant histopathological findings were seen in major organs (heart, lungs, spleen, kidney, liver and pancreas) on examination after acute oral toxicity study following a single oral dose of 2000 mg/kg of *Moringa oleifera* leaf extract.

**Table 4.1: Effect of Administration of 2,000 mg/kg of *Moringa oleifera* Leaf Extract on Body Weights of Rats in Acute Toxicity Studies (OECD Limit Test)**

<b>Percentage Increase From Initial Body Weight (%)</b>			
<b>Rats No</b>	<b>Day 3</b>	<b>Day7</b>	<b>Day 14</b>
<b>1</b>	7	4	7
<b>2</b>	4	3	0
<b>3</b>	6	7	7
<b>4</b>	4	2	5
<b>5</b>	13	16	15

## **4.2 Effect on Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Glycaemic Control Parameters**

### **4.2.1 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on fasting blood glucose**

The co-administration of Sitagliptin (50 mg/kg) and ethanol leaf extract of *M. oleifera* (300 mg/kg) brought about a statistically significant ( $p < 0.01$ ) decrease (60%) in fasting blood glucose after 14 days of drug administration and up to the 28<sup>th</sup> day (38%) compared to day 1. However, the blood glucose further increased on the 35<sup>th</sup> day (3%) compared to day one (Table 4.2). After commencement of drug administration on day 14 in the ameliorative group, a statistically significant ( $p < 0.05$ ) increase (16%) was observed in FBG on day 42 compared to days 21 and 28 (Table 4.2). There was significant decrease in FBG ( $p < 0.01$ ) from day 14 (58%) to day 42 (55%) compared to day 1 in rats that received only Sitagliptin. Significant decrease (45%), was also observed in FBG ( $p < 0.01$ ) on day 21 compared to day 1, in rats treated with *M. oleifera* alone. Thereafter, the level of decrease declined from day 28 (29%) up to day 42 (3%) (Table 4.2).



**Table 4.2: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Fasting Blood Glucose in Rats**

Group	Blood Glucose Concentration (g/dl)							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56
NC	87.71±21.81	66.14±34.82	67.14±9.77	57.42±8.85	69.00±15.66	72.00±17.26	73.92±22.06	
DC	179.83±23.56	224.75±37.61	152.50±10.55	128.33±9.56	198.16±16.92	213.83±18.64	144.16±23.82	
ST	246.42±21.81	159.42±34.82	103.28±9.77** (56%)	146.92±8.85* (40%)	130.28±15.66* (47%)	169.28±17.26* (31%)	110.42±22.06** (55%)	
MO	239.40±25.81	123.60±41.20*	149.60±11.56*	131.20±10.47** (45%)	169.80±18.53	202.15±20.42	217.80±26.10	
SM	226.85±21.81	172.28±34.82	90.00±9.77** (60%)	112.64±8.85** (50%)	138.57±15.66* (38%)	235.25±17.26	217.71±22.06	
PC	86.50±23.56	174.33 37.61* (50%)	93.00±10.5	87.16±9.56	94.83±16.92	109.66±18.64	176.75±23.82* (50%)	104.17±7.42
PPSM	78.71±21.81	96.71±34.82	88.14±9.77	83.14±8.858	87.85±15.66	93.28±17.26	105.14±22.06* (25%)	65.00±6.87

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

NC= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Ameliorative = group that received treatment at onset of complication

Values are mean ± SEM. Significant difference (decrease) \* =  $p < 0.05$ ; \*\* =  $p < 0.01$  Vs day1, for Ameliorative group Vs day 14, 21 and 28 and for PC Vs day 1. Split Plot ANOVA (Bonferoni Post Hoc test). **Duration of treatment = 42 days.**

Overall group effect also showed significant increase  $p < 0.01$ , in blood glucose levels comparing normal control to all diabetic control and drug treated groups

#### **4.2.2 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on random blood glucose**

The co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* brought about a statistically significant ( $p < 0.001$ ) decrease (24%) in random blood glucose after 42 days of drug administration compared with day 1. The combination also showed statistically significant ( $p < 0.001$ ) decrease in random blood glucose in the ameliorative group on day 42 (40%) and day 56 (62%) compared to day 1 (Table 4.3). There was a statistically significant decrease ( $p < 0.05$ ) in random blood glucose in Sitagliptin alone group following 7 days (20%) and 42 days (27%) of drug administration compared to day 1. No significant difference was seen in RBG levels in rats treated with *M. oleifera*.

**Table 4.3: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Random Blood Glucose in Rats**

Group	Blood Glucose Levels (g/dl)							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56
NC	122.6±22.40	96.37±29.59	116.62±30.62	112.12±28.72	110.12±29.16	104.25±32.54	142.12±22.15	
DC	535.33±25.86	554.16±34.17	533.16±35.36	473.16±33.16	502.66±33.67	390.16±37.58	447.33±25.57	
ST	557.71±23.94	457.42±31.64* (20%)	525.00±32.74	531.42±30.70	487.57±31.17	452.42±34.79	405.00±23.68** (27%)	
MO	512.60±28.33	510.80±37.43	548.40±38.73	582.80±36.33	459.80±36.88	492.26±41.17	470.73±28.01	
SM	564.33±25.86	515.83±34.17	474.66±35.36	557.25±33.16	534.66±33.67	536.77±37.58	429.72±25.57** (24%)	
PPC	459.33±25.86	440.16±34.17	579.66±35.36	566.00±33.16	490.16±33.67	480.91±37.58	366.25±25.57	214.83±11.05**
PPSM	466.85±23.94	407.28±31.64	386.71±32.74	403.57±30.70	368.85±31.17	347.42 ±34.79	279.85±23.68** (40%)	177.42±10.23** (62%)

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

NC= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Ameliorative = group that received treatment at onset of complication

Values are mean ± SEM. Significant difference (decrease) \* =  $p < 0.05$ , \*\* =  $p < 0.001$  Vs day1, Split Plot ANOVA ((Bonferoni Post Hoc test).

**Duration of treatment = 42 day**

Overall group effect also showed significant increase  $p < 0.01$ , in blood glucose levels comparing Normal control to all Diabetic control and drug treated groups.

#### **4.2.3 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on weekly weights of rats**

The co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* did not show any significant difference, although a slight increase in the body weights of the rats comparing day 42 to day1 was observed. The diabetic controls and other diabetic treated groups also showed no statistically significant alterations in body weights during the course of the experiment (Table 4.4).

**Table 4.4: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Weekly Weight of Rats**

Group	Weekly Weight (g)							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 56
<b>NC</b>	164.12±7.03	168.50±7.36	174.12±7.50	182.12±8.71	188.00±9.52*	194.87±9.21**	198.25±9.62**	
<b>DC</b>	137.80±8.90	146.20±9.31	135.80±9.48	147.60±11.02	150.00±12.04	153.80±11.66	157.50±12.17	
<b>ST</b>	139.42±7.52	135.14±7.87	137.85±8.01	136.42±9.31	146.71±10.18	144.85±9.85	146.07±10.28	
<b>MO</b>	149.40±8.90	140.20±9.31	146.00±9.48	149.60±11.02	154.20±12.04	157.60±11.66	162.60±12.17	
<b>SM</b>	139.50±9.95	127.75±10.41	140.00±10.60	132.25±12.32	138.66±13.46	142.00±13.03	145.91±13.61	
<b>PPC</b>	167.00±8.12	175.16±8.50	186.00±8.66	195.16±10.06	181.83±10.99	178.16±10.64	195.91±11.11	206.5±14.97
<b>PPSM</b>	177.57±7.52	180.00±7.87	187.00±8.01	191.85±9.31	193.71±10.18	193.71±9.85	190.42±10.28	194.28±13.86

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

NC= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

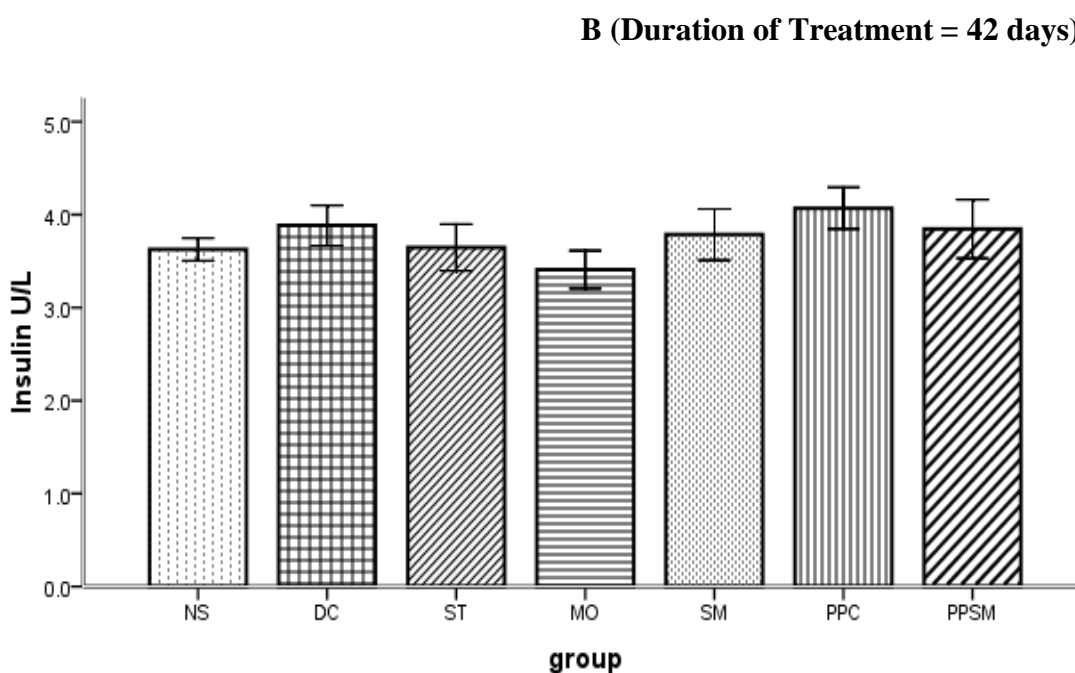
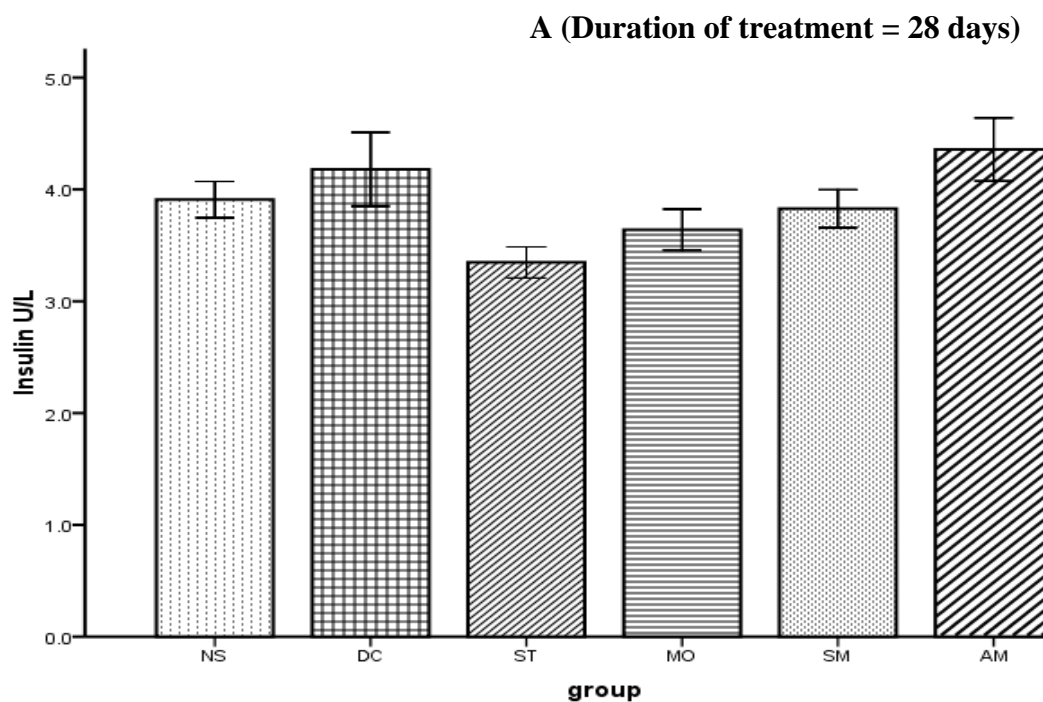
Ameliorative = group that received treatment at onset of complication

Values are mean ± SEM. Significant difference (increase) \* =  $p < 0.05$ , \*\* =  $p < 0.001$  Vs day1. Split Plot ANOVA (Bonferoni Post Hoc test).

**Duration of treatment = 42 days.**

#### **4.2.4 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on levels of insulin in alloxan-induced diabetic rats**

The co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* did not bring about any statistically significant difference in the mean levels of insulin in the rats compared to diabetic control and also Sitagliptin and *M. oleifera* as single agents respectively, following both 28 days (Figure 4.1A) and 42 days (Figure 4.1B) of drug administration. However rats with post prandial hyperglycaemia had the highest mean insulin levels in both phases.



**Figure 4.1: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Mean levels of Insulin in Rats after 28 days (A) and 42 days (B) of Treatment**

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg). NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, AM and PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

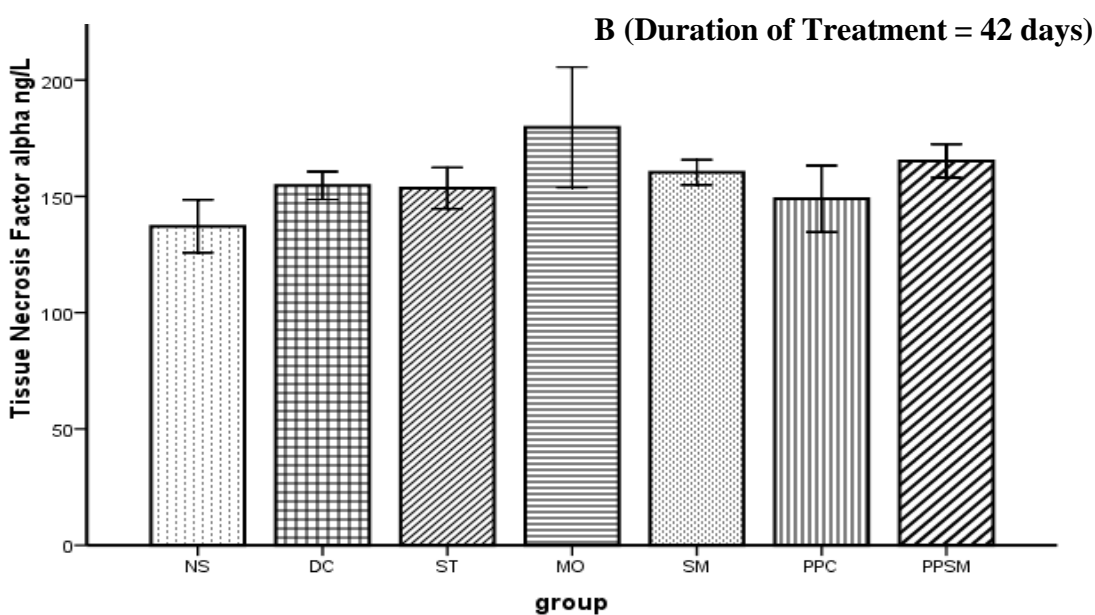
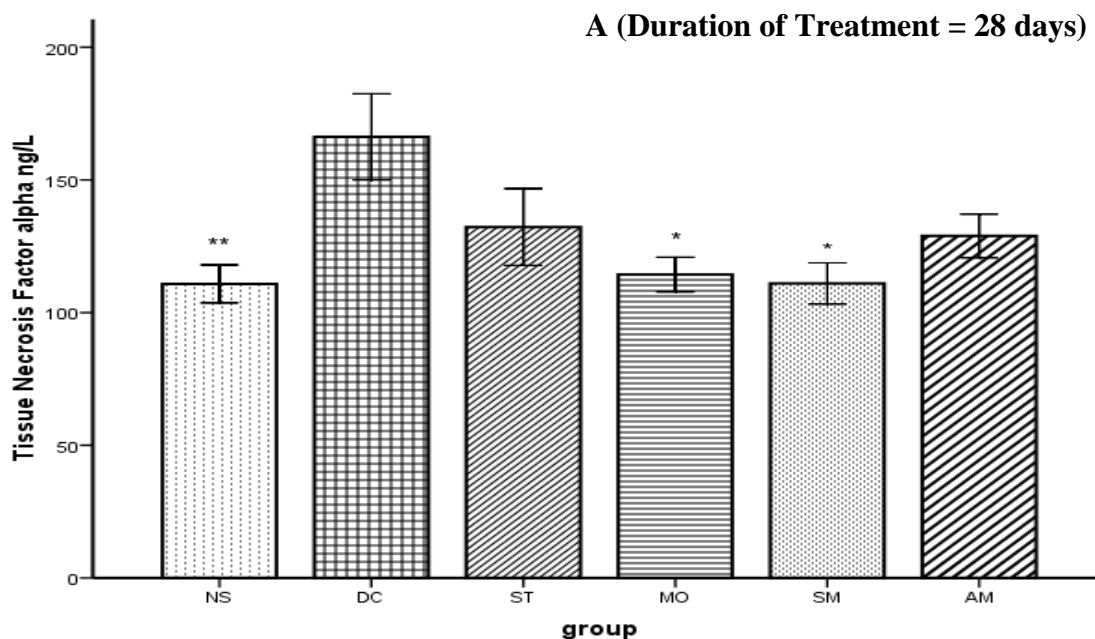
Values are mean  $\pm$  SEM. No significant difference between groups  $p > 0.05$  (One Way ANOVA) in both Phases.

### **4.3 Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Progression and Possible Amelioration of Diabetic Cardiomyopathy and on Biomarkers of Macroangiopathy in Rats**

#### **4.3.1 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on serum levels of tissue necrosis factor alpha in rats**

The mean serum levels of (TNF $\alpha$ ) of rats treated with Sitagliptin and *M. oleifera*, *M. oleifera* alone, and rats in normal control group showed statistically significant decrease ( $p < 0.05$ ) compared to diabetic control following 28 days drug administration (Figure 4.2A). However following 42 days of drug administration, there was no statistically significant difference in mean serum levels of TNF $\alpha$  in all drug treated groups compared to diabetic control (Figure 4.2B) but rats treated with *M. oleifera* had the highest serum level.





**Figure 4.2: Effect of the Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Mean Serum Levels of Tissue Necrosis Factor Alpha (TNF $\alpha$ ) in Rats after 28 days (A) and 42 days (B)**

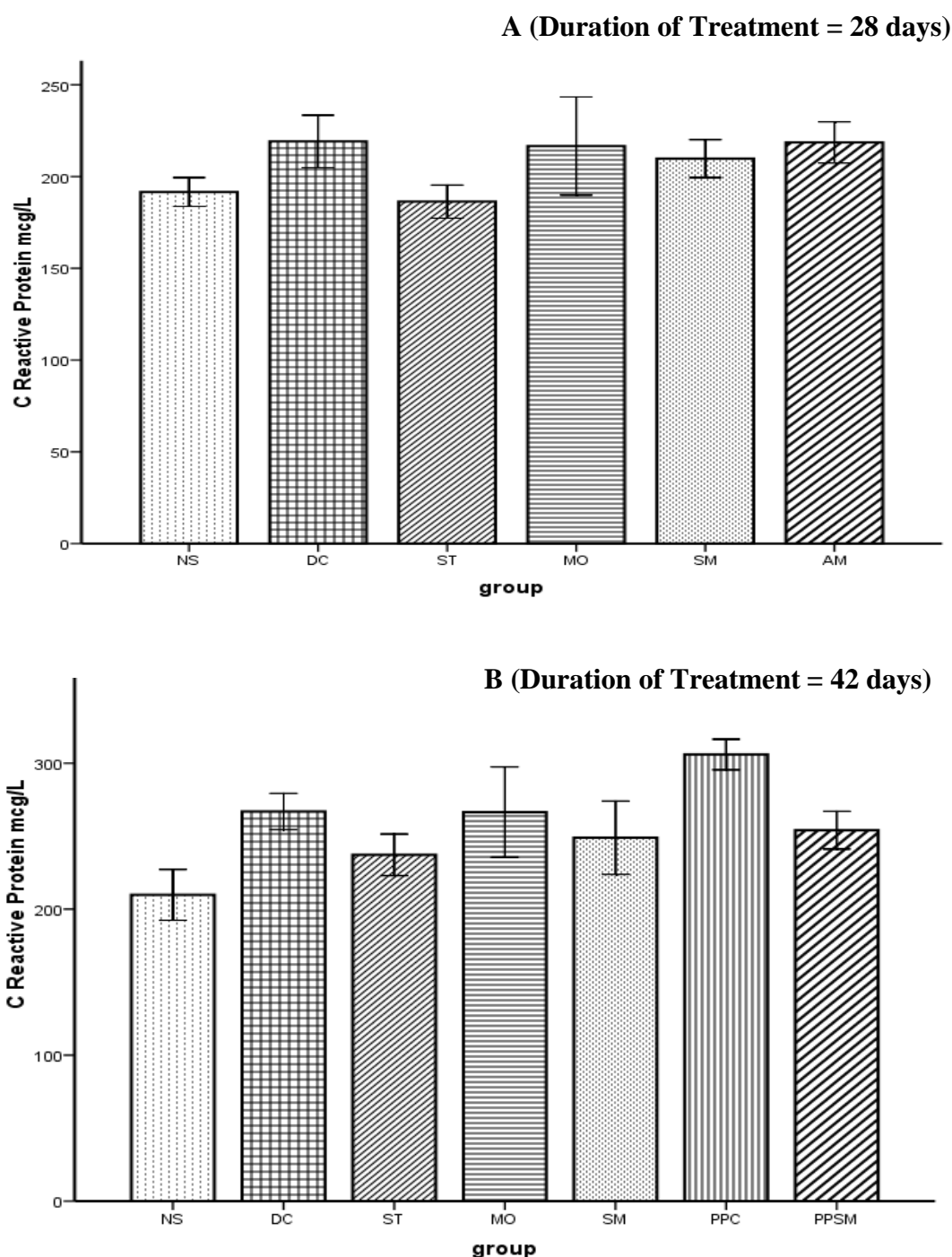
Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

Key: NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC= Post prandial control, AM and PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Values are mean  $\pm$  SEM. Significant difference (A = 28 days) \* =  $p < 0.05$ , \*\* =  $p < 0.01$  compared with diabetic control, One Way ANOVA (Hochberg Post Hoc test). No Significant difference (B = 42days),  $P > 0.05$  comparing drug treated groups with diabetic control.

#### **4.3.2 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean levels of C-reactive proteins in rats**

The co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera*, did not cause any statistically significant alterations in the mean serum levels of CRPs compared to diabetic control following 28 days (Figure 4.3A) and 42 days (Figure 4.3B) of drug administration. Nevertheless, rats in the diabetic controls and *M. oleifera* alone groups had the highest level of CRPs in both phases with levels of CRPs in post prandial control being statistically significantly higher ( $p<0.05$ ) than in the normal control group and no statistical significance with post prandial treated group (Figure 4.3B).



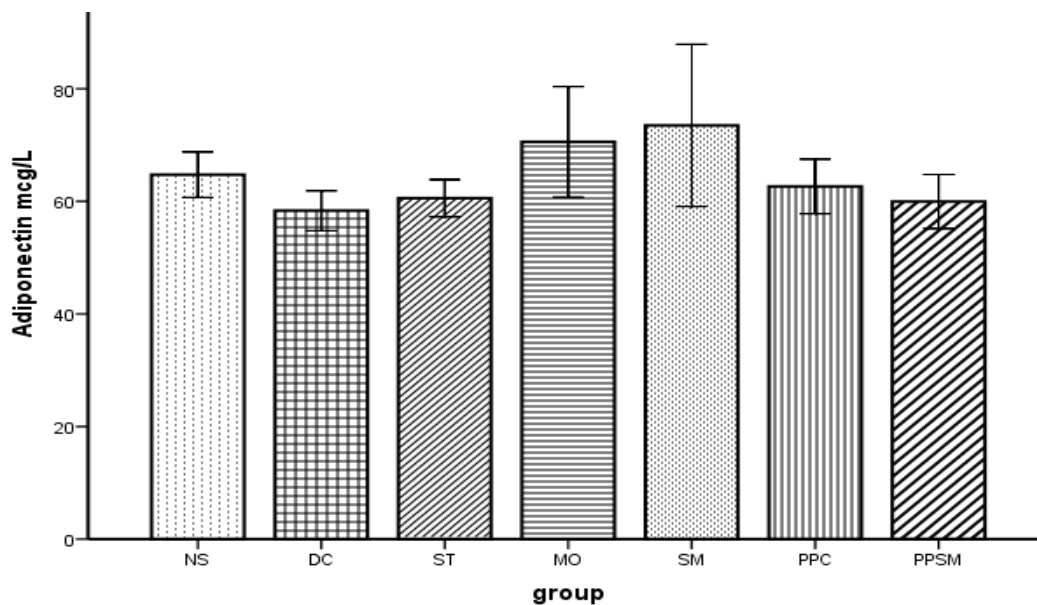
**Figure 4.3: Mean Levels of C Reactive Proteins (CRPs) Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats after 28 days (A) and 42 days (B) of Treatment**

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg). Key: NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, AM and PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Values are mean  $\pm$  SEM. No significant difference (**A = 28 days**)  $p > 0.05$ , ANOVA. Significant difference (**B = 42 days**) \* =  $p < 0.05$ , PPC Vs NS, ANOVA (Games Howell Post Hoc Test).

#### **4.3.3 The Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean levels of adiponectin in rats**

There was no statistically significant difference in the mean serum levels of adiponectin following the co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* in the rats compared to diabetic control and in post prandial treated group compared to post prandial control (Figure 4.4)



**Figure 4.4: Mean Levels of Adiponectin (ADP) Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats**

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

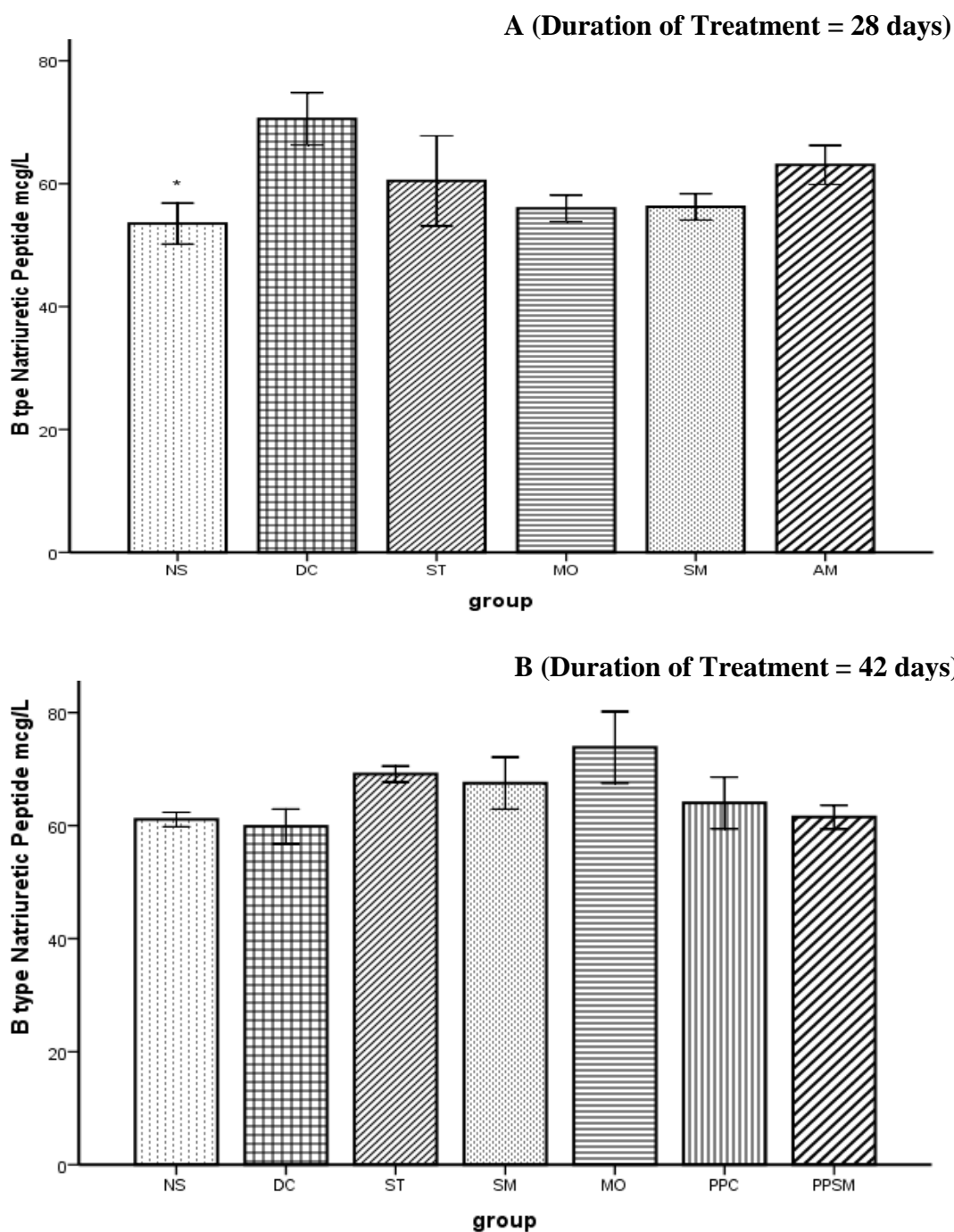
Key: NS= Normal control, DC=Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Values are mean  $\pm$  SEM, no significant difference ( $p>0.05$ ), One Way ANOVA).

**Duration of Treatment = 42 days.**

#### **4.3.4 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean levels of B type natriuretic peptide in rats**

There was a considerable decrease though not statistically significant in the mean serum levels of BNP in rats treated with Sitagliptin and ethanol leaf extract of *M. oleifera* and rat treated with *M. oleifera* alone compared to diabetic control following 28 days of drug administration (Figure 4.5A). After 42 days of drug administration in contrast to 28 days, rats treated with only *M. oleifera*, and Sitagliptin with *M. oleifera* combination had higher levels of serum BNP compared to diabetic control, with post prandial treated group showing no statistical significance compared to control (Figure 4.5B).



**Figure 4.5: Mean Levels of B type Natriuretic Peptide (BNP) Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats after 28 days (A) and 42 days (B) of Treatment**

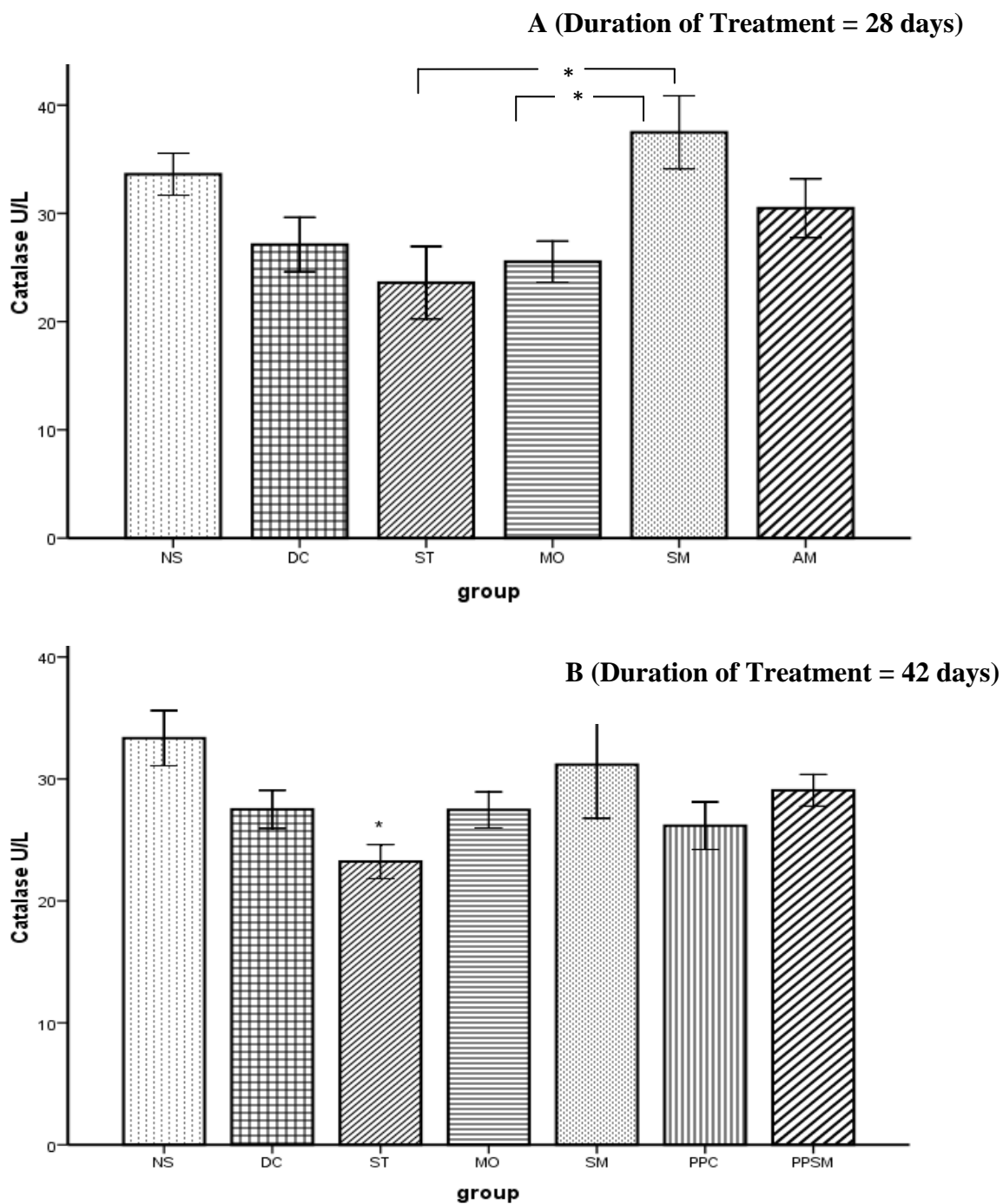
Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg). Key: NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO= *M. oleifera*, ST/MO= Sitagliptin and *M. oleifera*, PPC= Postprandial control, AM and PPSM= Ameliorative on Sitagliptin and *M. oleifera*..

Values are mean  $\pm$  SEM. Significant difference (**A = 28 Days**) \*=  $p < 0.05$  Normal control compared to Diabetic Control. ANOVA (Games Howell Post Hoc test). No significant difference ( $p > 0.05$ ), (**B = 42 Days**).

#### **4.3.5 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean levels of catalase in rats**

There was a statistically significant increase ( $p < 0.05$ ) in the mean serum levels of CAT in rats treated with Sitagliptin and ethanol leaf extract of *M. oleifera* compared to rats treated with only *M. oleifera* and only Sitagliptin following 28 days of drug administration (Figure 4.6A). After 42 days of drug administration, the levels of CAT in rats treated with Sitagliptin and *M. oleifera* in both SM and PPSM groups was still higher than the levels in rats that received the single agents (Figure 4.6B).





**Figure 4.6: Mean Levels of Catalase (CAT) Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats after 28 days (A) and 42 days (B) of Treatment**

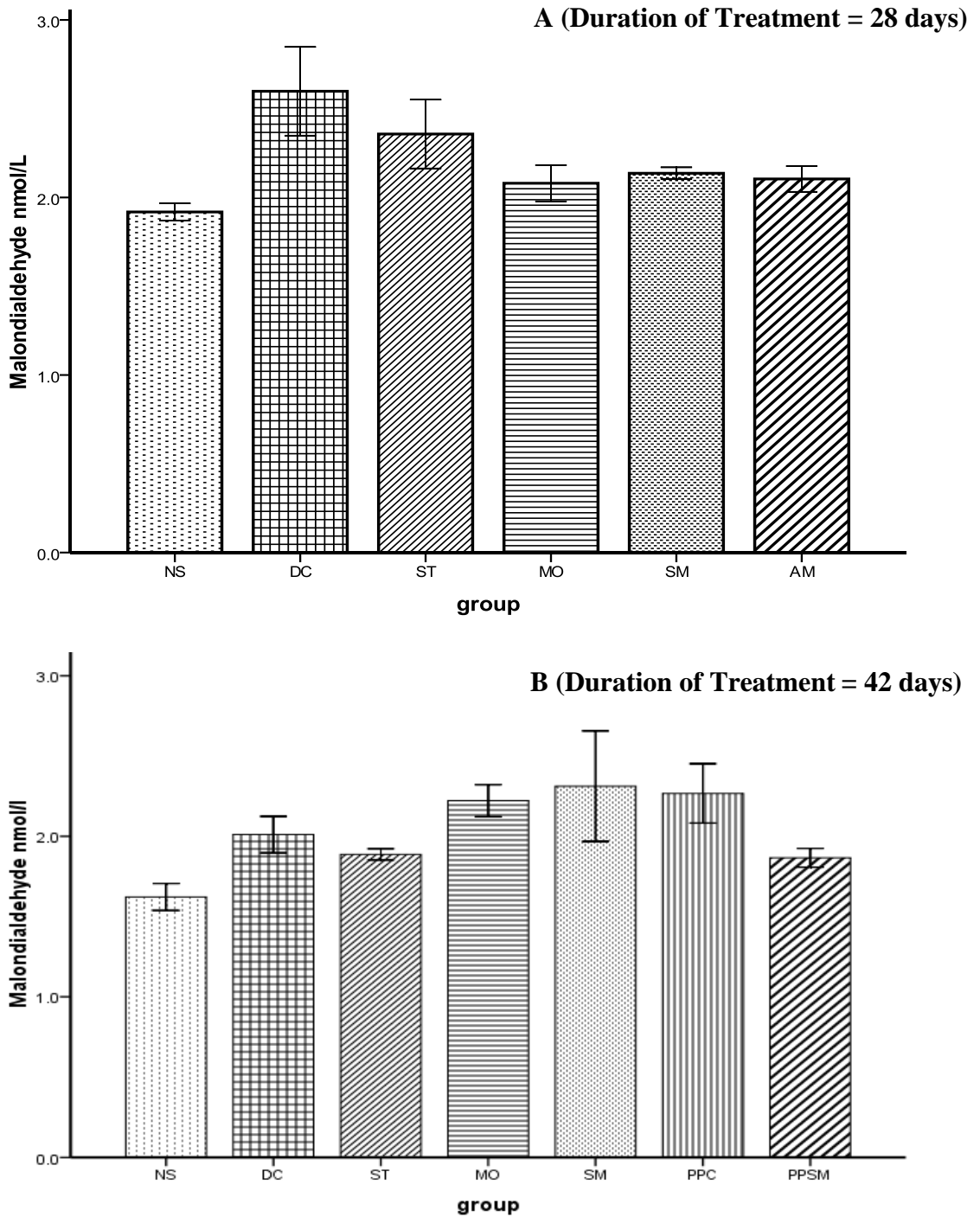
Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg).

Key: NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, AM and PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Values are mean  $\pm$  SEM. Significant difference \* =  $p < 0.05$ . MO and ST compared to SM (A= 28days) and Sitagliptin Vs Normal Control (B = 42 days). ANOVA (Horchberg Post Hoc test).

#### **4.3.6 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean levels of malondialdehyde in Rats**

The co-administration of Sitagliptin and *M. oleifera* for 28 days produced a non significant reduction in the serum levels of malondialdehyde compared to diabetic control (Figure 4.7A). Animals treated with only *M. oleifera* also showed a non significant decrease in serum MDA levels compared to diabetic control. Treatment for 42 days in Sitagliptin and *M. oleifera* group however showed slight increase compared to diabetic control, contrary to post prandial treated group being relatively reduced compared to post prandial control (Figure 4.7B).



**Figure 4.7: Mean Levels of Malondialdehyde (MDA) Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats after 28 days (A) and 42 days (B)**

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg). Key: NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Values are mean  $\pm$  SEM. No significant difference ( $p > 0.05$ ), Brown-Forsythe robust test of means (Games-Howell Post Hoc test) for **A = 28 days and B=42 days.**

#### **4.3.7 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on lipid profile in rats**

There was a statistically significant increase in the level of triglycerides in rats treated with Sitagliptin and *M. oleifera* compared to rats treated with only Sitagliptin and rats in normal saline, diabetic and post prandial control groups. There was no statistically significant difference in the levels of total cholesterol, high density lipoproteins and low density lipoprotein in the drug treated groups compared to control. Considerable, but non significant decreases in total cholesterol, triglycerides and low density lipoproteins in post prandial treated group compared to post prandial control (Table 4.5).

**Table 4.5: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Lipid Profile in Rats**

Groups	Serum lipids (mmol)			
	TC	TG	HDL	LDL
NC	2.55±0.14	0.56±0.10**	0.74±0.04	1.49±0.10
DC	3.23±0.21	0.85±0.11*	0.74±0.06	2.10±0.18
ST	3.12±0.25	0.73±0.09*	0.84±0.45	1.82±0.22
MO	3.28±0.46	1.60±0.51	0.69±0.62	1.86±0.44
SM	3.63±0.19	1.35±0.10	0.78±0.94	2.23±0.22
PPC	3.15±0.33	0.82±0.07*	0.76±0.07	2.01±0.28
PPSM	2.71±0.03	0.77±0.10*	0.79±0.07	1.63±0.06

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

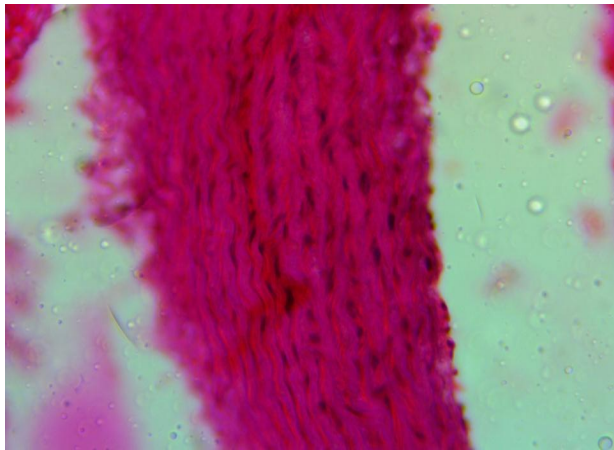
NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*. TC= Total Cholesterol, TG = Triglyceride, HDL = High Density Lipoproteins, LDL = Low Density Lipoprotein.

Ameliorative = group that received treatment at onset of complication.

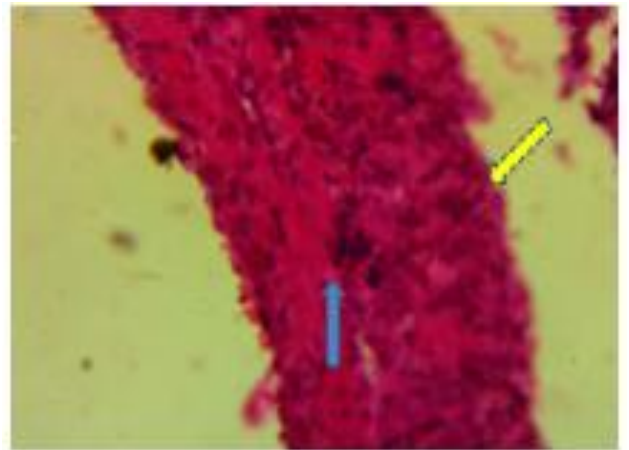
Values are mean ± SEM. Significant decrease \* =  $p < 0.05$ , \*\* =  $p < 0.005$  compared with SM. One way ANOVA, (Games-Howell Post Hoc test). **Duration of treatment = 42days.**

#### **4.3.8 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on histology of the aorta of rats**

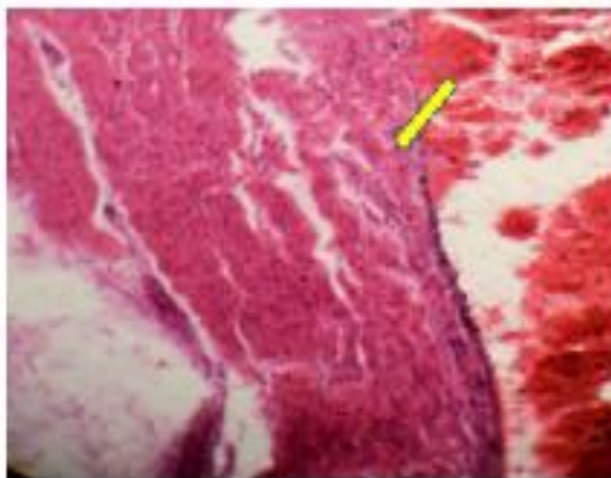
Sections in Normal control show well defined aortic wall. No significant histopathological findings seen, following the co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* after 42 days compared to the localized aortic vasculitis and thickening of the aortic wall seen in the diabetic control. However there was no amelioration of the lesions (thickening of the aortic wall) in the ameliorative group (Plate VI).



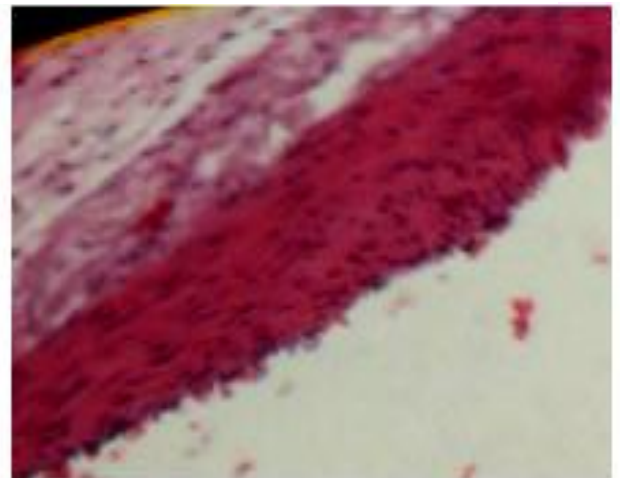
Normal Control



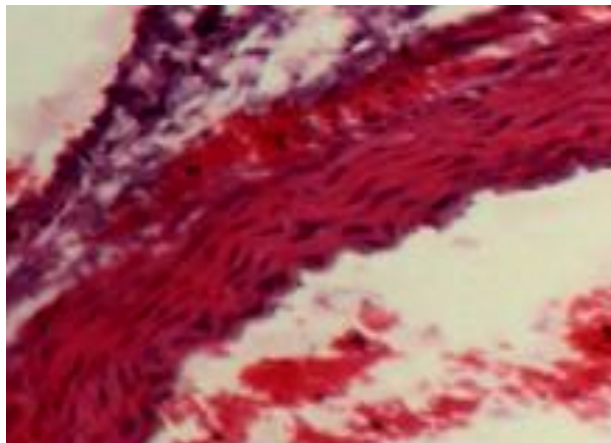
Diabetic Control



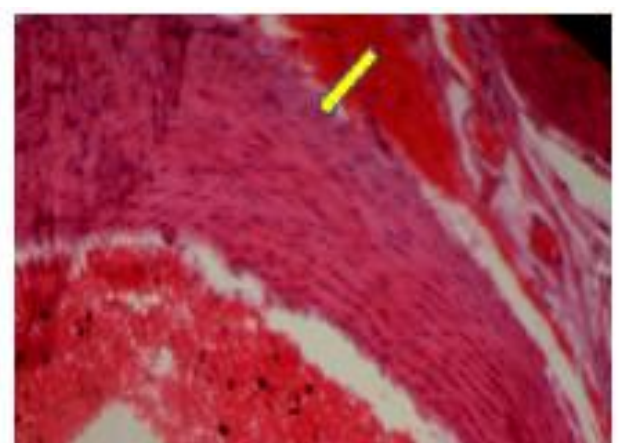
Sitagliptin treated rat



*M. oleifera* treated rats (NSHF)



Sitagliptin & *M. oleifera* treated rat (NSHF)



Ameliorative (Sitagliptin & *M. oleifera*)

**Plate VI: Photomicrographs of sections of the aorta of controls and drug treated rats (H&E X 400).**

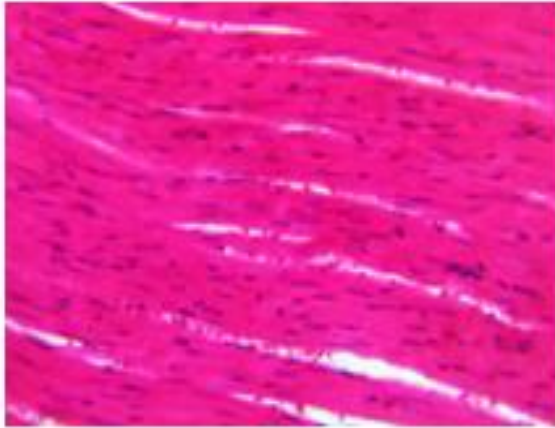
(↑) = localized aortic vasculitis, (⇩) = thickening of the aortic wall NSHF = No significant histopathological findings.

Details in Plates XI-XVII (Appendix VIII).

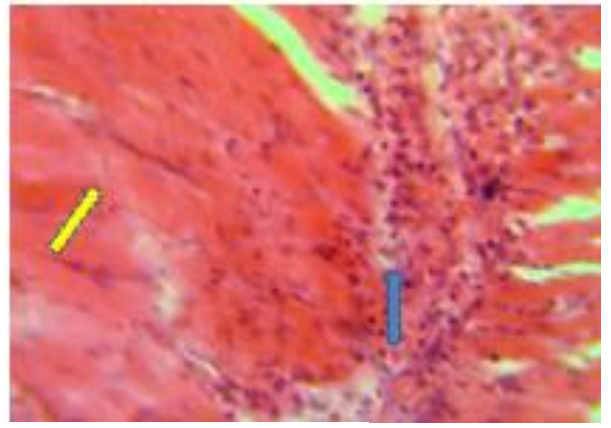
#### **4.3.9 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on histology of the heart of rats**

Sections in normal control show well arranged striated myofibrils and nucleated cells. The sections of the heart of rats that received Sitagliptin and *M. oleifera* leaf extract, and Sitagliptin as single agent, showed areas of congestion between myofibrils, but no necrosis. However, sections in rats in the ameliorative group, showed areas of mononuclear cellular infiltration and necrosis of myofibrils (myocarditis) as seen in the diabetic controls (Plate VII). Only sections of the heart in rats that received *M. oleifera* as single agent showed no histopathological findings.

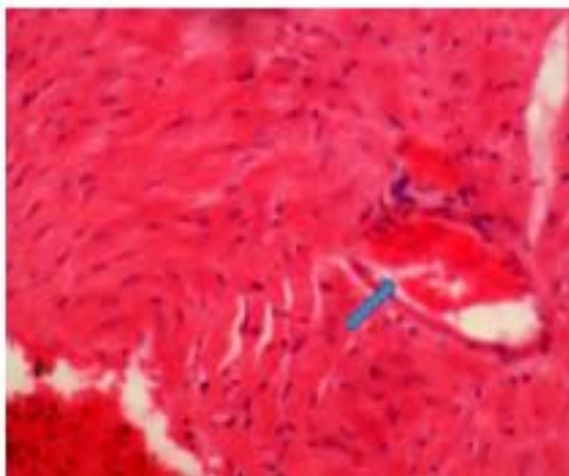




Normal Control



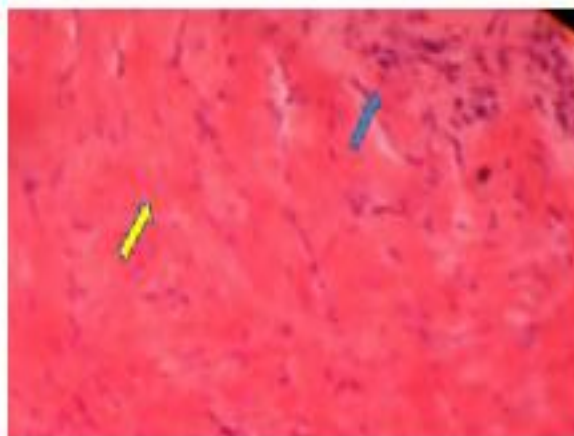
Diabetic Control



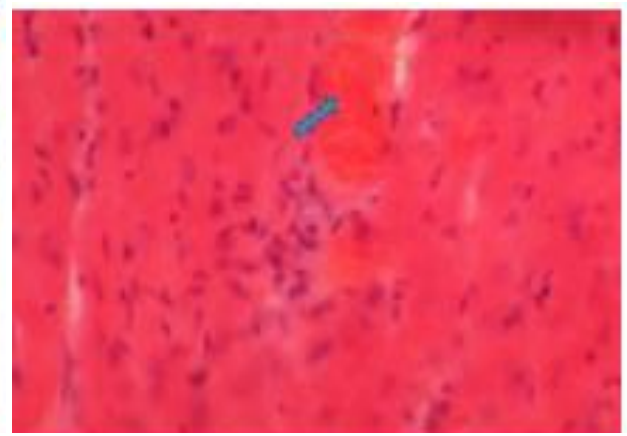
Sitagliptin treated rat



*M. oleifera* treated rat (NSHF)



Sitagliptin & *M. oleifera* treated rat



Ameliorative (Sitagliptin & *M. oleifera*)

**Plate VII: Photomicrographs of sections of the heart of controls and drug treated rats (H&E X 400).**

Normal control show well arranged striated myofibrils and cells. (↑) = areas of focal mononuclear cellular infiltration/congestion, (↓) = necrosis of myofibrils (myocarditis). NSHF = No significant histopathological findings. Details in Plates XVII-XXII (Appendix VIII).

#### **4.4 Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Progression and Possible Amelioration of Diabetic Nephropathy in Rats**

##### **4.4.1 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on serum electrolytes in rats**

The co-administration of Sitagliptin and *M. oleifera* did not bring about statistically significant changes in the serum levels of electrolytes compared to diabetic controls and the single agents (Table 4.6).

**Table 4.6: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Serum Electrolytes in Rats**

Serum Electrolytes (mmol/L)				
Group	Potassium (K <sup>+</sup> )	Sodium (Na <sup>+</sup> )	Chloride (Cl <sup>-</sup> )	Bicarbonate (HCO <sub>3</sub> <sup>-</sup> )
NC	4.57± 0.08	137.53 ± 1.03	108.86 ± 1.31	22.66 ± 0.98
DC	5.11 ± 0.12	138.62 ±2.23	106.92 ± 1.81	23.55 ± 1.47
ST	4.76 ± 0.33	138.54 ±2.49	109.53 ± 1.74	22.53 ± 1.70
MO	4.74 ± 0.22	130.42 ± 5.85	110.53 ± 3.74	21.48 ± 2.95
SM	5.13 ± 0.14	129.35 ± 3.61	102.00 ± 3.28	20.22 ±2.33
PPC	4.88 ± 0.16	135.10 ±1.91	106.58 ± 1.25	17.34 ± 4.03
PPSM	4.81 ± 0.82	138.07 ± 0.75	112.20 ± 4.92	22.75 ± 2.34

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

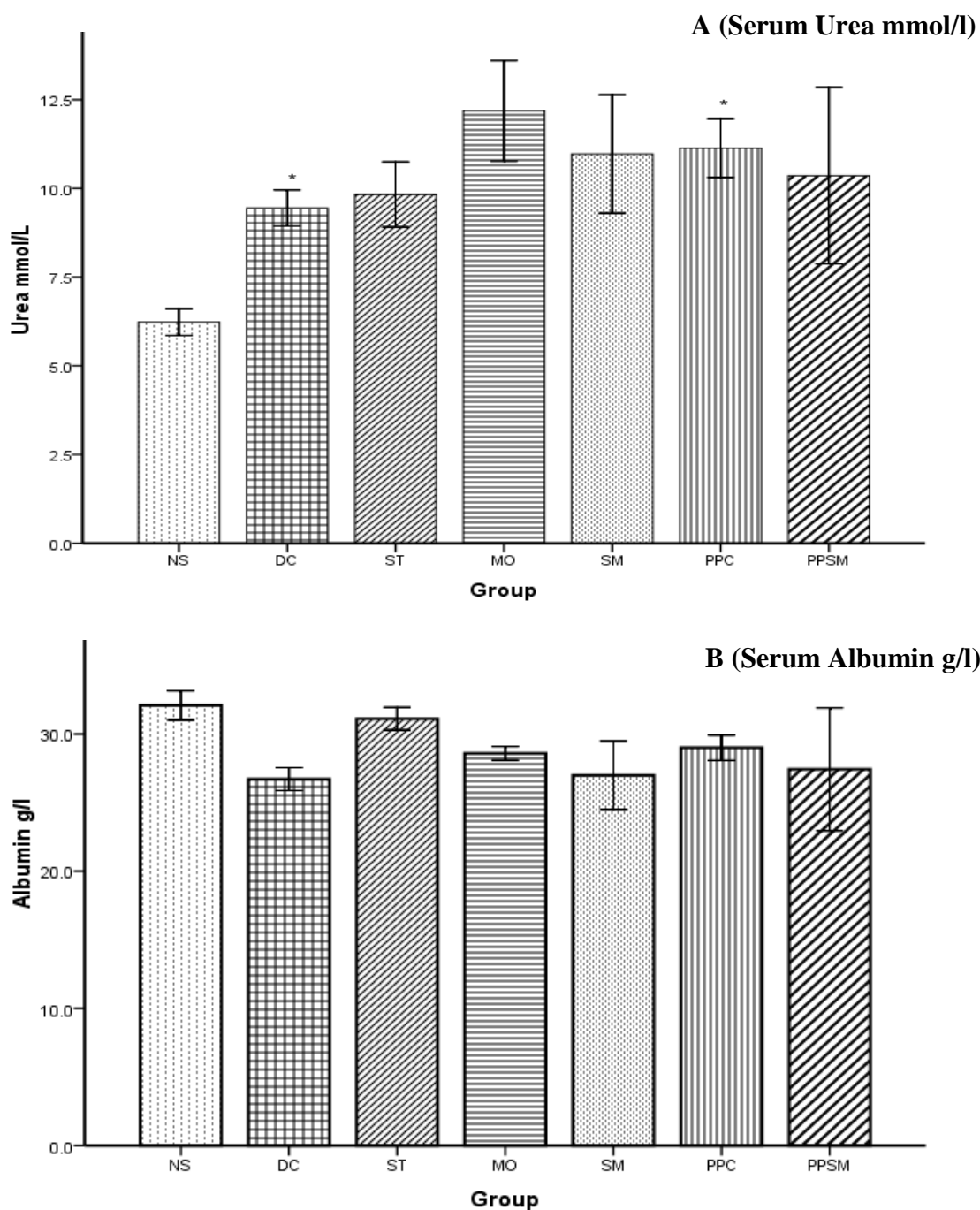
NC= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M.oleifera*, SM= Sitagliptin and *M.oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M.oleifera*.

Ameliorative = group that received treatment at onset of complication.

Values are mean ± SEM. No Significant difference ( $p>0.05$ ) between groups (One Way ANOVA).  
**Duration of treatment =42 days.**

#### **4.4.2 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mean serum levels of urea and albumin in rats**

The co-administration of Sitagliptin and *M. oleifera* increased (not significantly) serum urea levels in rats compared to diabetic control. However a statistically significant increase ( $p<0.05$ ) was seen in serum urea levels of the diabetic and post prandial controls compared to normal control (Figure 4.8A). There was also an increase in the serum urea levels of rats that were treated with *M. oleifera* alone compared to diabetic control. There was no statistically significant difference in serum albumin levels in the drug treated groups compared to controls (Figure 4.8B).



**Figure 4.8: Mean Serum Levels of Urea and Albumin Following Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* in Rats**

NS= Normal control, DC= Diabetic control, ST= Sitagliptin, MO= *M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC= Postprandial control, PPSM = Ameliorative on Sitagliptin and *M. oleifera*. Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg).

Values are mean  $\pm$  SEM. Significant difference (**A: Urea mmol/l**)  $p < 0.05$ , Normal control compared to Daibetic and Postprandial control (One Way ANOVA) No significant difference between groups (**B: Albumin g/l**). **Duration of treatment = 42 days.**

#### **4.4.3 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on relative heart and kidney weights of rats**

There was a statistically significant increase ( $p < 0.001$ ) in the relative kidney weight of rats treated with Sitagliptin and *M. oleifera* and rats treated with only Sitagliptin and only *M. oleifera* ( $p < 0.05$ ) compared to normal control (Table 4.7). However, there was no statistically significant decrease in the relative heart weights of rats treated with Sitagliptin and *M. oleifera* compared to diabetic control (Table 4.7). There was also no significant decrease in relative kidney weight in the post prandial drug treated group compared to post prandial control.

**Table 4.7: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Relative Heart and Kidney Weight of Rats**

Relative Organ Weights		
Group	Heart	Kidney
Normal control	0.44 ± 0.020	0.30 ± 0.007
Diabetic control	0.55 ± 0.028	0.40 ± 0.020*
Sitagliptin	0.56 ± 0.038	0.41 ± 0.017*
<i>M. oleifera</i>	0.56 ± 0.024	0.50 ± 0.023*
Sitagliptin & <i>M. oleifera</i>	0.48 ± 0.032	0.42 ± 0.007**
Postprandial control	0.45 ± 0.008	0.40 ± 0.030
Ameliorative (Sitagliptin & <i>M. oleifera</i> )	0.54 ± 0.067	0.41 ± 0.040

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

Ameliorative = group that received treatment at onset of complication.

Values are mean ± SEM. Significant difference \*= $p < 0.05$ , \*\*= $p < 0.001$  compared to Normal control. One Way ANOVA (Games-Howell Post Hoc test). **Duration of Treatment = 42 days.**

#### **4.4.4 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on liver enzymes in rats**

Statistically significant increase in serum levels of alanine transaminase ( $p < 0.05$ ) and non significant increase in aspartate transaminase in rats treated with Sitagliptin and *M. oleifera* compared to normal control (Table 4.8). Increased serum levels of alanine transaminase and aspartate transaminase in rats treated with *M. oleifera* alone compared to diabetic and normal control. Rats in the ameliorative group, also showed increased levels of aspartate transaminase compared to post prandial control.



**Table 4.8: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Liver Enzymes in Rats**

Groups	ALT U/L	AST U/L
Normal control	40.29 ± 1.36	147.41 ± 5.71
Diabetic control	48.17 ± 3.03	182.82 ± 12.04
Sitagliptin	45.36 ± 4.88	152.46 ± 12.99
<i>M. oleifera</i>	58.40 ± 7.67	187.10 ± 37.44
Sitagliptin+ <i>M. oleifera</i>	66.00 ± 14.49*	226.65 ± 50.76
Postprandial control	47.97 ± 5.81	148.74 ± 21.07
Ameliorative (Sitagliptin+ <i>M. oleifera</i> )	39.23 ± 8.82	190.07 ± 34.07

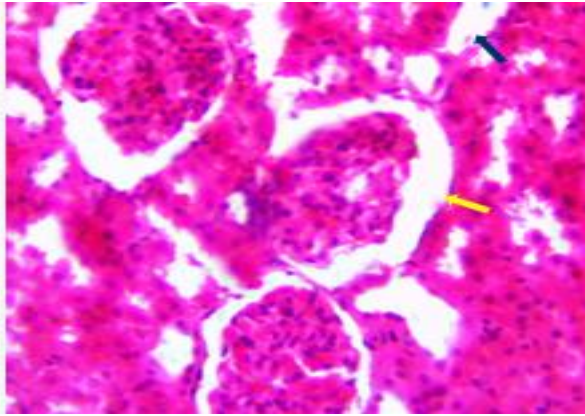
Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

Ameliorative = group that received treatment at onset of complication.

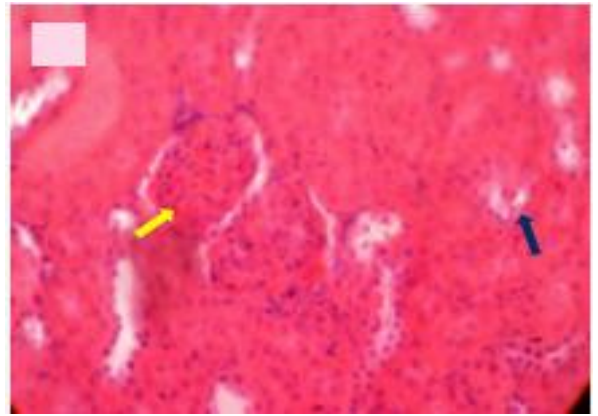
Values are mean ± SEM. Significant difference between groups,  $p < 0.05$ . (One Way ANOVA).  
Horchberg Post hoc test. **Duration of Treatment = 42 days**

#### **4.4.5 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on histology of the kidney of rats**

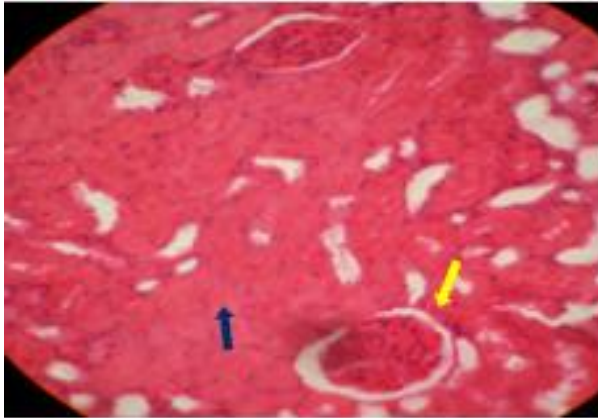
Section in normal control shows well defined Bowmans space and normal tubules. There was a mild difference in the severity of pathologic lesions seen in sections of kidney of rats treated with Sitagliptin and *M. oleifera*, as this showed necrosis of most renal tubular epithelial cells with thin Bowmans space characterized as glomerulosclerosis grade 1 compared to diabetic control. Rats in diabetic control had marked necrosis of renal tubular cells, with the Bowmans capsule adhered to the glomerulus, and graded as glomerulosclerosis grade 3 (Plate VIII). However, rats in the post prandial diabetic control showed the most severe pathologic lesions evident by a complete obliteration of the Bowmans space and this is graded as glomerulosclerosis grade 4. There was no significant amelioration of the lesions in the Ameliorative group as the Bowmans space was also obliterated with significant tubular necrosis, graded as glomerulosclerosis grade 3 (Plate VIII).



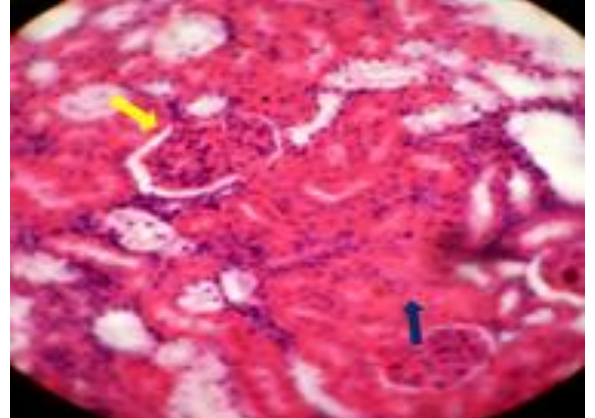
Normal Control



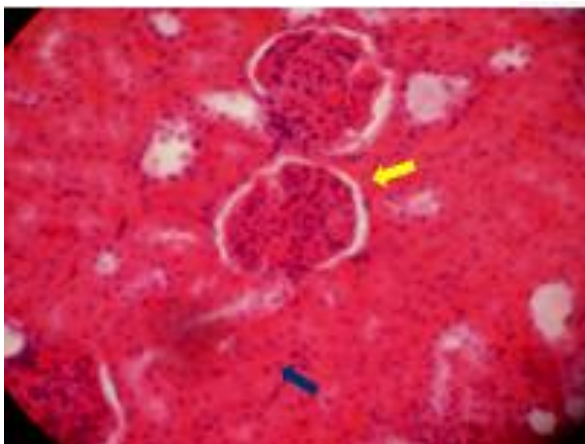
Diabetic Control



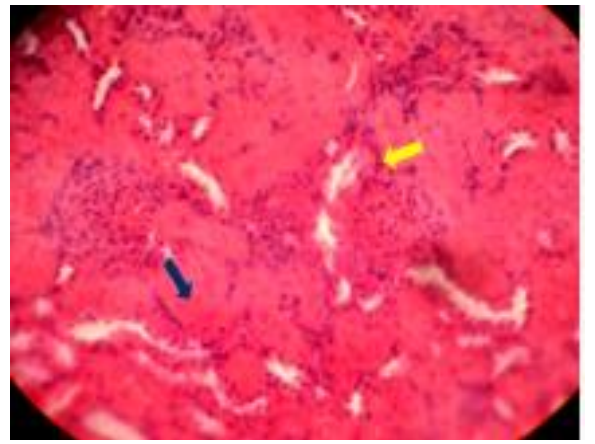
Sitagliptin treated rat



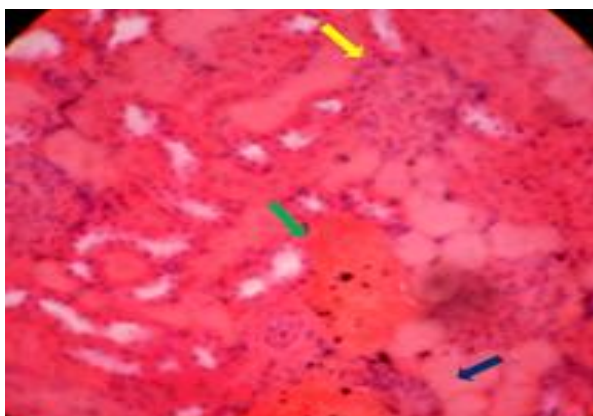
*M. oleifera* treated rat



Sitagliptin & *M. oleifera* treated rat



Ameliorative (Sitagliptin & *M. oleifera*)



Post prandial control

**Plate VIII: Photomicrographs of sections of the kidney of controls and drug treated rats (H&E X 400).**

(↑) = Tubular necrosis, filled with pink amorphous material. (↑) = Bowmans space is clear in normal control, thin in drug treated groups and obliterated in diabetic controls. (↑) = congestion in the kidney.

Details in Plates XXIII-XXIX (Appendix VIII).

#### **4.5 Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Onset and Progression of Diabetic Neuropathy in Rats**

##### **4.5.1 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on thermal hyperalgesia (Latency for Paw Withdrawal) in rats**

There was a statistically significant decrease ( $p < 0.05$ ) in latency for paw withdrawal following the co-administration of Sitagliptin and *M. oleifera* at the 5<sup>th</sup> week compared to week 2. However at the 6<sup>th</sup> week the latency further increased ( $p < 0.005$ ) and this was same for rats treated with only Sitagliptin and rats in the Ameliorative group (Table 4.9). Rats that received only *M. oleifera* showed a statistically significant decrease in latency for paw withdrawal from the 3<sup>rd</sup> week to the 6<sup>th</sup> week compared with week 2 (Table 4.9). There was overall, a highly significant difference ( $p < 0.001$ ) comparing the normal control with the diabetic control and diabetic treated groups at all the time points.

**Table 4.9: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Thermal Hyperalgesia (Latency for Paw Withdrawal) in Rats**

Latency for Paw Withdrawal (Secs)					
Group	Week 2	Week 3	Week 4	Week 5	Week 6
NC	3.05 ± 0.15	2.56 ± 0.12	3.42 ± 0.19	2.40 ± 0.10	2.80 ± 0.16
DC	1.50 ± 0.18	1.67 ± 0.14	1.26 ± 0.22	1.44 ± 0.11	1.74 ± 0.18
ST	1.64 ± 0.17	1.74 ± 0.13	1.77 ± 0.11	1.25 ± 0.11**	1.74 ± 0.17
MO	2.23 ± 0.18	1.49 ± 0.14*	1.44 ± 0.22*	1.45 ± 0.12**	1.24 ± 0.18**
SM	1.94 ± 0.18	1.78 ± 0.14	1.58 ± 0.22	1.30 ± 0.12*	1.54 ± 0.18
PPC	2.04 ± 0.18	1.66 ± 0.14	2.00 ± 0.22	1.33 ± 0.12*	1.94 ± 0.18
PPSM	1.81 ± 0.17	1.72 ± 0.13	1.70 ± 0.20	1.15 ± 0.11*	1.38 ± 1.17

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

NC= Normal control, DC= Diabetic control, ST= Sitagliptin, MO= *M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC= Postprandial control, PPSM = Ameliorative on Sitagliptin and *M. oleifera*

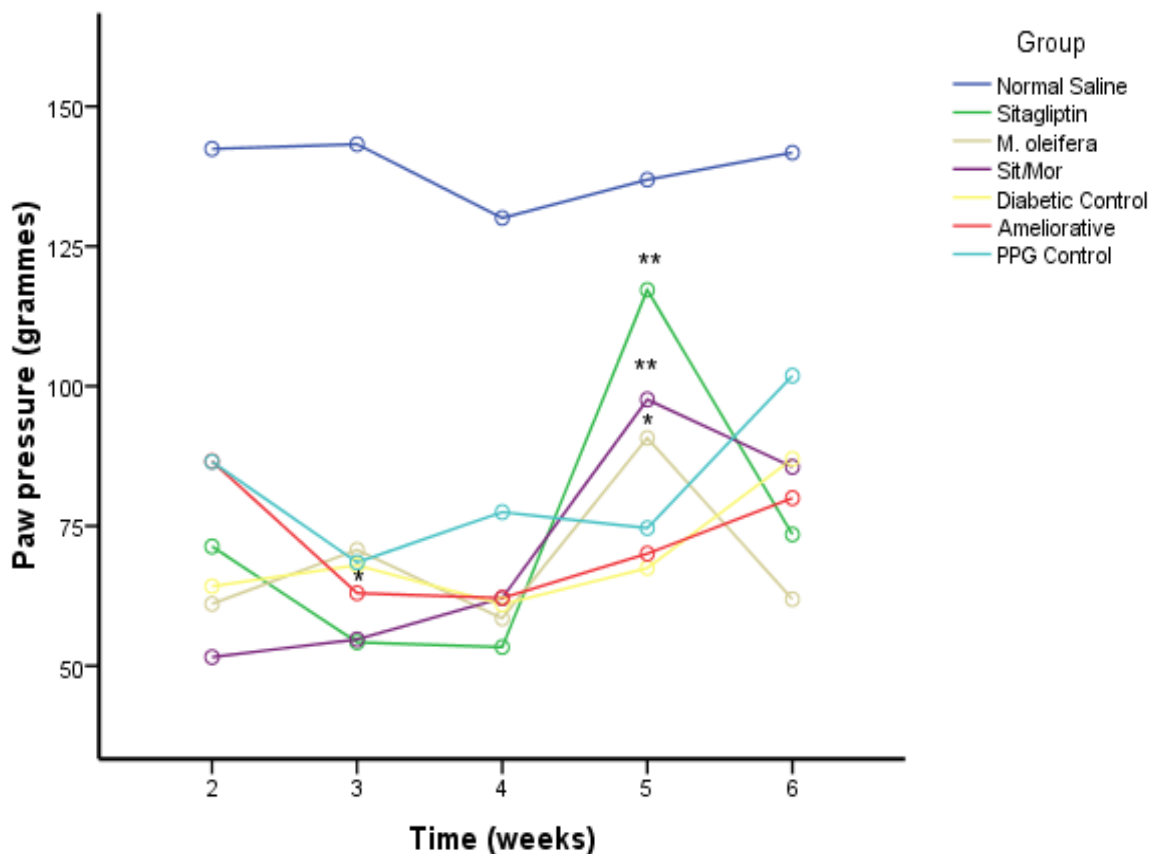
Ameliorative = group that received treatment at onset of complication.

Values are mean ± SEM. Significant difference \* $p < 0.05$ , \*\* $p < 0.005$  Split Plot ANNOVA . Sitagliptin (comparing week 3 to 5), *M. oleifera* (compared to week 2). Sitagliptin + *M. oleifera* (compared to weeks 2 and 3), Postprandial control and Ameliorative group is in comparison to week 1 (Bonferoni Post Hoc test). **Duration of Treatment = 42 days**

Overall group effect also showed significant decrease  $p < 0.001$ , in latency for paw withdrawal comparing Normal control to Diabetic control and Drug Treated groups.

#### **4.5.2 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on mechanical hyperalgesia (Paw Pressure Test) in Rats**

There was a statistically significant increase ( $p<0.05$ ) in the pressure in force that rats could tolerate on their paws, following the co-administration of Sitagliptin and *M. oleifera* in week 5 compared to weeks 3 and 4. This trend was similar following administration of the Sitagliptin and *M. oleifera* as single agents. However rats in the Ameliorative group showed a statistically significant decrease ( $p<0.05$ ) in the force for paw pressure only in week 3 in comparison with week 2 and thereafter an increase was further seen as the weeks progressed (Figure 4.9). There was also an overall highly significant difference ( $p<0.001$ ) comparing the normal control, with diabetic control and diabetic treated groups at all the time points.



**Figure 4.9: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Mechanical Hyperalgesia (Paw Pressure Test)**

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg).

.NS= Normal control, DC= Diabetic control, ST= Sitagliptin, MO= *M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC= Postprandial control, PPSM = Ameliorative on Sitagliptin and *M. oleifera*.  
Ameliorative = group that received treatment at onset of complication

Values are mean  $\pm$  SEM. Significant difference  $*$ =  $p < 0.05$ ,  $**$ =  $p < 0.001$  Split Plot ANNOVA. Sitagliptin (compared to weeks 2, 3 4 and 6) and *M. oleifera* (compared to Week 4). Sitagliptin + *M. oleifera* (compared to weeks 2 and 3). Ameliorative group is in comparison to week 2 (Bonferoni Post Hoc test) **Duration of Treatment = 42 days.**

Overall Group Effect also showed significant decrease  $p < 0.001$ , in latency for paw withdrawal comparing Normal control to Diabetic control and all Drug Treated groups.

#### **4.5.3 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on alodynia (Latency for Tail Flick) in rats**

There was a statistically significant decrease in the latency for tail flick for thermal alodynia at the 4<sup>th</sup> week ( $p < 0.05$ ) and the 6<sup>th</sup> week ( $p < 0.005$ ) following the co-administration of Sitagliptin and *M. oleifera* in comparison with week 2, with a similar trend in rats treated with only *M. oleifera* (Table 4.10). The diabetic control and post prandial control showed a steady decrease in latency for tail flick throughout the study period with a statistically significant decrease at the 6<sup>th</sup> week compared to week 2. There was no statistically significant difference across time points in the latency for tail flick throughout the study period in the Ameliorative group (Table 4.10).



**Table 4.10: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Alodynia (Latency for Tail Flick) in Rats**

Latency for Tail Flick (Secs)					
Group	Week 2	Week 3	Week 4	Week 5	Week 6
DC	6.11 ± 1.23	5.7 ± 1.8	5.5 ± 0.66	3.82 ± 0.82	3.65 ± 0.7*
ST	9.43 ± 1.17	7.46 ± 0.92	5.90 ± 0.61	6.45 ± 0.75	7.0 ± 0.64
MO	11.08 ± 1.15	8.02 ± 1.0	5.6 ± 0.66**	7.07 ± 0.81	4.44 ± 0.70**
SM	10.42 ± 1.23	6.82 ± 1.0	6.13 ± 1.0*	8.62 ± 0.82	4.95 ± 0.70**
PPC	9.42 ± 1.23	7.21 ± 1.0	6.63 ± 0.66	5.28 ± 0.81*	5.15 ± 0.70**
PPSM	8.64 ± 1.16	9.31 ± 0.92	6.23 ± 0.61	6.39 ± 0.76	7.38 ± 0.64

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

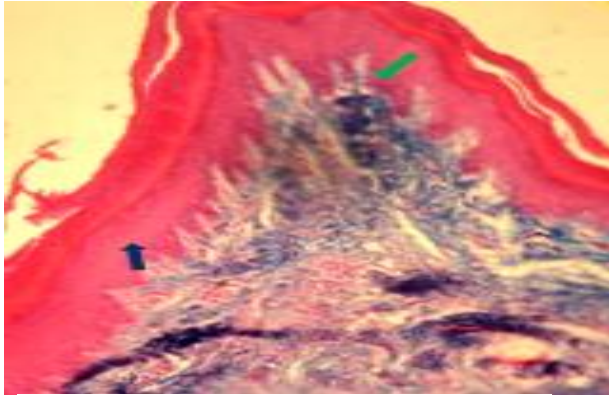
NC= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M. oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Ameliorative = group that received treatment at onset of complication.

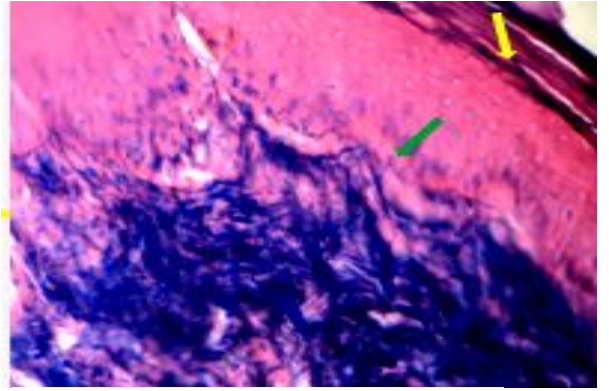
Values are mean ± SEM. Significant difference \*= P<0.05, \*\*= P<0.005 compared to week 2, Split Plot ANOVA (Bonferoni Post Hoc test). **Duration of Treatment = 42 days.**

#### **4.5.4 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* on histology of the skin of hind paw of rats**

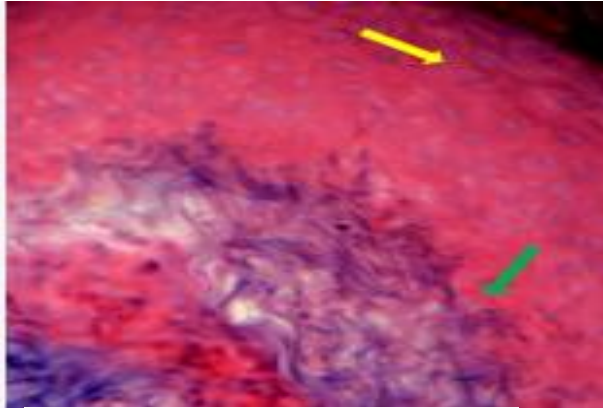
Section in Normal control shows high density of intraepidermal nerve fibres projecting from the dermis into the epidermis, in addition to a well defined keratinized layer. There was no much difference in the pathology of sections of skin of the hind paw in rats treated with Sitagliptin and *M. oleifera*, as moderate intra epidermal nerve fibre density was seen, and similarly, in the diabetic control (Plate IX). Sections in the post prandial control, however, had very low intraepidermal nerve fibre density.



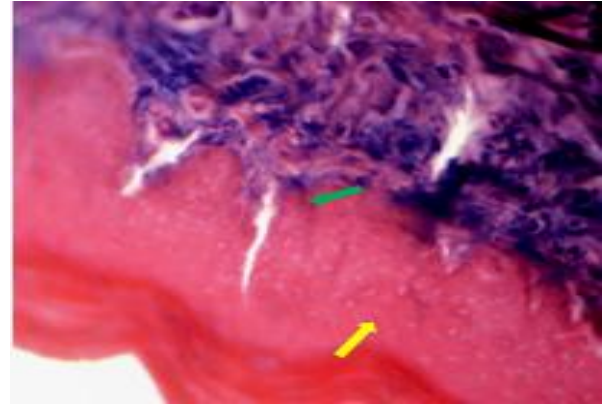
Normal Control



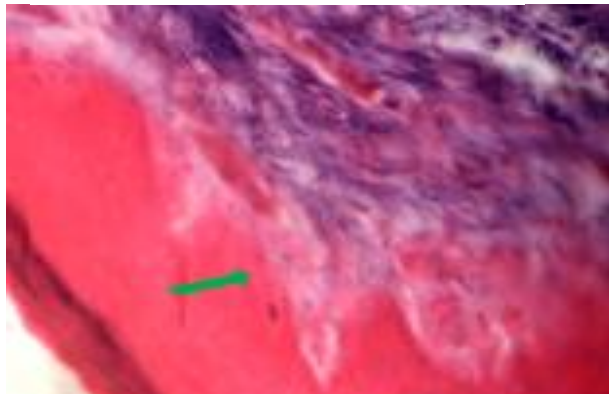
Diabetic Control



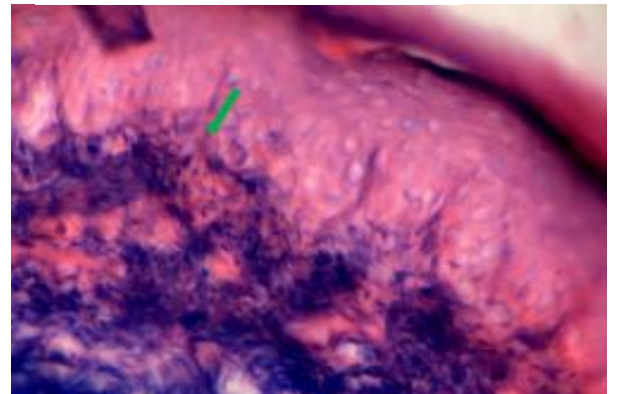
Sitagliptin treated rat



*M. oleifera* treated rat



Sitagliptin & *M. oleifera* treated rat



Ameliorative (Sitagliptin & *M. oleifera*)



Post prandial control

**Plate IX: Photomicrographs of sections of the skin of hind paw of controls and drug treated rats (LFB & E X 400).**

(↑) = Squamous epidermis. (↑) = Intra epidermal nerve fibres projecting into the epidermis from the dermis.

Details in Plates XXX-XXXVI (Appendix VIII).

#### **4.6 Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *Moringa oleifera* on Onset and Progression of Diabetic Retinopathy**

##### **4.6.1 Effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on lenticular opacity**

Only one rat that received Sitagliptin and *M. oleifera* showed vacuoles (subcapsular cataract) at the end of the 4<sup>th</sup> and 7<sup>th</sup> week post diabetic induction (Table 4.11). Rats in the negative control and *M. oleifera* groups showed clear lenses with no vacuoles, while diabetic control group had 4 rats already with vacuoles forming a subcapsular cataract after 4 weeks of diabetic induction. At the end of the 7<sup>th</sup> week post diabetic induction 3 rats from the diabetic control still showed subcapsular cataract while only one had a hazy cortex with dense nuclear opacity (Table 4.11).

**Table 4.11: Effect of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Lenticular Opacity**

Group	Week 3				Week 6			
	Stages (I-IV) / Number of Rats							
	I	II	III	IV	I	II	III	IV
NS		Clear				Clear		
DC	4				3	2		
ST				1				1
MO		Clear				Clear		
SM		1				1		
PPC	1					1		
PPSM	1				1			

Sitagliptin (50 mg/kg), *Moringa oleifera* extract (300 mg/kg)

NS= Normal control, DC= Diabetic control, ST =Sitagliptin, MO =*M.oleifera*, SM= Sitagliptin and *M. oleifera*, PPC =Postprandial control, PPSM= Ameliorative on Sitagliptin and *M. oleifera*.

Ameliorative = group that received treatment at onset of complication.

#### **Staging as described by Suryanarayana *et al.* (2005)**

Clear: clear lenses and no vacuoles present

Stage 1: vacuoles cover part of the cortex forming a subcapsular cataract

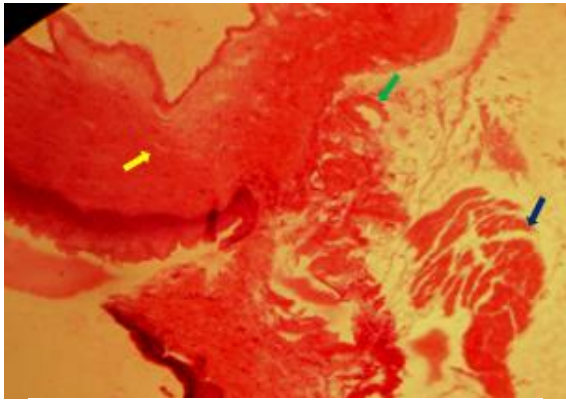
Stage 2: some vacuoles have disappeared and the cortex exhibits a hazy opacity

Stage 3: a hazy cortex remained and dense nuclear opacity is present

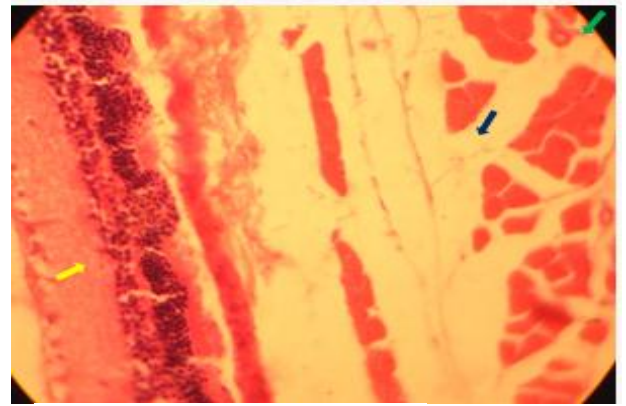
Stage 4: a mature cataract is observed as a dense opacity in both cortex and nucleus.

#### **4.6.2 Effect of the co-administration of Sitagliptin and ethanol leaf extract of *M. oleifera* on histology of the retina of rats**

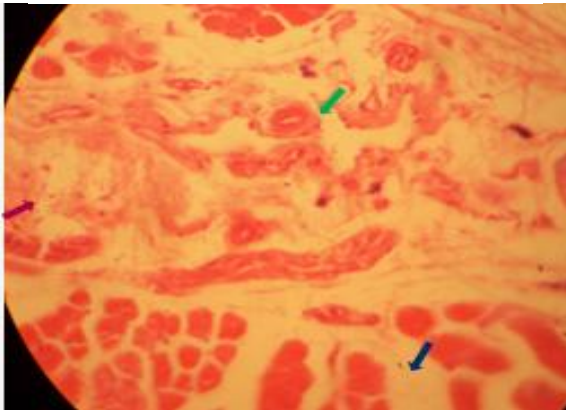
Section in normal control shows intact musculature, normal retinal cell layer arrangement and capillary wall. Moderate distortion in retinal cell layer arrangement, thickened capillary wall and oedema between the layers of the myofibrils were seen in sections of the retina and its associated structures following the co-administration of Sitagliptin and *M. oleifera* for 42 days in diabetic rats (Plate X). The diabetic controls, and other drug treated groups except the group that received *M. oleifera* alone had similar pathologic findings with associated congestion and haemorrhage. Rats that received *M. oleifera* alone showed no significant histopathological findings (Plate X).



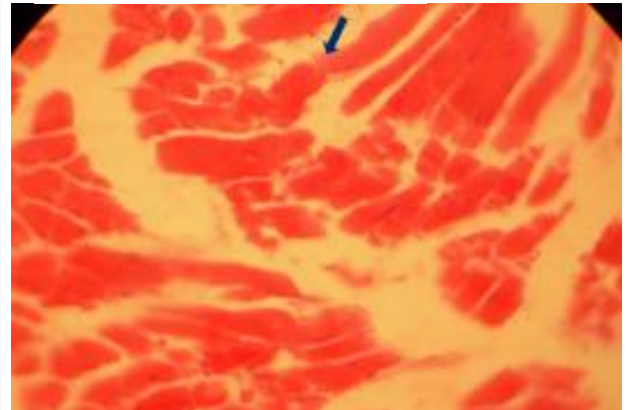
Normal Control



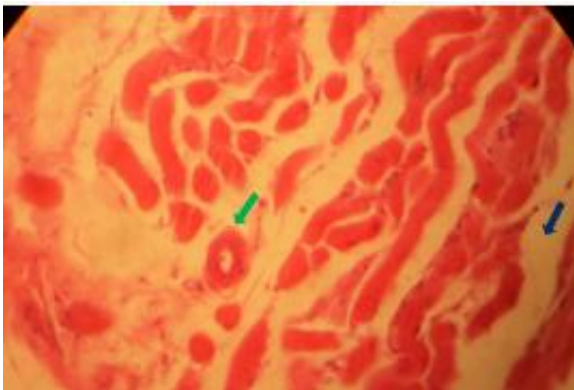
Diabetic Control



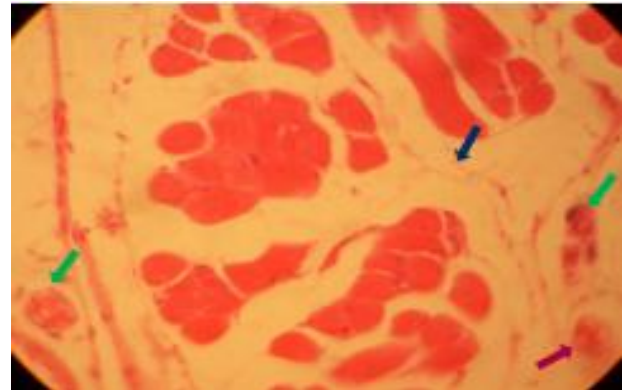
Sitagliptin treated rat



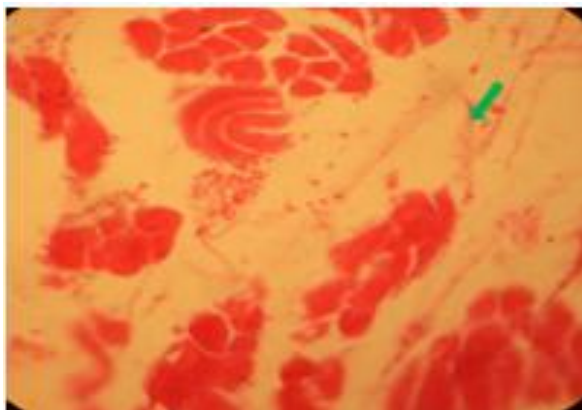
*M. oleifera* treated rat



Sitagliptin & *M. oleifera* treated rat



Ameliorative (Sitagliptin & *M. oleifera*)



Post prandial Control

**Plate X: Photomicrographs of sections of the adjoining structures of the retina of controls and drug treated rats (H&E X 400).**

(↑) = Retinal cell layer. (↓) = Normal capillary wall /thickened wall in diabetic and treated groups. (↕) = Intact musculature in normal control/oedema between layers of myofibrils in diabetic and treated groups. (⊥) = congestion/haemorrhage.

Details in Plates XXXVII-XLVIII (Appendix VIII).

**Table 4.12: Summary of the Effects of Co-administration of Sitagliptin and Ethanol Leaf Extract of *M. oleifera* on Pathologic Lesions of the Kidney, Heart, Eyes and Skin of Hind Paw of Rat**

<b>Group</b>	<b>Kidneys</b>	<b>Heart</b>	<b>Aorta</b>	<b>Skin</b>	<b>Eye</b>
<b>Diabetic control</b>	Necrosis of many tubules and are filled with PM. Bowmans capsule adhered to glomeruli	focal areas of necrosis and congestion	localized aortic vasculitis and thickening of the aortic wall	Low nerve fibre density and reduced keratin layer with a number of fat globules.	Thickened capillary wall and oedema between layers of myofibrils and between retina and myofibrils
<b>Sitagliptin</b>	Tubules show necrosis and are filled with PM. Thin Bowmans space	congestion of spaces between myofibrils with no necrosis	Thickening of the aortic wall	Moderate intraepidermal nerve fibre density	Thickened capillary wall, oedema between layers of myofibrils and haemorrhage
<b><i>M. oleifera</i></b>	Necrosis of tubules and are filled with PM. Thin Bowmans space.	NSHF	NSHF	Moderate intraepidermal nerve fibre density	NSHF
<b>Sitagliptin &amp; <i>M. oleifera</i></b>	Necrosis of most tubules and are filled with PM. Thin Bowmans space. Intact glomerulus	congested heart	NSHF	Moderate intraepidermal nerve fibre density	Moderate distortion in retinal cell layer arrangement, thickened capillary wall and oedema
<b>Postprandial control</b>	Necrosis of tubules, filled with PM. Total obliteration of Bowmans capsule	focal areas of necrosis and congestion	No distinct aorta could be viewed	Very low intraepidermal nerve fibre density	Moderate distortion in retinal cell layer arrangement, thickened capillary wall and mild oedema
<b>Ameliorative Sitagliptin &amp; <i>M. oleifera</i></b>	Necrosis of renal tubular epithelial cells. Obliteration of Bowmans capsule	mononuclear cellular infiltration and necrosis of myofibrils (myocarditis)	Thickening of the aortic wall	Moderate intraepidermal nerve fibre density	Thickened capillary wall, oedema between layers of myofibrils and congestion

NSHF: No significant histopathological findings, PM: Proteineous Material



## CHAPTER FIVE

### 5.0 DISCUSSION

Potential herb-drug interactions are a major safety concern as they may represent a potential risk or cause adverse clinical effects to patients under conventional pharmacotherapy. As such, a strong need exists to describe these important interactions. Sitagliptin and ethanol leaf extract of *M. oleifera* were investigated for potential drug herb interactions.

The oral LD<sub>50</sub> of *M. oleifera* estimated to be greater than 2,000 mg/kg shows that the plant is non toxic and this is similar to the results obtained by Das and Kanodia (2012). All the organs examined did not show any significant histopathology, further confirming its non toxic nature when used acutely as also demonstrated by Chivapat *et al.* (2011). However, an acute toxicity study using doses of 1600mg/kg and 2000mg/kg led to 1/6 and 2/6 of deaths in rats (Adedapo *et al.*, 2009). Nonetheless, the authors concluded that nutritional and therapeutic consumption of *M. oleifera* is safe at doses below 2000mg/kg.

Significant reductions in fasting and random blood glucose after co-administration of Sitagliptin and *M. oleifera* in this study followed the pattern seen in rats that received only Sitagliptin (onset after 2 weeks of administration). However, on chronic administration for greater than 4 weeks a reduced antihyperglycaemic effect was seen in FBG following the pattern as seen in rats treated with only *M. oleifera*. Significant reductions in FBG in animal and PPG in human studies have been demonstrated following Sitagliptin administration (Aschner *et al.*, 2006; Rizzo *et al.*, 2012; Abu-Amara and Gebaly, 2012). Previous studies indicate that DPP-4 inhibitors have a

small effect on post meal glycaemic levels (DeFronzo *et al.*, 2008; Vilsboll *et al.*, 2010), which probably was why the effect of Sitagliptin on RBG in this study was seen only on day 42. The majority of the HbA1c lowering effect of DPP-4 inhibitors results instead from reductions in FPG. The speculation is that DPP-4 inhibitors may exert more of an effect on FPG through augmentation of the portal signal with modest stimulation of insulin secretion compared to GLP-1 agonist (Fineman *et al.*, 2012). This probably explains the effect seen more on FBG than on RBG on co-administration of Sitagliptin and *M. oleifera*. This effect is very important as there is evidence that at the early stages of type 2 diabetes, even when fasting glucose and HbA1c are within normal ranges, post meal hyperglycemia is implicated in both macrovascular complications and microvascular complications (Ceriello *et al.*, 2004; Leiter *et al.*, 2005). This is because, hyperglycemic spikes after meals, induces endothelial dysfunction and inflammatory reactions leading to progression of atherosclerosis (Cavalot *et al.*, 2006; Node and Inoue, 2009).

Co-administration of *M. oleifera* with Sitagliptin did not alter this reduced effect of Sitagliptin on RBG (onset seen after 6 weeks of administration) but on prolonged administration reduced the fasting glycaemic effect. Animal studies have shown significant reduction in blood glucose concentration following administration of *M. oleifera* in diabetic rats (Abd El Latif *et al.*, 2014). However, another study on *M. oleifera* concludes on exercising caution on long term administration of *M. oleifera* following increased levels of urea and malondialdehyde in treated rats (Awodele *et al.*, 2012).

No demonstrable effect was also seen in insulin levels following the co-administration of Sitagliptin and *M. oleifera* even after prolonged administration. This suggests that blood glucose lowering effect of *M. oleifera*, was probably through other mechanisms. This include, reducing glucose absorption, a glucosidase inhibitory effect or reduction in hepatic gluconeogenesis, as Sitagliptin shows no effect on fasting insulin levels. However, hyperinsulinaemia which is indicative of insulin resistance and early pancreatic beta-cell impairment is seen in diabetes mellitus. Insulin resistance has been associated with accelerated atherosclerosis, especially in coronary heart disease (Fang *et al.*, 2004) implying negative effects in cardiomyopathy. *In vitro* study using cell culture lines reveal the insulinotropic potential of *M. oleifera* leaf extract (Ojo, 2014) although other data suggests that quercetin, a flavonoid responsible for the antihyperglycaemic activity in *M. oleifera* controls blood glucose without any alteration in serum insulin levels (Chang *et al.*, 2013). In addition, DPP-4 inhibitors also show modest stimulation of insulin secretion compared to GLP-1 agonist (Fineman *et al.*, 2012). In this study, diabetic controls and post prandial control rats, showed non-significant increase in insulin levels as similarly demonstrated in previous animal and human studies (Lele *et al.*, 2006; AL-Zahrani *et al.*, 2012). This is of paramount importance as baseline and fasting hyperinsulinaemia have been associated with major cardiovascular events in both prospective studies and meta analysis (Ruige *et al.*, 1998; Lempiainen *et al.*, 1999) which remained significant after 15 years following adjustment for other cardiovascular risk factors. (Pyorala *et al.*, 1998).

Weight neutrality is an important consideration for antidiabetic agents especially when obese patients with an increased risk of cardiovascular complications are involved. The co-administration of Sitagliptin and *M. oleifera* displayed weight neutrality in this study. Sitagliptin unlike GLP-1 agonists do not appear to have an effect on gastric emptying which promotes satiety bringing about moderate weight loss or neutrality (Drucker and Nauck, 2006; Vella *et al.*, 2007). The administration of *M. oleifera* in non-diabetic animals has also shown non-significant increase in body weight (Awodele *et al.*, 2012).

Alloxan induced diabetic cardiomyopathy has been studied using glycaemic control parameters, oxidative stress markers and morphological alterations in the heart (Bhatti *et al.*, 2011). But the pathophysiology actually involves an initial onset of cardiac inflammation and then progression which is characterized by increased levels of proinflammatory cytokines (Westermann *et al.*, 2007). Increase in serum levels of inflammatory cytokines like TNF- $\alpha$  and CRPs has been associated with DCM in humans.

The beneficial effect of co-administration of Sitagliptin and *M. oleifera* manifested as decreased serum levels of TNF- $\alpha$  following 28 days administration was not observed in rats treated for a longer period of 42 days. This, from the study is attributed to *M. oleifera*, having the highest level of TNF- $\alpha$  alpha on chronic administration. Previous studies had also showed increased level of TNF- $\alpha$  in rats following Sitagliptin administration for 6 weeks (Ferreire *et al.*, 2010). Levels of TNF- $\alpha$  is generally increased in overweight and obese individuals, playing a key role in atherosclerosis. TNF- $\alpha$  exacerbates insulin resistance and overexpression has been

associated with cardiac hypertrophy and fibrosis, as well with left ventricular dysfunction (Yokoyama *et al.*, 1997; Sun *et al.*, 2004). The increase in serum TNF- $\alpha$  levels seen on co-administration of Sitagliptin and *M. oleifera* in the present study, might suggest undesirable side effects in the heart and arteries and may contribute to development of cardiomyopathy and atherosclerosis rather than amelioration on prolonged use.

The serum levels of CRPs another very important inflammatory biomarker was not significantly reduced following co-administration of Sitagliptin and *M. oleifera* compared to diabetic controls in both phases of the study. Although previous animal studies have shown a decrease in CRPs levels following sitagliptin administration (Ferreira *et al.*, 2010), human studies revealed an increase in CRPs levels instead (Rizzo *et al.*, 2012). Elevated levels of CRPs predicts onset of diabetic complications (Swellam *et al.*, 2009) as it is an important contributor in endothelial dysfunction and atherosclerosis (Verma *et al.*, 2004). Overexpression of human CRPs also exacerbates left ventricular dysfunction and remodeling in diabetic cardiomyopathy (Palmiere *et al.*, 2003). Though, GLP-1 has beneficial effects on the cardiovascular system, a school of thought postulates that the inhibitory effect of DPP-4 inhibitor may negate the beneficial effect of GLP-1 which is mediated through its metabolite (GLP 9-39). As such, the potential use of DPP-4 inhibitors, such as Sitagliptin, as a therapeutic strategy to augment endogenous GLP-1 in CVD may be counter productive. However *M. oleifera* has been documented as having cardioprotective potentials (Faizi *et al.*, 1994, Edwards *et al.*, 2007), with most studies using lipids and blood pressure as indices (Ghasi *et al.*, 2000; Nambiar *et al.*, 2010). Mbikay (2012) in a review of the pharmacological potential of *M. oleifera* is of the opinion that a rather more extensive

analysis on circulating biochemical markers of inflammation would be justified, as the balance of pro and anti-inflammatory cytokines could influence the course of both diabetes, atherosclerosis and CVD risk. *M. oleifera* did not significantly reduce the proinflammatory biomarkers in this study. Nevertheless, the inability of the drug and herb to reduce significantly CRPs (most validated inflammatory biomarker) in diabetic rats also indicates the probability of not offering adequate protection against the risk of onset and progression of atherosclerosis and diabetic cardiomyopathy.

The anti-inflammatory cytokine adiponectin was slightly increased in the combination treated group compared to control, indicating a beneficial effect. Rizzo *et al.* in 2012, had shown a slight and non significant increment of adiponectin levels following Sitagliptin use in human studies for 12 weeks. However, this was not the case in this study following Sitagliptin administration for 6 weeks in diabetic rats. Adiponectin suppresses the attachment of monocytes to endothelial cells which is a fundamental step in experimental vascular damage as well as an early event in the atherosclerotic process. Human studies show that plasma ADP concentrations are reduced among patients with atherosclerotic complications as ADP levels were inversely related to body mass index values and directly related to high density lipoproteins (HDL) (Zoccali *et al.*, 2002, Hsu *et al.*, 2012).

The reduction in serum levels of the cardioactive peptide B type natriuretic peptide (BNP) following 28 days of co-administration of Sitagliptin and *M. oleifera* (using this biomarker) reflects a delay in the onset of left ventricular dysfunction, as it is one of the most relevant molecular markers of cardiac hypertrophy (Vanderheyden, 2004). A reduction in BNP levels (up to 30% during the first 24 hours of treatment) reflects

an adequate response in management of heart failure (Pfisterer *et al.*, 2009). However just as shown with the inflammatory biomarkers, continued administration of the drug and herb appear to have diminishing effect on the peptide level which can also be attributed to *M. oleifera* as evident from higher levels of BNP in rats that received only *M. oleifera* in phase 2. This apparent increase seen in BNP levels on progression of the disease has negative implication as higher BNP levels (even in the normal range) are associated with a higher prevalence of peripheral artery disease and atherosclerosis in T2DM patients (Ashley *et al.*, 2008; Jin *et al.*, 2014). Over expression of BNP also seems to be the best early marker of diabetic cardiomyopathy (Nunes *et al.*, 2013).

The contributions of oxidative stress to macro and microvascular complications of T2DM are well established. Malondialdehyde (MDA) (lipid peroxidation product) is one of the oxidative biomarkers that have been shown to have prognostic significance in cardiovascular disease (Ho *et al.*, 2013). The co-administration of Sitagliptin and *M. oleifera* reduced serum MDA levels considerably but with a non significant increase observed as the disease progressed. In addition, the synergistic beneficial effect in levels of the antioxidant enzyme catalase seen after 28 days of drug administration indicates the possibility of the combination reducing the onset of oxidative stress in T2DM. This was the exact trend seen with rats that received only *M. oleifera*. Awodele *et al.* (2012) similarly demonstrated a non significant increase in MDA levels and a decrease in catalase with 500 mg/kg of *M. oleifera* leaf extract in Wistar rats with no effect seen in these biomarkers at 250 mg/kg after 60 days, indicating, an ability to induce free radical with high doses on prolonged use. There was however no difference in serum levels of MDA following Sitagliptin

administration as similarly reported by Ferreira *et al.* (2010). Nevertheless the study also revealed a possibility of the combination ameliorating oxidative stress induced by long standing postprandial hyperglycaemia using the above biomarkers.

Adhesion of lipids in arteriosclerosis, with the resultant formation of plaques which eventually dislodges to block blood vessels remains an important contributory factor in cardiovascular complications in T2DM. Most antidiabetic agents do not possess lipid lowering effects making antilipidaemics an additional recommendation for T2DM patients. Multiple lipid lowering medications that effectively reduce fasting concentrations of low density lipoproteins, cholesterol and triglycerides have been developed. Although several of these medications, particularly the statins, routinely reduce cerebrovascular disease (CVD) risk by 25-35%, there remains substantial residual and absolute risk in higher CVD risk populations, such as in T2DM (Ansar *et al.*, 2011). In this study, the co-administration of Sitagliptin and *M. oleifera* increased serum levels of triglycerides with respect to levels in normal and diabetic controls and in sitagliptin treated rats. Studies have demonstrated that high concentrations of triglycerides and glucose are inversely related to endothelial dysfunction (Bae *et al.*, 2001; Ceriello *et al.*, 2002) which is an important link between the postprandial state, arteriosclerosis and cardiovascular disease. Decreased levels of triglycerides in Sitagliptin treated group could be due to increased GLP-1 concentration from DPP-4 inhibition, which in addition to slowing gastric emptying, may also decrease intestinal lymph flow, triglyceride absorption, and apolipoprotein synthesis thereby adding to the multifactorial mechanisms that may limit the release of triglycerides into the circulation after lipid-containing meals (Qin *et al.*, 2005). This study, recorded increased levels of triglycerides following *M. oleifera*



administration. On the contrary, studies with *M. oleifera* show reductions in TC and TG levels but with no effect on HDL and LDL levels (Ghasi *et al.*, 2000; Atsukwei *et al.*, 2014). This effect has been linked to kaempferol, one of its major bioactive compounds (Siddhuraju and Becker, 2003) that is proven to possess hypolipidaemic and antiatherosclerotic properties by the inhibition of LDL oxidation. But with a higher dose of 600 mg/kg, Atsukwei *et al.* (2014) in non diabetic rats fed with high fat diets recorded significant differences in HDL and LDL levels. However, the significant cholesterol lowering effect seen in these studies seems to be more evident in settings of post prandial hyperlipidaemia. This effect could be attributed to the presence of a bioactive phytoconstituent in *M. oleifera*, i.e.  $\beta$ -sitosterol (Frawley, 2009). This antihyperlipidaemic effect could be of clinical benefit when *M. oleifera* is administered with Sitagliptin in T2DM especially as this disease is strongly associated with hyperlipidaemia. More so, their concurrent administration in this study also reflect the probability of a beneficial effect on lipid levels in settings of established high postprandial hyperglycaemia which is normally characterized with hyperlipidaemia in T2DM.

The lack of significant difference in the relative heart weights of the drug treated and diabetic groups with respect to control did not preclude the absence of significant pathologic lesions in the heart commonly attributed to diabetes as seen in the diabetic control. Myocardial fibrosis and myocyte hypertrophy are the most frequently proposed mechanisms to explain cardiac changes in diabetic cardiomyopathy (Fang *et al.*, 2004). Myocyte cell death being a consequence of apoptosis or necrosis or both and have been identified in diabetic heart disease (Frustaci *et al.*, 2000). The concurrent administration of Sitagliptin and *M. oleifera* reduced the progression of the

lesions and necrosis seen in the diabetic control. There was however no significant histopathological findings in the heart of rats treated with only *M. oleifera* as similarly reported by Asiedu-Gyekye *et al.* (2014) while only Sitagliptin treated rats showed mild congestion between myofibrils. The effect can be attributed to quercetin, a flavanoid in *M. oleifera* documented to reduce cardiac hypertrophy in hypertensive rats (Duarte *et al.*, 2001). These findings also revealed the positive contribution of *M. oleifera* to the effect seen in the combination, though congestion in the heart did not ameliorate when treatment was delayed.

Thickening or otherwise hardening being reflected by the increase in diameter of the aortic wall is a major characteristic of arteriosclerosis. Enhanced arteriosclerosis in the coronary arteries is directly related to myocardial ischemia, increased oxidative stress, and vascular endothelial dysfunction, which may promote the progression of diabetic cardiomyopathy. Thickening of the aorta in addition to aortic vasculitis, was seen in the diabetic control but was not evident on co-administration of Sitagliptin and *M. oleifera*. This result also strongly indicates the beneficial effect of the combination in maintaining vascular integrity from the histologic point of view. Nonetheless, just as seen in the heart, the case was not the same on delaying treatment for a further two weeks as this failed to ameliorate the lesions that had already set in. This is to further emphasize the importance of instituting treatment as early as possible after diagnosis.

There was no alteration in the serum levels of electrolytes following treatment as values remained as high as in diabetic control. Diabetic rats have shown increase in serum sodium and potassium levels compared to negative saline control (Al-Malki and El Rabey, 2015). Increased serum levels of potassium and sodium is characteristic

of T2DM and this is a consequence of reduced erythrocyte  $\text{Na}^+\text{-K}^+$  ATPase activities and is implicated in pathogenesis of nephropathy and neuropathy (Shahid *et al.*, 2005). Careful monitoring of serum levels of electrolytes is indicated in diabetes especially where medications that alter  $\text{Na}^+\text{-K}^+$  ATPase pump are used as this could have deleterious implications. Chivapat *et al.* (2011) had recorded a decrease in serum potassium level with high dose of *M. oleifera* and suggested that the long term consumption of high dose of *M. oleifera* may affect potassium level, and therefore it may not be suitable for patients with hypokalemia and arrhythmia. At the dose of 300 mg/kg used in this study for 6 weeks this effect was not seen, neither did it contribute negatively to the result of the combination treatment. Nevertheless the report from the above study calls for caution on prolonged administration.

An increased serum level of urea is another diagnostic criterion for renal damage. The level of urea was increased following co-administration of Sitagliptin and *M. oleifera* compared to normal and diabetic control, indicating the possibility of onset of functional anomalies in the kidneys. The very high levels of urea seen in *M. oleifera* alone group as similarly reported by Oyagbemi *et al.* (2013) and also in non diabetic rats by Awodele *et al.*, (2012), could be contributory, as no difference was recorded with rats that received only Sitagliptin. Although, very low doses of Sitagliptin (5 and 10 mg/kg) had produced decreases in serum levels of urea in diabetic rats (Kamble and Bodhankar, 2013; Marques *et al.*, 2014). The combination treatment did not also increase serum albumin levels in the diabetic animals. Lower baseline levels of serum albumin have been associated with a rapid decline in kidney function (Keller *et al.*, 2010). This lower level indicates a higher clearance by the kidneys and human and animal studies showed serum albumin decreased significantly in patients with reduced

creatinine clearance which is another important biomarker in renal function (Viawanathan *et al.*, 2004; Al-Malki and El Rabey, 2015).

Histopathological findings of the kidneys showed a mild tendency of the co-administration of the drug and extract to delay the progression of tubulo-interstitial damage (renal tubular necrosis) and adherence of glomerulus to Bowmans capsule (glomerulosclerosis). Nonetheless, mild morphological alterations seen may not necessarily imply an absence of functional anomalies, evident from increased levels of urea seen above. In addition, there was no ameliorative effect on the lesions in already damaged kidneys following delayed treatment in post prandial hyperglycaemia. Administration of Sitagliptin also showed mild reduction in severity of tubular necrosis and glomerulosclerosis and this has been similarly demonstrated for Sitagliptin (Marques *et al.*, 2014). Marked reductions in renal tubule, glomerular lesions and other renal biomarkers have only been recorded with very low doses of Sitagliptin i.e. 10 mg/kg, which could also be subclinical as an antidiabetic agent (Mega *et al.*, 2011). The mild effect seen in progression of kidney lesions from the co-administration could be consequent of Sitagliptin effect as *M. oleifera* has also been associated with kidney toxicity especially in high doses and on prolonged use (Awodele *et al.*, 2012). Also, this study further confirms the important role of postprandial hyperglycaemia in the etiology of diabetic nephropathy as seen in severity of glomerular and tubular lesions with complete sclerosis and synechia of the Bowmans capsule in the post prandial hyperglycaemic control than even in the diabetic control. The co-administration had little or no effect in ameliorating these lesions.

As regards kidney trophism, the co-administration did not decrease kidney trophism compared to diabetic control, but it was significantly higher than normal control. Rats that received only Sitagliptin followed the same trend and this has been similarly reported by Mega *et al.* (2011). In patients with diabetic nephropathy, the initial physiological change is glomerular hyperfiltration, while the initial morphological change is glomerular hypertrophy evidenced as kidney hypertrophism. Using the effect on renal lesions, one could say that the co-administration of the drug and extract, slightly delayed the progression of renal injury but not ameliorate the lesions in evident diabetic nephropathy with functional anomalies already seen.

The liver is the major organ of metabolism of biochemicals and xenobiotics and is also highly involved in glucose homeostasis. Any damage or insults in the liver will obviously compromise these important functions thereby complicating management of T2DM. Aspartate amino transaminase (AST) and Alanine amino transaminase (ALT) are major markers of liver function. Toxic injury to the liver leads to elevation in levels of these liver enzymes. Thus, the significant increase in ALT and higher levels of AST following co- administration of Sitagliptin and *M. oleifera* even greater than the diabetic control, would suggest a potential adverse effect on the liver. These values were closely followed by values in rats that received only *M. oleifera*. Significant increases in liver enzymes have similarly been recorded following administration of *M. oleifera* in doses of 200-400 mg/kg in diabetic rats (Oyagbemi *et al.*, 2013; Asiedu-Gyekye *et al.*, 2014). Since ALT is localized primarily in the cytosol of hepatocytes, it is a more sensitive marker of hepatocellular damage than AST. Other studies have however demonstrated the hepatoprotective effects of *M. oleifera* in rats (Pari and Kumar, 2002; Fakurazi *et al.*, 2008; Buraimoh, 2011). The

difference seen could be due to other forms of chemically induced liver damage models employed by the authors i.e. anti-tuberculi drug induced model and acetaminophen induced liver damage. The above studies also used *M. oleifera* as pretreatment before induction of hepatic damage.

Hyperalgesia is a constant feature of sensory dysfunction in spontaneous and experimental models of diabetic neuropathy. Alloxan induced neuropathy is evidenced as slow nerve conduction velocity, thermal hyperalgesia, allodynia and increase in oxidative stress markers (Shaikh and Somani, 2010). Diabetic animals in both thermal and mechanical hyperalgesic models used clearly demonstrated an increased sensitivity to pain, further supporting the idea that hyperglycemia does contribute to a state of hyperalgesia in alloxan diabetic rats (Ibironke *et al.*, 2004). Diabetic amyotrophy (DA) may be a manifestation of diabetic mononeuropathy and is characterized by severe pain and muscle weakness and atrophy, usually in large thigh muscles. One out of seven sitagliptin treated diabetic rat did show signs of DA in this study and the drug has been previously associated with this (Kao *et al.*, 2008). The findings indicated an improvement in hot-plate response related to the increased pain threshold of diabetic animals treated with Sitagliptin and *M. oleifera* combinations. The co-administration delayed the onset of thermal hyperalgesia, but this eventually progressed with hypoalgesia setting in, at about the 6<sup>th</sup> week. Hypoalgesia is sometimes mistaken for improvement in symptoms due to increased pain threshold but it may actually be related to worsening of neuropathy because of loss of sensation to pain. In clinical settings, many patients ultimately experience a complete loss of sensation in their hands and feet, which can increase the risk of trauma leading to infection and eventual amputation. In alloxan induced neuropathy hypoalgesia sets in

around the sixth week following alloxan administration and this was probably the reason why the pain threshold increased after the sixth week of co-administration. This trend was same in the mechanical hyperalgesic model where the pain threshold of rats in the dug treated groups also increased significantly in the sixth week but thereafter returned to initial values. The continued administration of the drugs probably reduced the progression of hypoalgesia since the diabetic control groups still had a steady increase, thereafter.

It has been proposed that acute biochemical alterations in neural tissues might result from prolonged hyperglycemia and could contribute to the development of diabetic neuropathy (Lee *et al.*, 1990). The fact that the co-administration significantly reduced hyperglycaemia up to the 4<sup>th</sup> week of the study which was thereafter followed by a gradual rise in the FBG could have been causal to more degeneration in nerve tissues leading to hypoalgesia. The positive synergistic effect previously seen on oxidative stress markers is also probably contributory to this reduced onset and delayed progression as oxidative stress plays a key role in the pathogenesis of diabetic neuropathy (Anjaneyulu and Chopra, 2004). Sitagliptin appears to have contributed significantly in the effect seen in the combination group as rats that received only *M. oleifera* had significant increase in pain threshold (thermal hyperalgesia) throughout the period of the study. At doses of 100 and 200 mg/kg of *M. oleifera*, Kongrum *et al.* (2012) showed significant reversal of the withdrawal latency in hot plate tests, but similarly recorded non significant reversal at 300 mg/kg. The concurrent administration also delayed the onset of thermal allodynia but did not reduce the progression.

Assessment of cutaneous innervations in skin biopsies is emerging as a valuable means of both diagnosing and staging diabetic neuropathy. The moderate innervation of the rats foot pad following the administration of Sitagliptin and *M. oleifera* is also a confirmation of the reduced progression of diabetic neuropathy. The very low innervation seen in the post prandial control also further reiterates the important role of post prandial hyperglycaemia in the pathogenesis of diabetic neuropathy. The co-administration however showed little or no effect in the marked nerve degeneration that accompanied the post prandial state.

The delay in the onset of development of lenticular opacity seen in the study could be a result of the combined effect of the drug and the herb. Previous study on Sitagliptin 40 mg/kg showed a prolongation but not prevention in the development of cataract and retinopathy in diabetic rats (Pandit *et al.*, 2013). In addition, rats that received only *M. oleifera* showed neither signs of opacity in the lens (prevented the development) nor significant pathology in the retina. It is possible that a more prolonged administration of drugs would indicate a positive effect on progression of lenticular opacity as seen in review of previous studies by Lai and Lo (2013) where alloxan was used at different time points and at different doses. The co-administration however showed little effect on treated post prandial hyperglycaemic groups for possible reasons of dose and duration of study earlier mentioned above. As a multiple regression analysis had revealed that postmeal hyperglycaemia independently correlated with the incidence of diabetic retinopathy and neuropathy. (Shiraiwa *et al.*, 2005). Morphologically, the well laid out arrangement of the retinal cell layer was distorted, including thickened capillary wall and oedema following alloxan administration in the diabetic control. The co-administration of the drug and



extract did not reduce nor ameliorate the pathologic lesions in the treated groups. These lesions are signs indicative of hyperglycaemic damage, although the duration of the study did not show neovascularization and macrophage accumulation, the capillary basement membrane thickening, and oedema that were seen are characteristic of retinopathy as also shown in other studies of alloxan induced retinopathy following durations of 2-12 months (Schroeder *et al.*, 1991; Kern *et al.*, 2000; Kowluru *et al.*, 2001). However, *M. oleifera* extract appear to reduce the extent of hyperglycaemic damage in the rats eye as evident from both an intact lens and retina in the *M. oleifera* treated groups as earlier reported (Gupta *et al.*, 2013).

## CHAPTER SIX

### 6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

#### 6.1 Summary of Findings

In this study, the effect of co-administration of Sitagliptin and ethanol leaf extract of *Moringa oleifera* on glycaemic control and chronic complications were investigated in alloxan induced diabetic rats

The oral LD<sub>50</sub> of *M. oleifera* was estimated to be greater than 2,000 mg/kg in rats, and all the organs (heart, kidney, lungs, liver, pancreas and spleen) examined did not show any significant histopathologic findings following acute oral toxicity studies.

Sitagliptin (50 mg/kg) and ethanol leaf extract *Moringa oleifera* (300 mg/kg) clearly displayed antihyperglycaemic activities, when administered as single agents in alloxan induced diabetic rats. However, on prolonged co-administration of Sitagliptin and *Moringa oleifera*, a progressive decrease in antihyperglycaemic effect was seen which has negative implications in both macro and microvascular complications. No demonstrable effect was seen in insulin levels and the weights of the rats following the co-administration.

The beneficial effect of the co-administration of Sitagliptin and *M. oleifera* leaf extract on the serum levels of TNF $\alpha$  was not observed in rats treated for a longer period, suggesting undesirable side effect in the heart and arteries on prolonged use. No observable effect was seen in the levels of proinflammatory cytokine (CRP), indicating the probability of not offering adequate protection against the risk of progression of atherosclerosis and diabetic cardiomyopathy. The anti-inflammatory

cytokine adiponectin was also not significantly increased, but evidence shows the possibility of improvements on prolonged administration of Sitagliptin and *M. oleifera*. The reduction in serum levels of the cardioactive peptides (BNP) on co-administration of Sitagliptin and *M. oleifera* leaf extract probably reflects a delay in the onset of left ventricular dysfunction, but this effect was seemingly abolished after 4 weeks of treatment. The study also revealed a possibility of the combination ameliorating oxidative stress induced by long standing postprandial hyperglycaemia using malondialdehyde and catalase as biomarkers.

The co-administration also reflects the possibility of a beneficial effect on lipid levels in settings of established high postprandial hyperglycaemia. The combination also showed amelioration of pathologic lesions in the aorta of the rats indicating a propensity to maintain vascular integrity. Nonetheless, in the heart, the combination failed to ameliorate the lesions that had already set in.

The co-administration of Sitagliptin and *M. oleifera* leaf extract neither altered the serum levels of electrolytes, urea and albumin nor did it delay the progression or ameliorate significantly kidney hypertrophism in alloxan-induced kidney damage. In kidney histology, there was mild reduction in the progression of renal injury but no amelioration of the lesions already evident from post prandial hyperglycaemia.

Co-administration of Sitagliptin and *M. oleifera* leaf extract significantly reduced pain perception (mechanical testing) 5 weeks post administration but did not bring about any significant reversal in thermal hyperalgesia during the study period. However moderate improvement was seen in the intra epidermal nerve fibre density with little

or no effect in the marked nerve degeneration that accompanied the post prandial state. The possibility exists that prolonged use in clinical setting might contribute positively in management of peripheral diabetic neuropathy.

The combination of the agents delayed the onset of alloxan induced lenticular opacity (cataract) but neither reduced the progression nor ameliorated, hyperglycaemic induced pathologic lesions in the retina.

## **6.2 Conclusion**

*M. oleifera* decreased antihyperglycaemic effect of Sitagliptin. The co-administration of *M. oleifera* and Sitagliptin may delay the onset, but not progression of cardiomyopathy in Wistar rats. The combination however, appears to have a protective effect against the development of arteriosclerosis. The combination appears not to delay the progression nor ameliorate significantly the lesions associated with long standing diabetic nephropathy. A propensity to regenerate the intraepidermal nerve fibre density also indicates a delayed onset of peripheral diabetic neuropathy and that further use might also reduce its progression. The duration of the study could not reveal significant effects in retinopathy, but it appears that co-administration of Sitagliptin and *M. oleifera* delayed the onset but not progression. In the long run, beneficial effects seemed apparent on microvascular complications, but after a protracted time it resulted in negative cardiorenal consequences.

## **6.3 Recommendations**

From the foregoing, it is evident that the co-administration of Sitagliptin and *Moringa oleifera* especially for a prolonged period does not appear to be without some

negative consequences. Although the current study was carried out using animal models, data from animals have sufficient similarity with man (in many physiological responses and functions) and have been shown to be predictive in several clinical circumstances including T2DM. It is therefore recommended that caution be exercised on chronic and indiscriminate use of *Moringa oleifera* alongside Sitagliptin, with a suggestion of close monitoring of vital organs and their functions.

Suggestion for further investigation include isolation of the active antihyperglycaemic principle in *Moringa oleifera* for mechanistic studies, including possible DPP-4 inhibition and further interaction of the isolate with Sitagliptin for a longer period. This is with a view of possibly enhancing the drug-herb antihyperglycaemic effect, with consequent prevention of diabetic complication and reducing the negative effects seen in this study, which might be consequent of other active principles in the herb.

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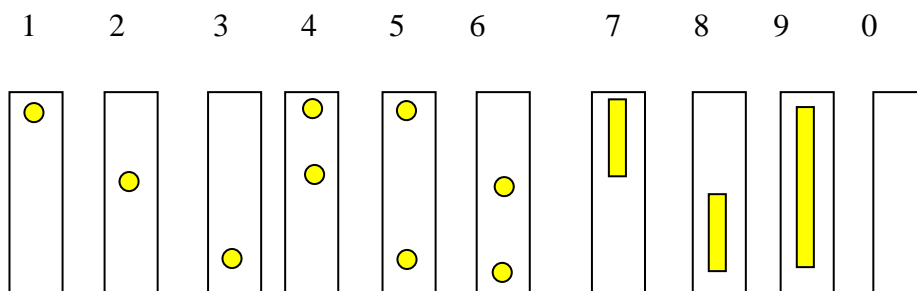
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## Appendix I

### Animal Numbering Code

Rats were numbered using a coding system that was based on the vertical division of the animal into three parts namely, the middle, the left and the right side of the rats. Visually dividing the rats into three for each of the sides, results in a total of nine divisions. A coding which utilizes the numbers 1-9 and 0, as shown below (with examples), was employed in marking the animals using pricric acid solution. This was re-applied if needed to reinforce the number code and prevents it from fading over time.



4



22



18

The numbers above represent the code of each rat.

## Appendix II

### **Procedure for preparation and calculation of Sitagliptin and 50% ethanol leaf extract of *M. oleifera***

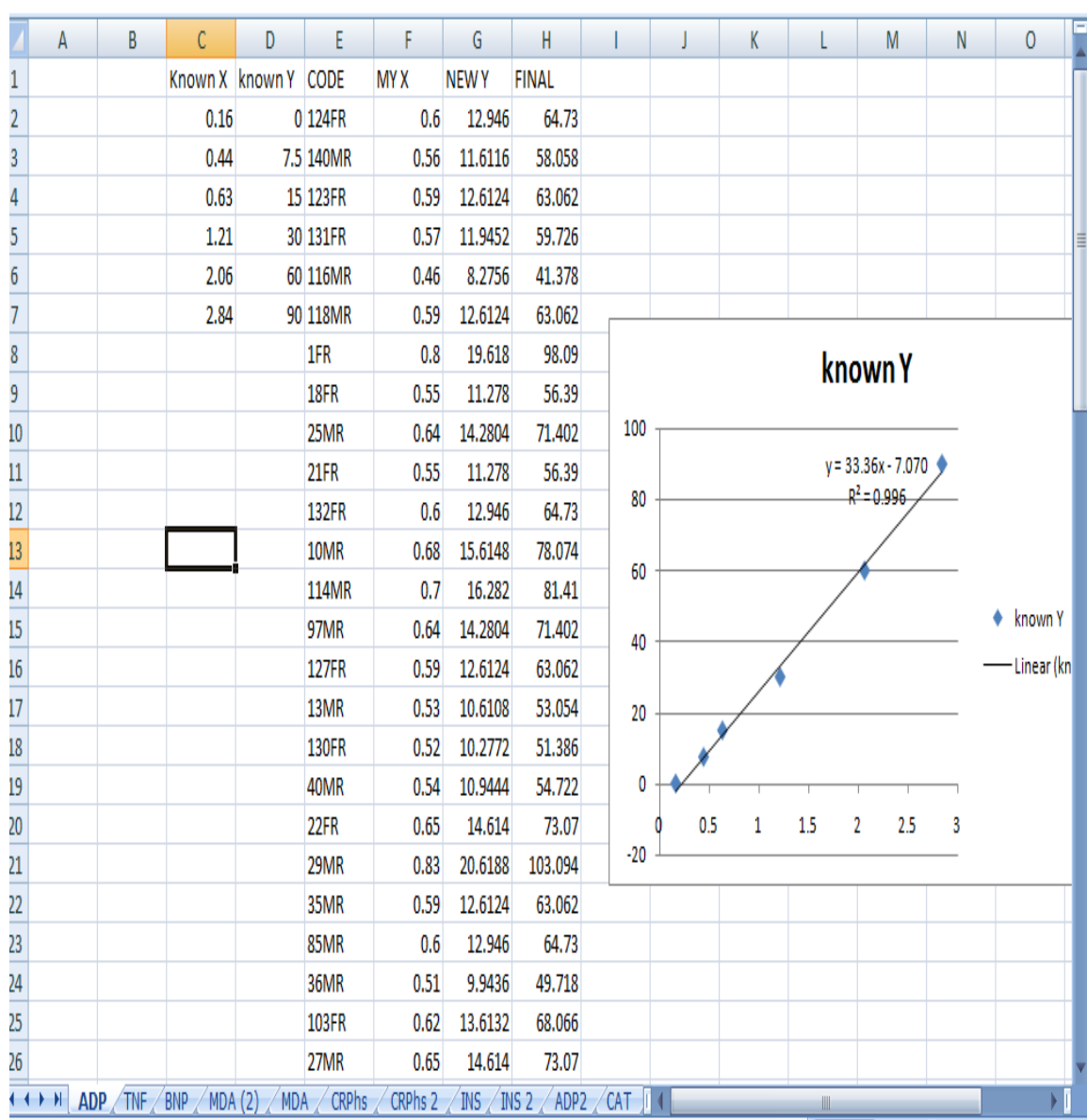
#### *Preparation of Sitagliptin Solution*

Sitagliptin is readily soluble in water and exhibits a pH dependent aqueous solubility. The solution was prepared freshly on a daily basis by dissolving a 100 mg in 4mls of distilled water to obtain a stock solution of 25 mg/ml. The dose of Sitagliptin used was 50 mg/kg, therefore a rat weighing an average of 200 g received 10 mg of Sitagliptin which was 0.4 ml of the stock solution.

#### *Preparation of Moringa oleifera Leaf Extract Solution*

*Moringa oleifera* leaf extract is also readily soluble in water. The dose used was 300 mg/kg, as such 20 rats weighing an average of 200 g each will receive a total of 1,200 mg of the extract. 1,200 mg was weighed and dissolved in 8 ml of distilled water to obtain a stock solution of 150 mg/ml. A rat weighing 200 g received 60 mg of the extract which was 0.4 ml of the stock solution.

### Appendix III



The absorbance (Known X) of the serial dilutions of standard (Known Y) of adiponectin used in plotting the regression graph and extrapolating the corresponding concentrations (New Y) of the sample absorbances (My X).

## Appendix IV



**Picture of an example of the various reagent and standard provided for the Rat specific ELISA kits.**

## Appendix V

### Calculation for the Biochemical parameter using absorbance values

$$\text{Conc of Test} = \frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times \text{Conc of Std}$$

Key:

O.D. = Optical density

Std = Standard

Conc = Concentration

## **Appendix VI**

### **Calculation of the force in grammes applied to Rats foot pad**

The force is measured on the scale calibrated in 10-gram steps, by a pointer riveted to the slide. For example 7.5 means 75 grams. The scale can however be multiplied by 2 or 3 by placing on the slide one or two discs. By adding one disc on the slide the force will be 240 g when the pointer indicates 12. With the pointer in the same position, by adding two discs on the slide the force will be 360 g.

The experiment was carried out using one disc. As such with the pointer for example on position 3, the force is calculated as  $3 \times 10 \times 2 = 60$  g.

## **Appendix VII**

### **1. Grading Scheme for Glomerulosclerosis and Tubular interstitial Damage**

According to the method described by Schafer *et al.* (2003)

Grade 1: Minimal (Single glomeruli affected and thickening of mesangium)

Grade 2: Mild (Multiple glomeruli affected and thickening of mesangium)

Grade 3: Moderate (Many glomeruli affected segmental or nodular thickening of the mesangium and synechia with one Bowmans capsule)

Grade 4: Marked (Majority of glomeruli affected with synechia of Bowmans capsule or complete sclerosis)

Grade 5: Severe (General sclerosis of glomeruli in all areas)

### **2. Staging of Lenticular Opacity**

According to the method described by Suryanarayana *et al.* (2005)

Clear: clear lenses and no vacuoles present

Stage 1: Vacuoles cover part of the surface of the anterior pole, forming a subcapsular cataract

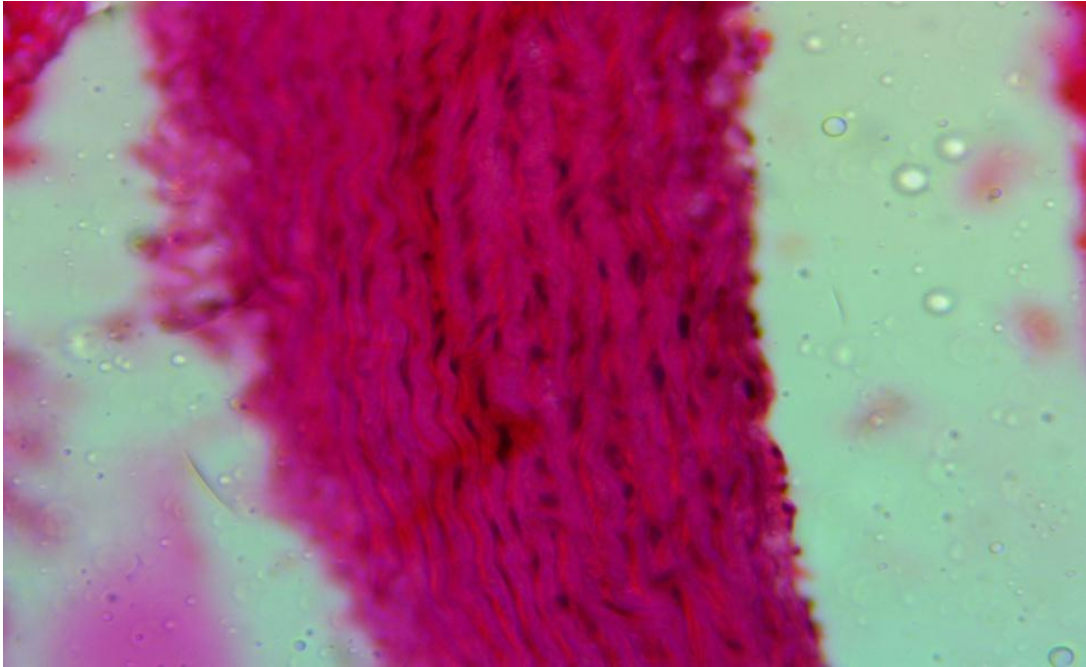
Stage 2: Some vacuoles have disappeared and the cortex exhibits a hazy opacity

Stage 3: A hazy cortex remained and dense nuclear opacity is present

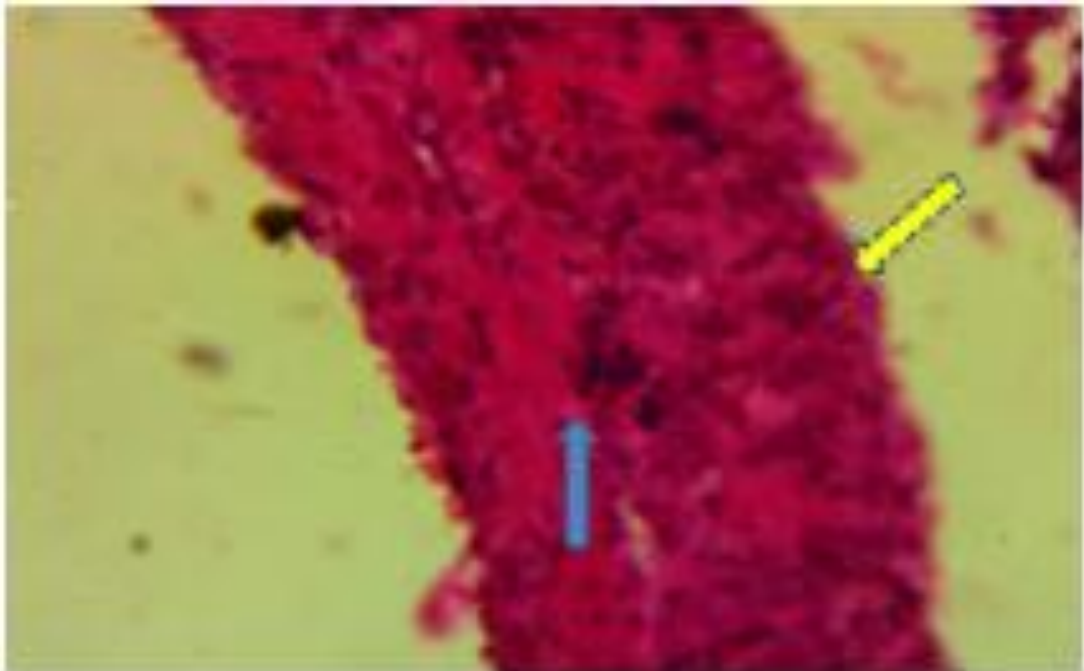
Stage 4: A mature cataract is observed as a dense opacity in both cortex and nucleus

## Appendix VIII

### Photomicrographs of sections of the Aorta, Heart, Kidney Skin of Hind Paw and Retina of Rats.



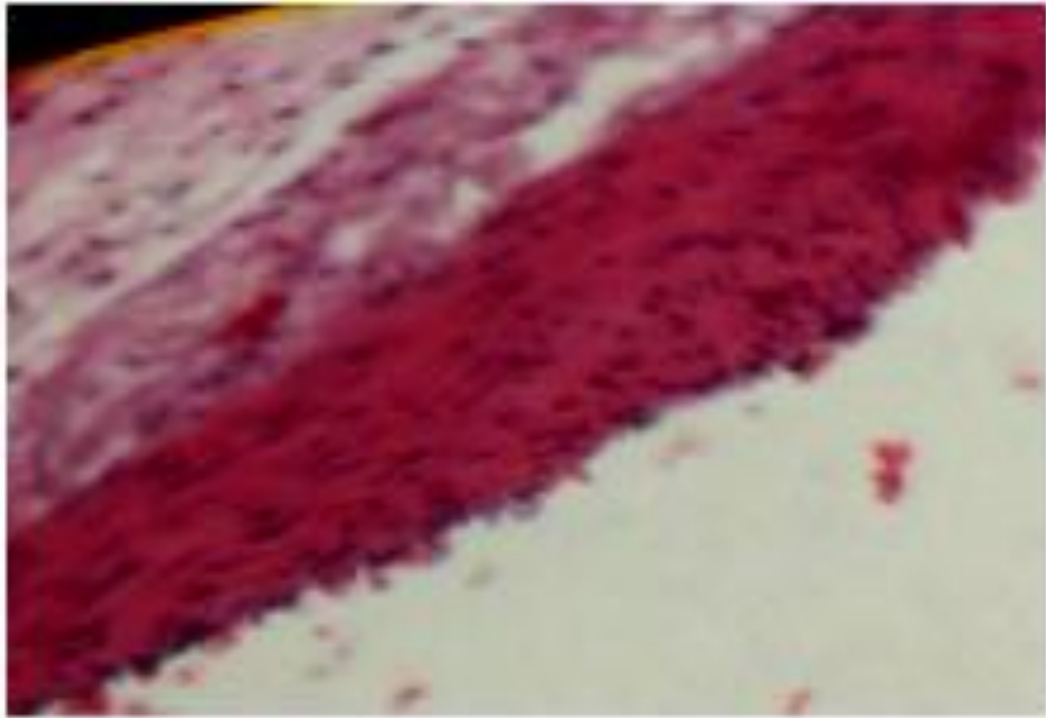
**Plate XI: Photomicrograph of a section of the aorta of a normal rat (H&E X 400).**



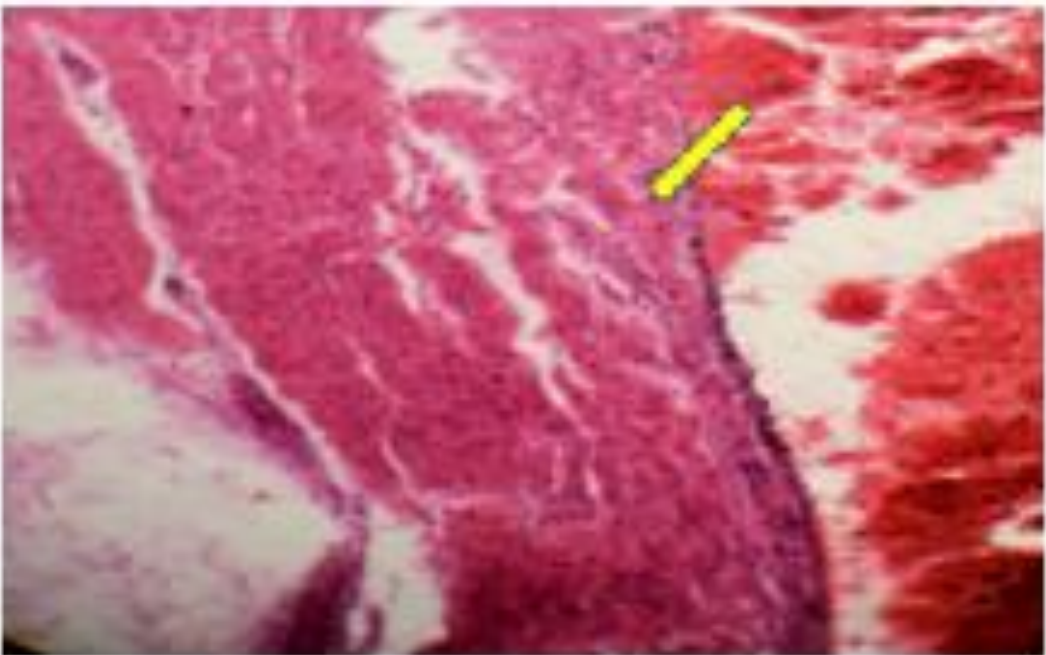
**Plate XII: Photomicrograph of a section of the aorta of a diabetic control rat (H&E X 400).**

The section shows localized aortic vasculitis (↑) and thickening of the aortic wall (↑).



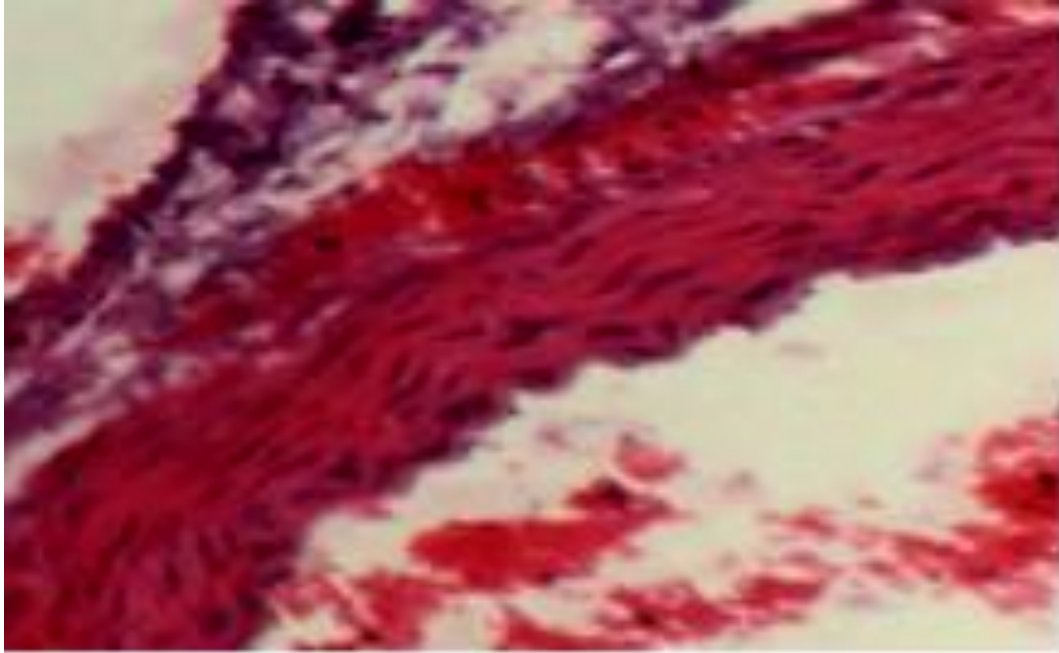


**Plate XIII: Photomicrograph of a section of the aorta of *M. oleifera* treated rat (H&E X 400). No significant histopathological findings.**

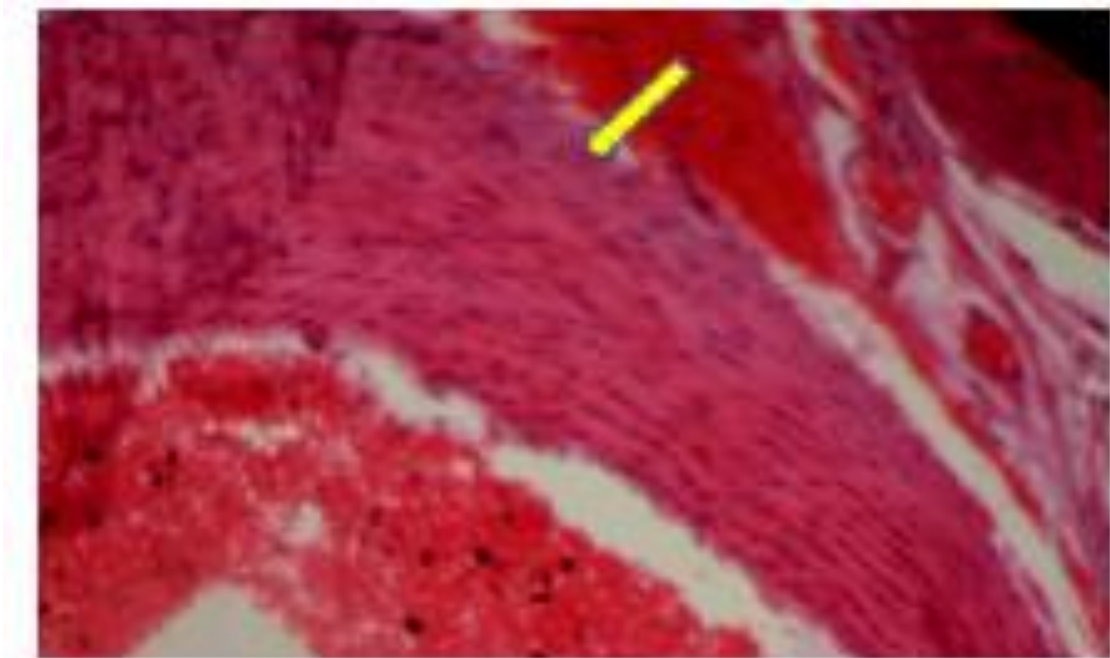


**Plate XIV: Photomicrograph of a section of the aorta of Sitagliptin treated rat (H&E X 400).**

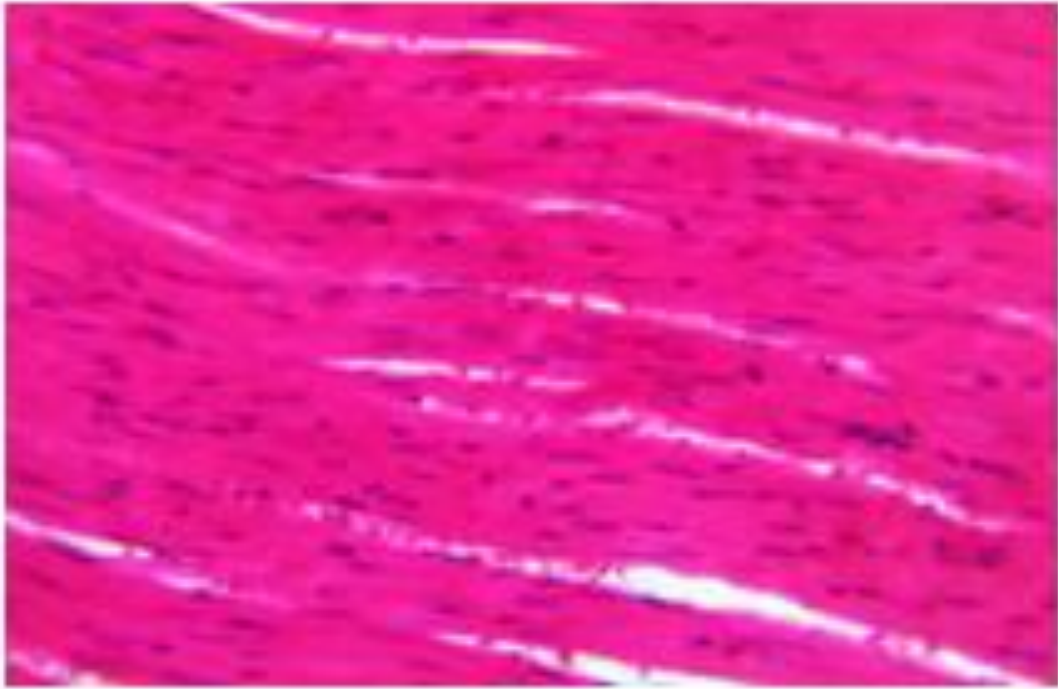
The section shows thickening of the aortic wall (↑).



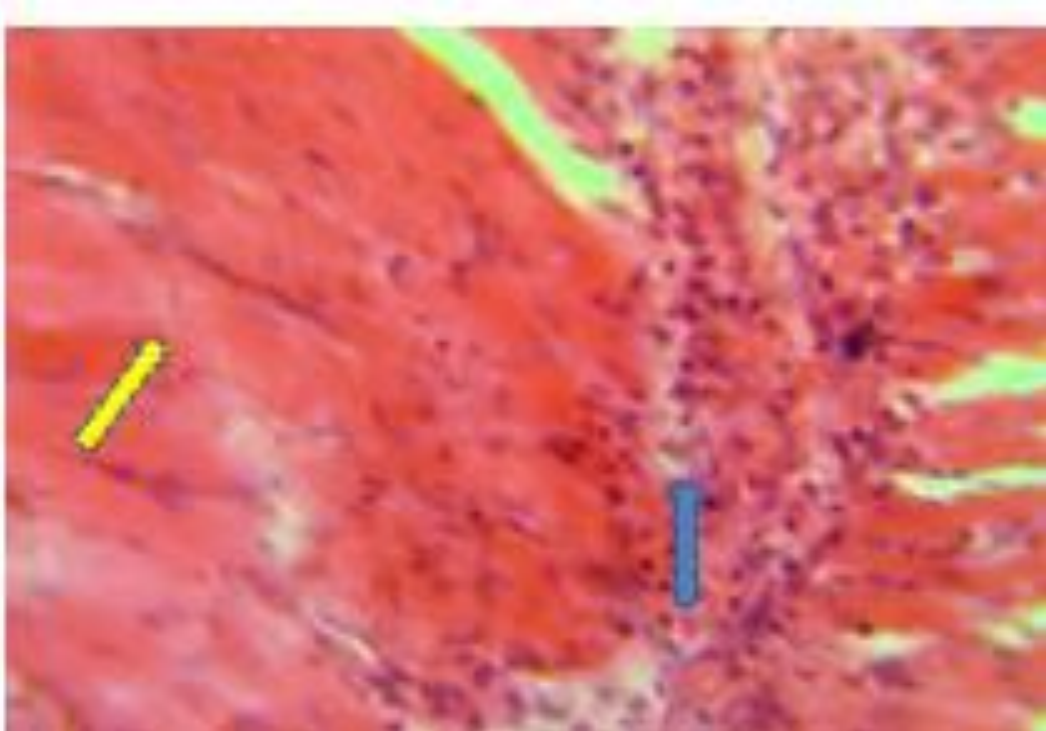
**Plate XV: Photomicrograph of a section of the aorta of Sitagliptin & *M. oleifera* treated rat (H&E X 400). No significant histopathological findings.**



**Plate XVI: Photomicrograph of a section of the aorta of a rat in the Ameliorative group treated with (Sitagliptin & *M. oleifera*) (H&E X 400). The section shows thickening of the aortic wall (↑).**



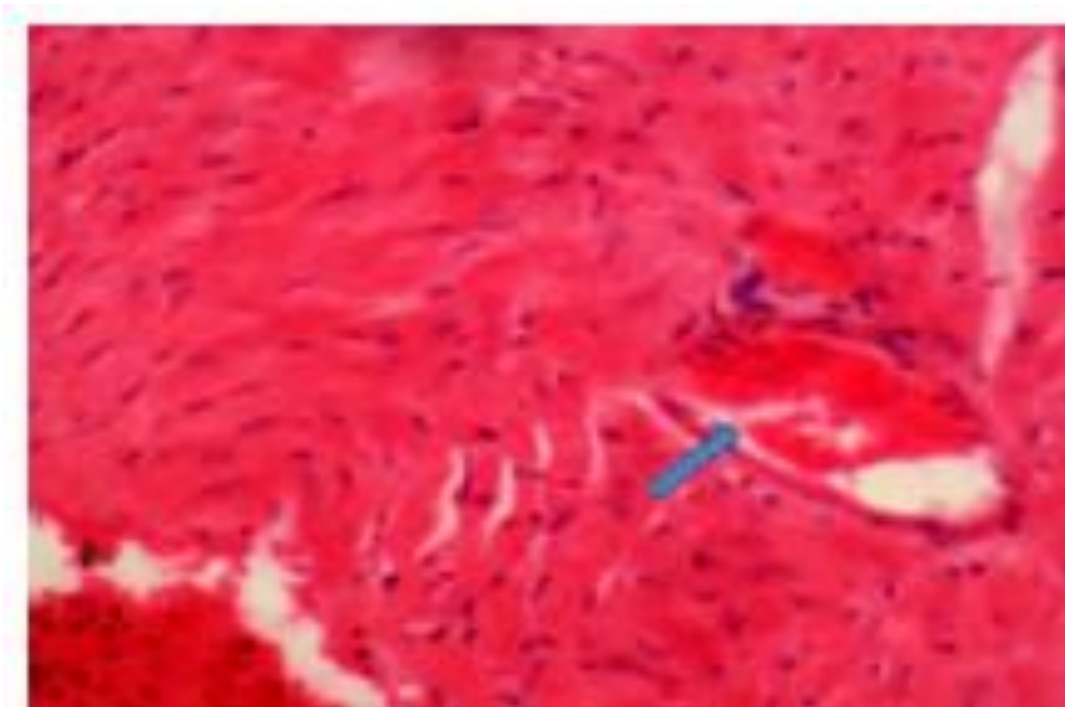
**Plate XVII: Photomicrograph of a section of the heart of a normal rat (H&E X 400)**



**Plate XVIII: Photomicrograph of a section of the heart of a diabetic control rat (H&E X 400).** The section shows focal areas of necrosis (↑) and infiltration of inflammatory cells (macrophages) and congestion (↓).

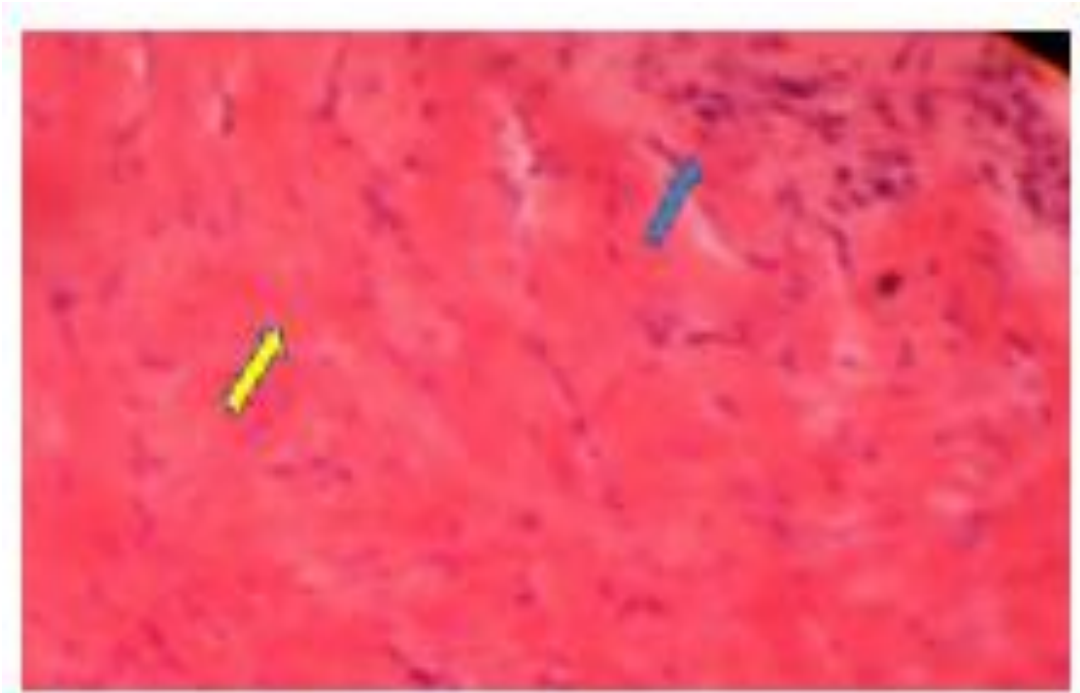


**Plate XIX: Photomicrograph of a section of the heart of a *M. oleifera* treated rat (H&E X 400). No significant histopathological finding seen.**

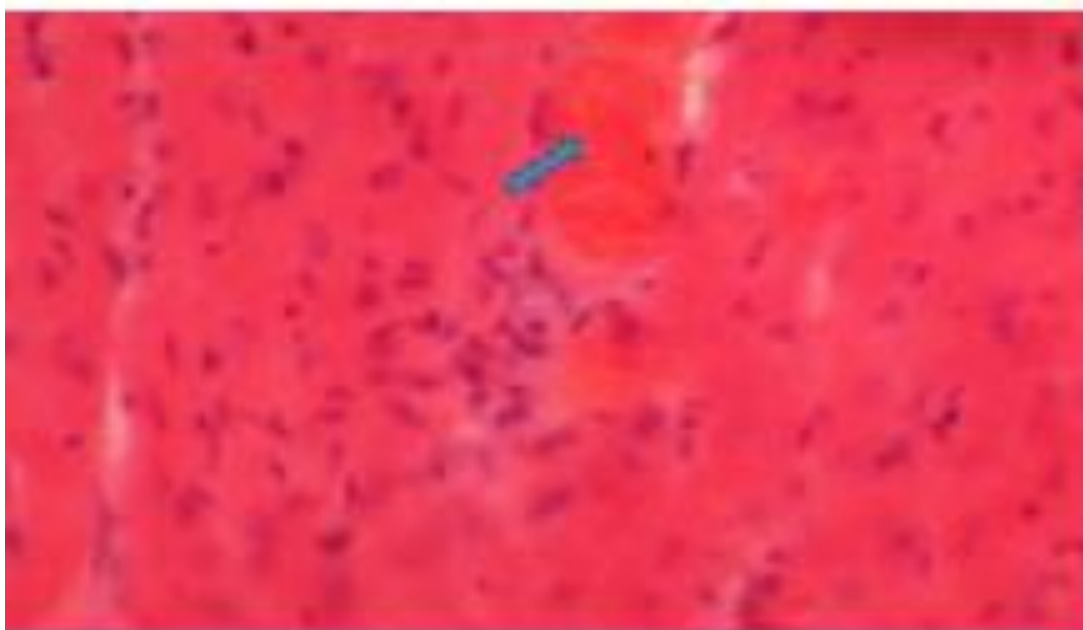


**Plate XX: Photomicrograph of a section of the heart of a Sitaliptin treated rat (H&E X 400).**

The section shows congestion of spaces between myofibrils (↑) with no necrosis.

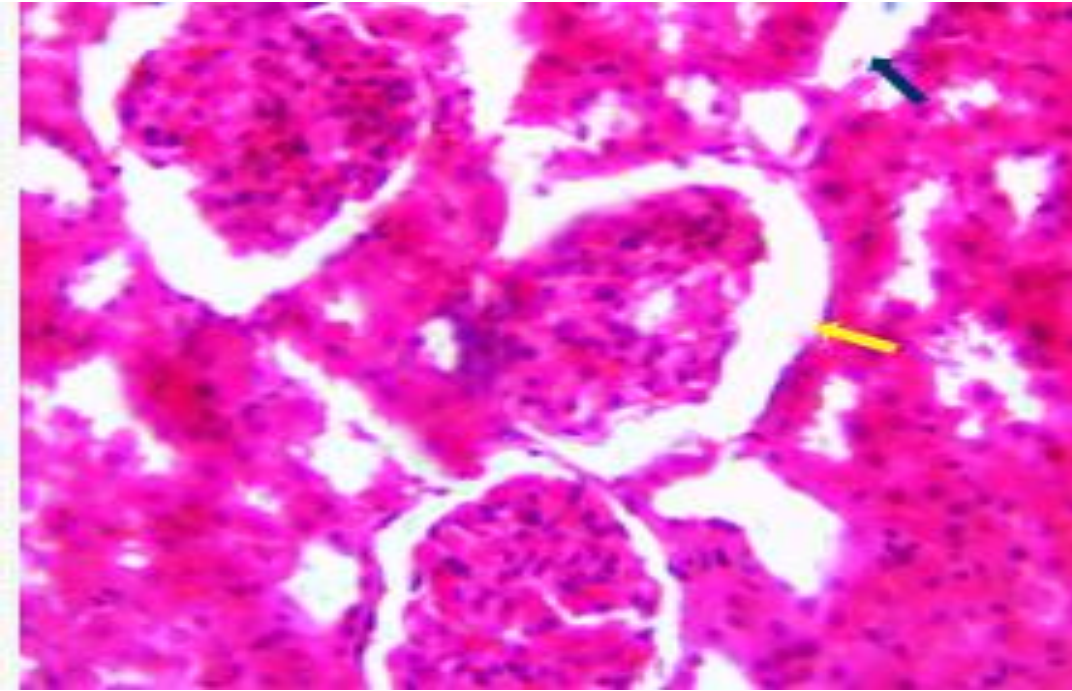


**Plate XXI: Photomicrograph of a section of the heart of a Sitagliptin & *M. oleifera* treated rat (H&E X 400).** The section shows area of focal mononuclear cellular infiltration (↑) and necrosis of myofibrils (myocarditis) (↑).

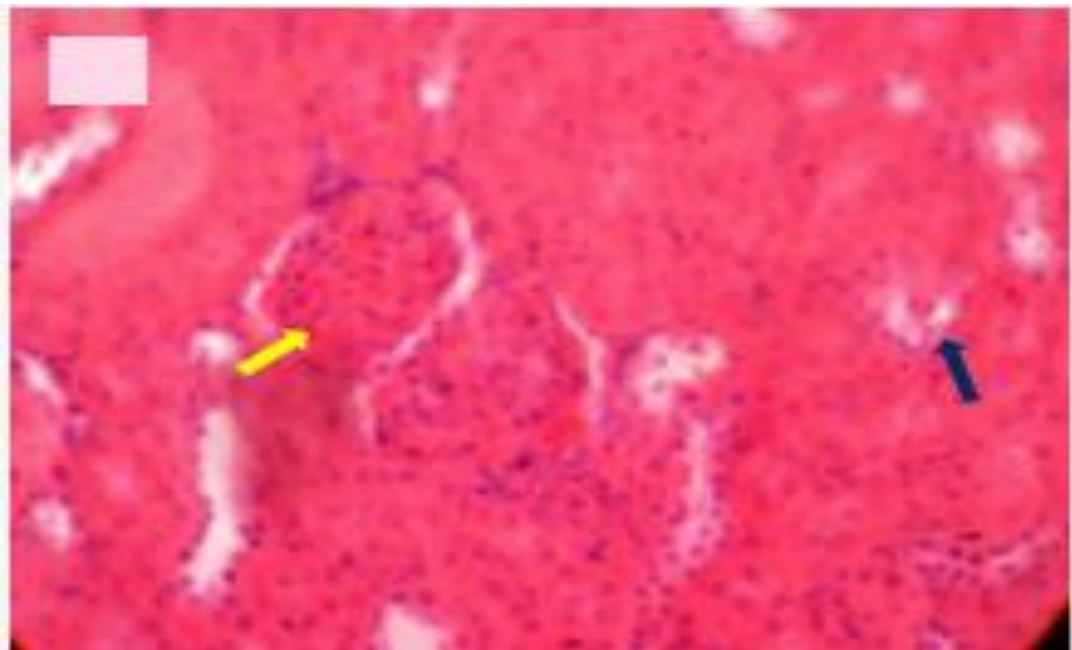


**Plate XXII: Photomicrograph of a section of the heart of a rat in the Ameliorative group treated with (Sitagliptin & *M. oleifera*) (H&E X 400).**

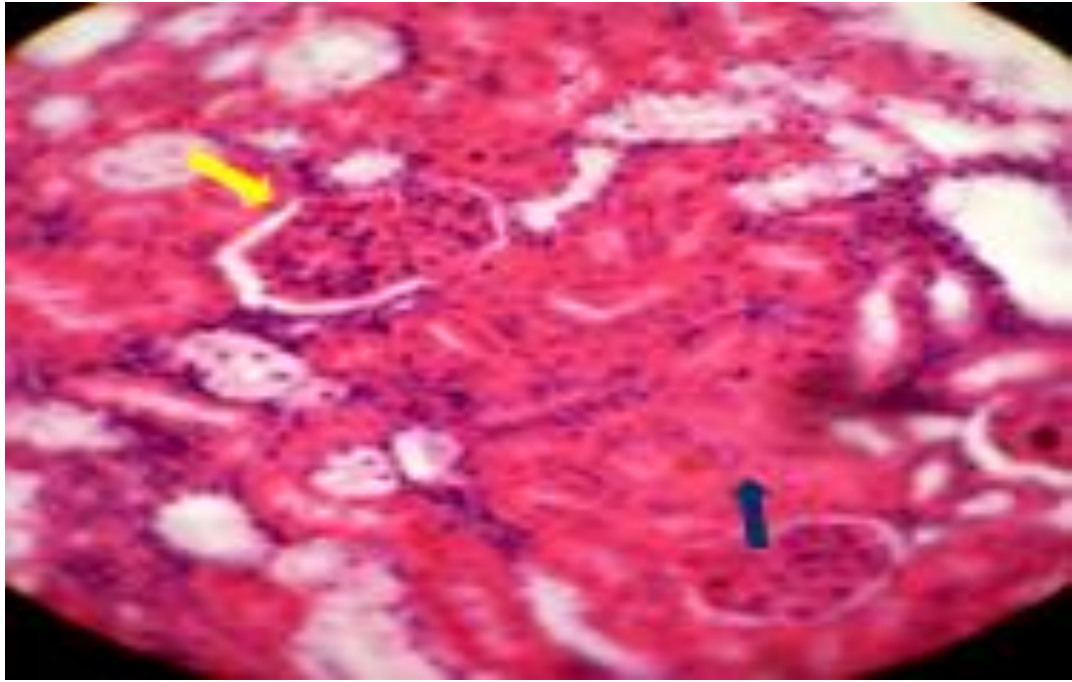
The section shows areas of congestion (↑).



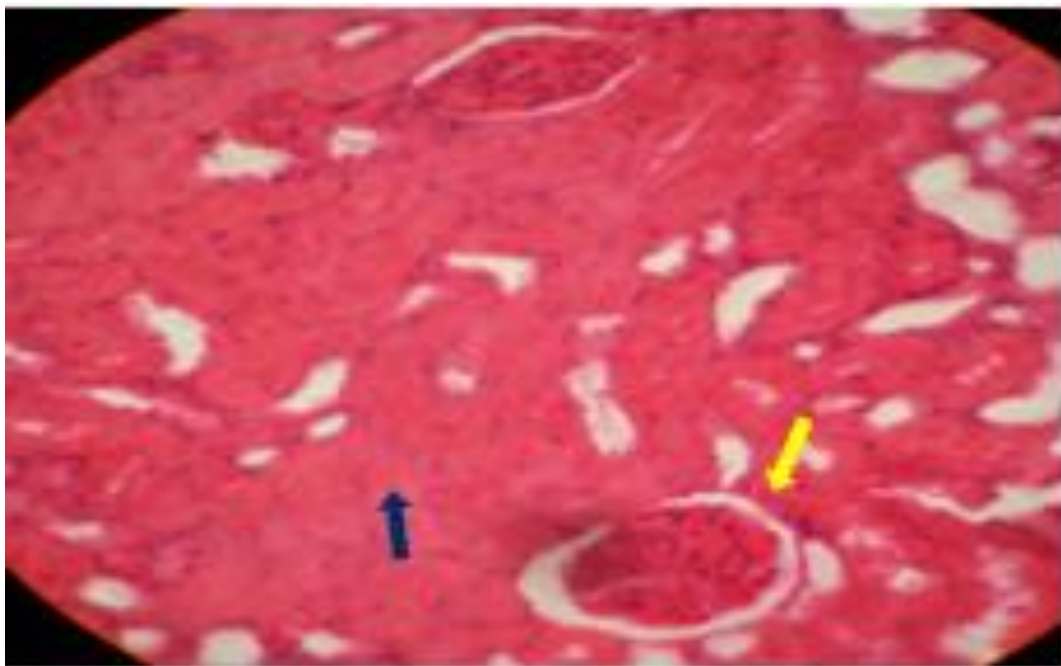
**Plate XXIII: Photomicrograph of a section of the kidney of a normal control rat (H&E X 400).** The section shows an intact Bowmans Capsule (↑), with well defined tubular lumen (↑).



**Plate XXIV: Photomicrograph of a section of the kidney of a diabetic control rat (H&E X 400).** The section shows distorted renal architecture with many tubules are filled with proteinaceous material. Bowmans capsule adhered to glomeruli and very thin (↑) and there is necrosis of renal tubular epithelium (↑). Glomerulosclerosis, (Grade 4).

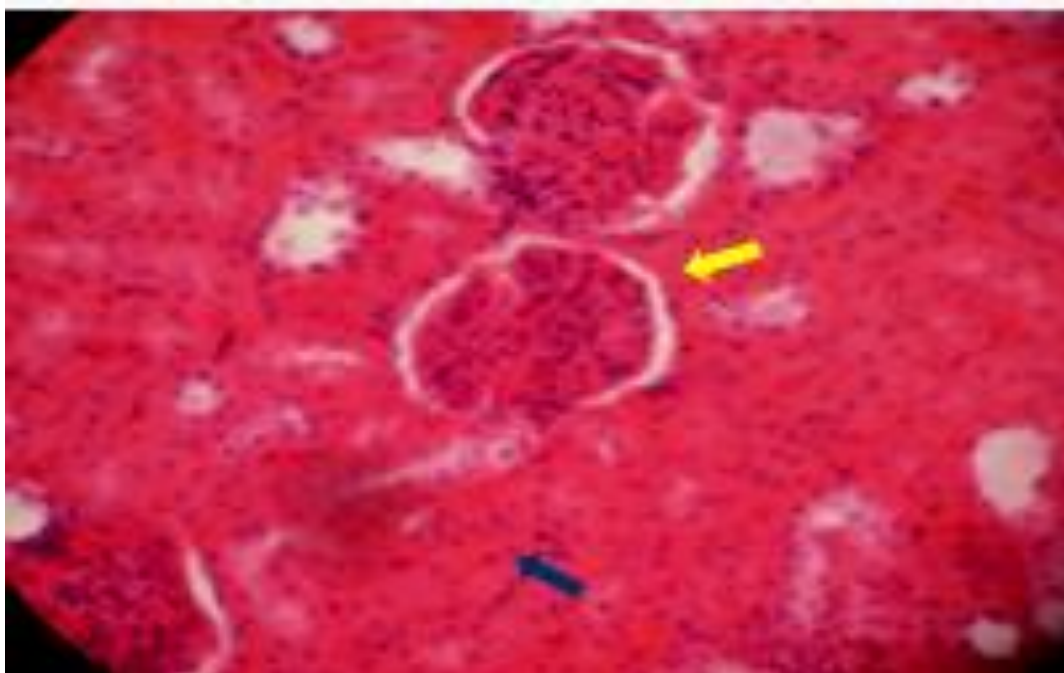


**Plate XXV: Photomicrograph of a section of the kidney of *M. oleifera* treated rat (H&E X 400).** The section shows thin bowmans capsule (↑) and tubules filled with pink amorphous material indicative of tubular necrosis (↑).Glomerulosclerosis, (Grade 1).

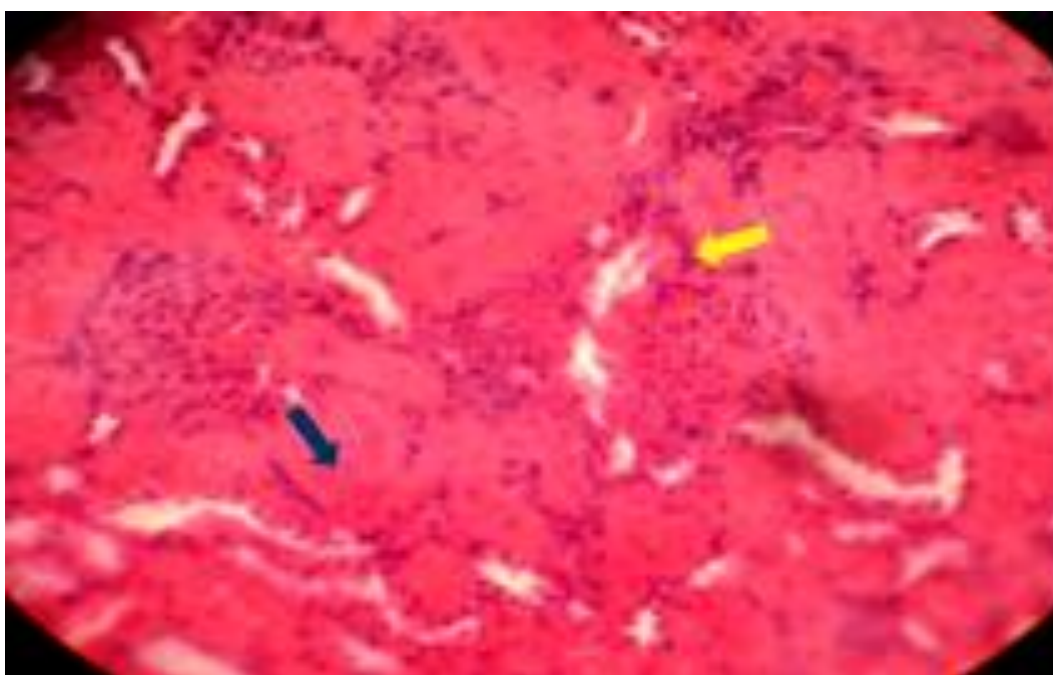


**Plate XXVI: Photomicrograph of a section of the kidney of a Sitagliptin treated rat (H&E X 400).**

The section shows tubules filled with pink amorphous material (↑). Bowmans space is clear (↑).Glomerulosclerosis, (Grade 1).



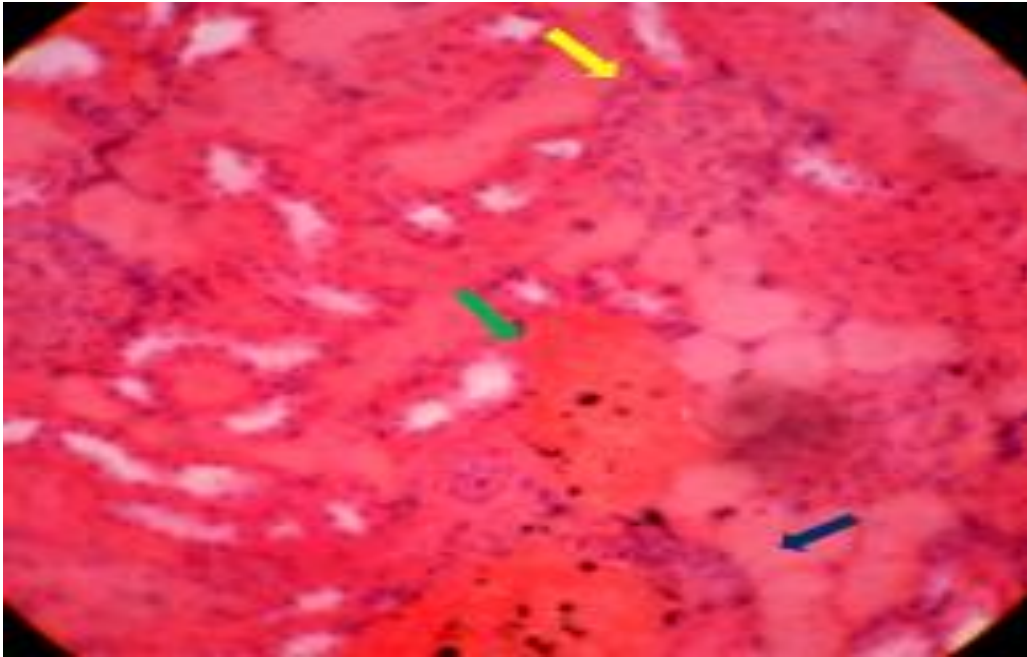
**Plate XXVII: Photomicrograph of a section of the kidney of a Sitagliptin & *M. oleifera* treated rat (H&E X 400).** The section shows necrosis of renal tubular epithelium with lumen of tubules filled with pink amorphous proteinaceous material (↓). Thin but clear Bowmans capsule (↑). Glomerulosclerosis (Grade 1).



**Plate XXVIII: Photomicrographs of a section of the kidney of a rat in the Ameliorative group treated with (Sitagliptin & *M. oleifera*) (H&E X 400).**

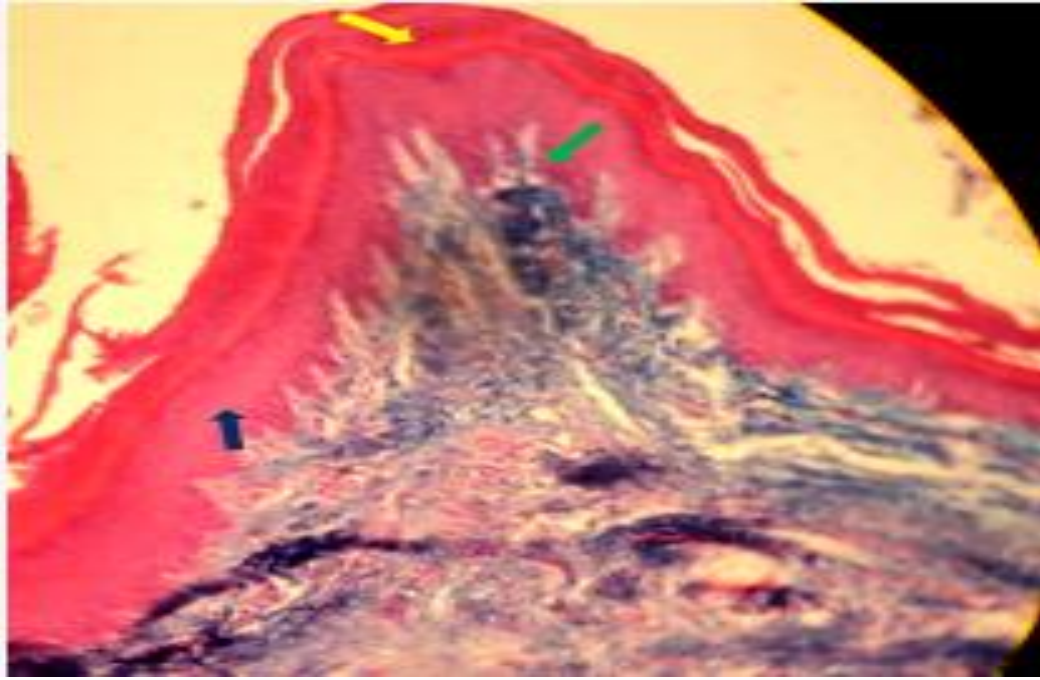
The section shows necrosis of renal tubular epithelium (↓) and obliterated Bowmans capsule (↑). Glomerulosclerosis, (Grade 3).





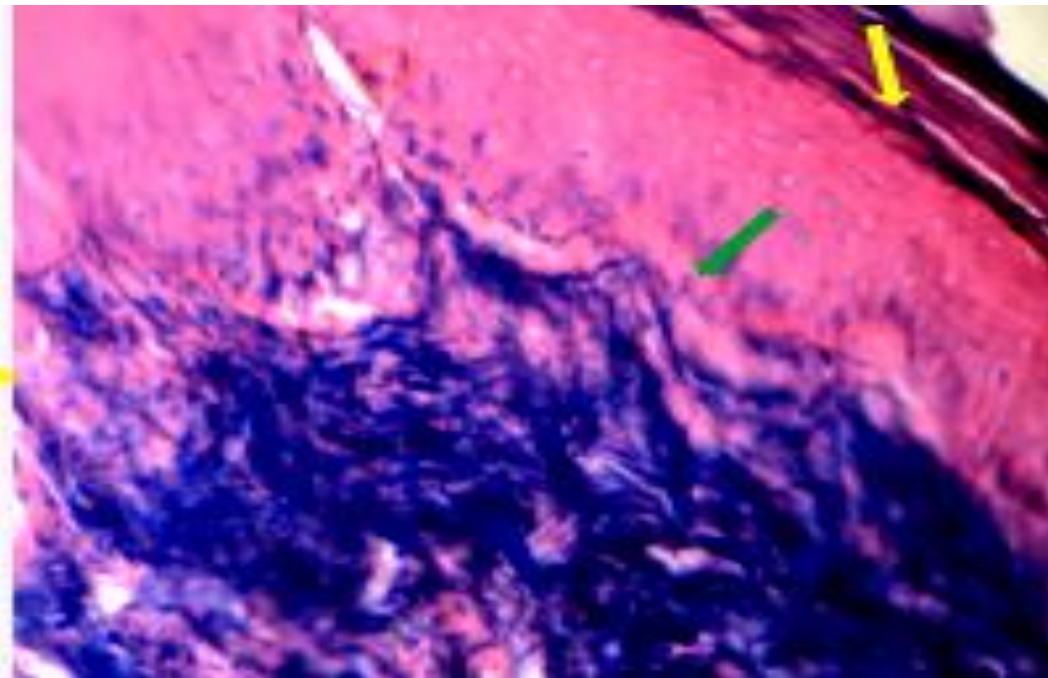
**Plate XXIX: Photomicrographs of a section of the kidney of a Post Prandial Diabetic Control Rat (H&E X 400).**

The section shows necrosis of renal tubular epithelium with pink amorphous material (↑), totally obliterated Bowmans capsule (↑) and congestion in the kidney (↑). Glomerulosclerosis, (Grade 4).



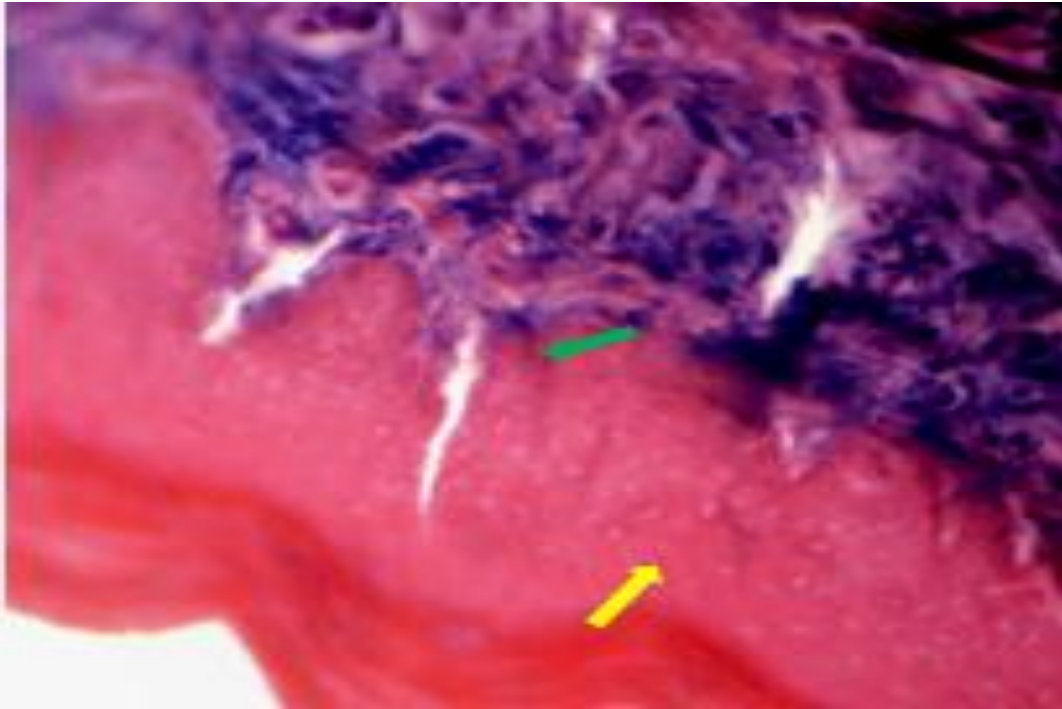
**Plate XXX: Photomicrograph of a section of hind paw of a normal control rat (LFB & E X 400).**

Distinct squamous epidermis (↑), intra epidermal nerve fibres projecting into the epidermis from the dermis (↑). High nerve fibre density.

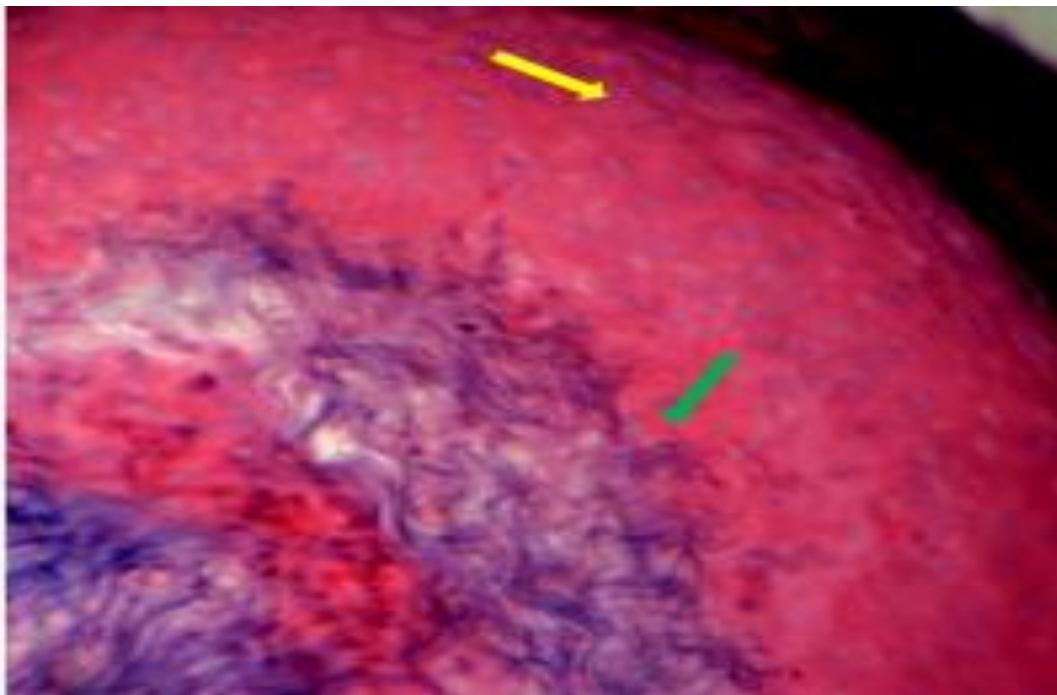


**Plate XXXI: Photomicrograph of a section of hind paw of a diabetic control rat (LFB & E X 400).**

It shows reduced keratin layer (↑), intra epidermal nerve fibres projecting into the epidermis from the dermis (↑). Low nerve fibre density.

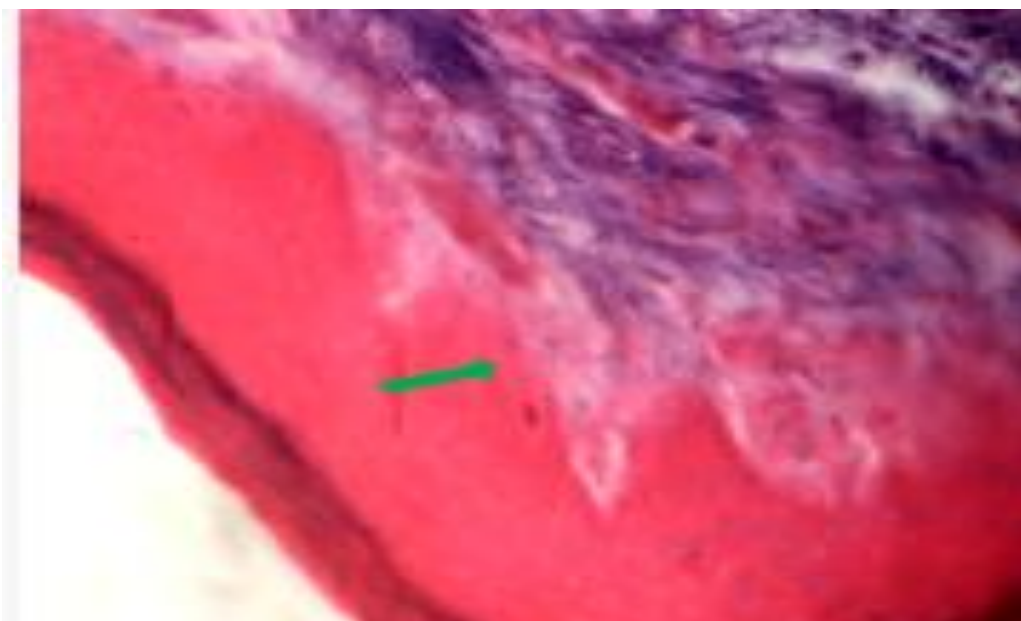


**Plate XXXII: Photomicrograph of a section of hind paw of an *M. oleifera* treated rat (LFB & E X 400).** Intra epidermal nerve fibres projecting into the epidermis from the dermis (↑), Epidermal layer (↑). Moderate intraepidermal nerve fibre density.



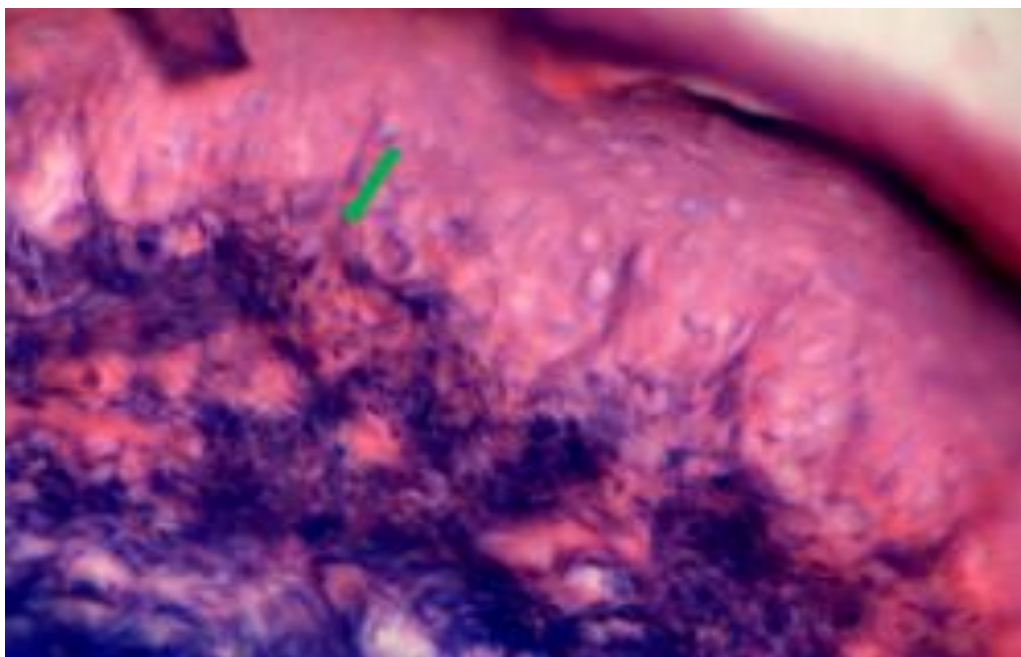
**Plate XXXIII: Photomicrograph of a section of hind paw of a Sitagliptin treated rat (LFB & E X 400).**

Intra epidermal nerve fibres projecting into the epidermis from the dermis (↑), Epidermal layer (↑). Moderate nerve fibre density.



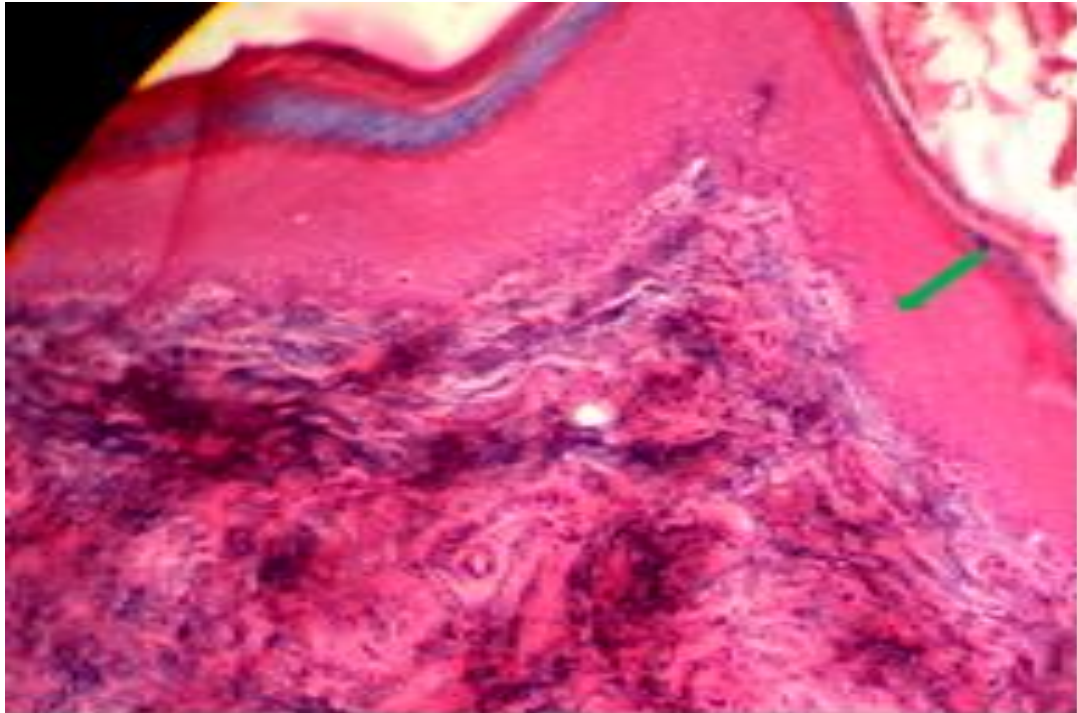
**Plate XXXIV: Photomicrograph of a section of hind paw of a Sitagliptin & *M. oleifera* treated rat (LFB & E X 400).**

Intraepidermal nerve fibres projecting into the epidermis from the dermis (↑).  
Moderate intraepidermal nerve fibre density.



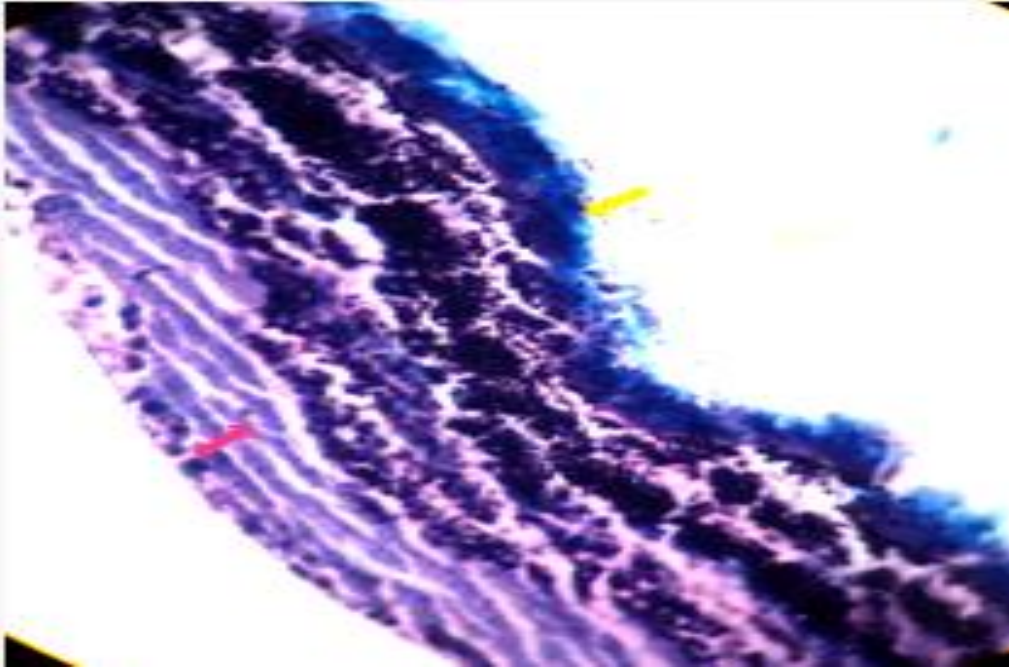
**Plate XXXV: Photomicrograph of a section of the hind paw of a rat in the Ameliorative group treated with (Sitagliptin & *M. oleifera*) (LFB & E X 400).**

Intra epidermal nerve fibres projecting into the epidermis from the dermis (↑).  
Moderate intraepidermal nerve fibre density.



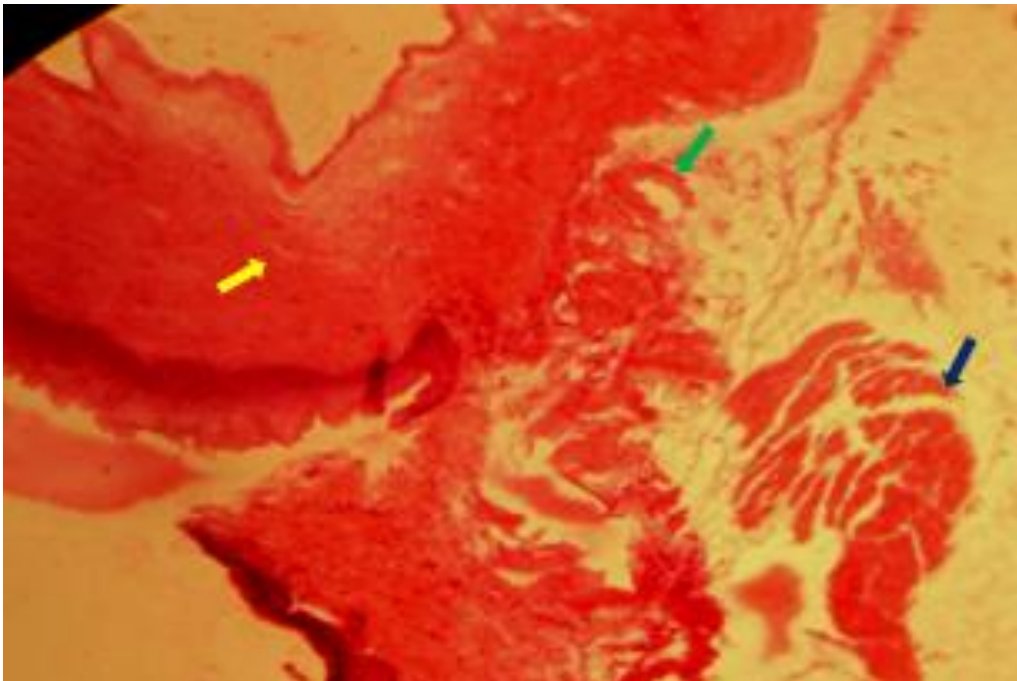
**Plate XXXVI: Photomicrograph of a section of hind paw of a post prandial control rat (LFB & E X 400).**

Intra epidermal nerve fibres (↑). Very low intraepidermal nerve fibre density.

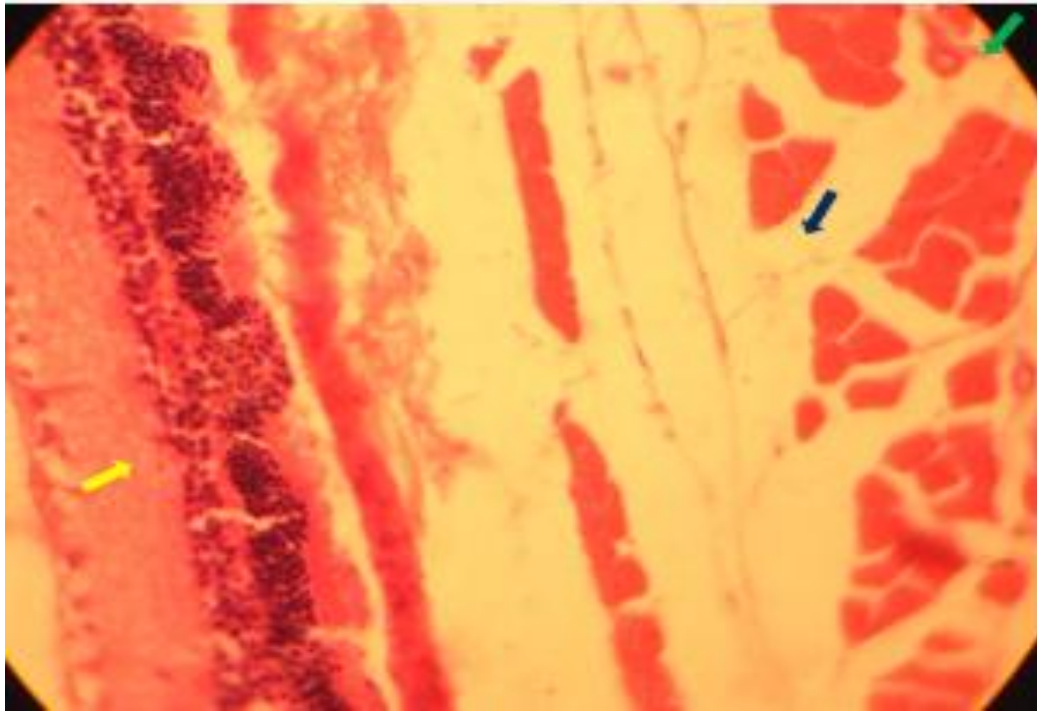


**Plate XXXVII: Photomicrograph of a section of the retina of a normal control rat (LFB X 400).**

It show regular arrangements of retinal cell layers including nerve fiber layer, ganglion cell later, inner plexiform layer, nuclear cell layer and pigmented epithelium (↑).

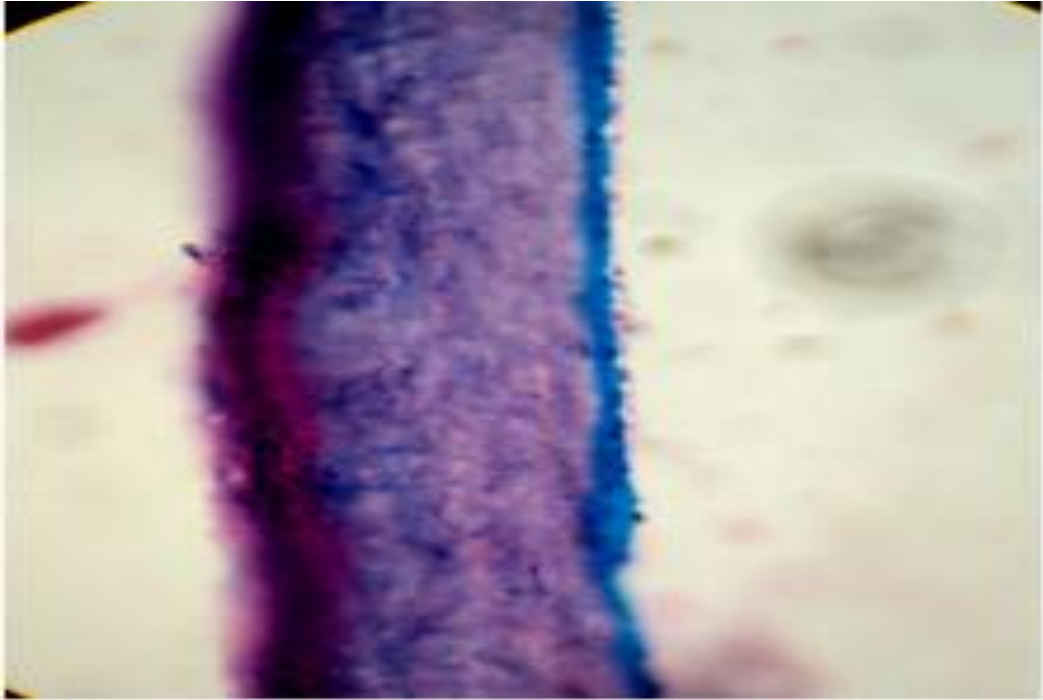


**Plate XXXVIII: Photomicrograph of a section of the retina and its adjoining structure of a normal control rat (H&E X 400).** It shows an intact capillary (↑), and musculature (myofibrils) (↑).

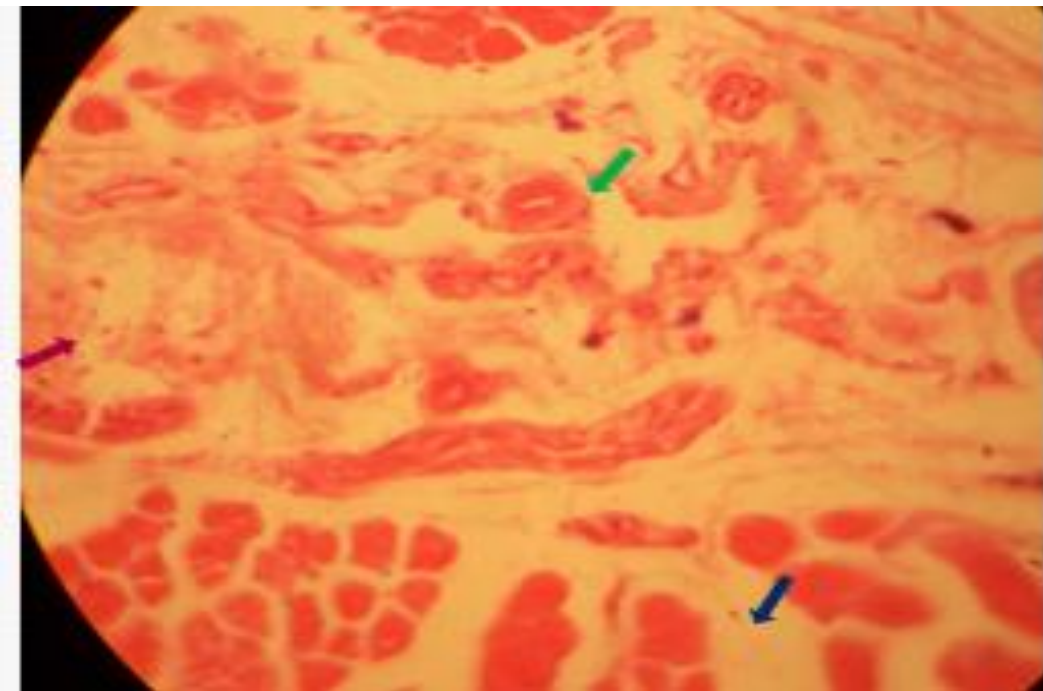


**Plate XXXIX: Photomicrographs of a section of the retina and its adjoining structure of a diabetic control rat (H&E X 400).**

It shows thickened capillary wall (↑) and oedema between layers of myofibrils and between retina and myofibrils (↑).



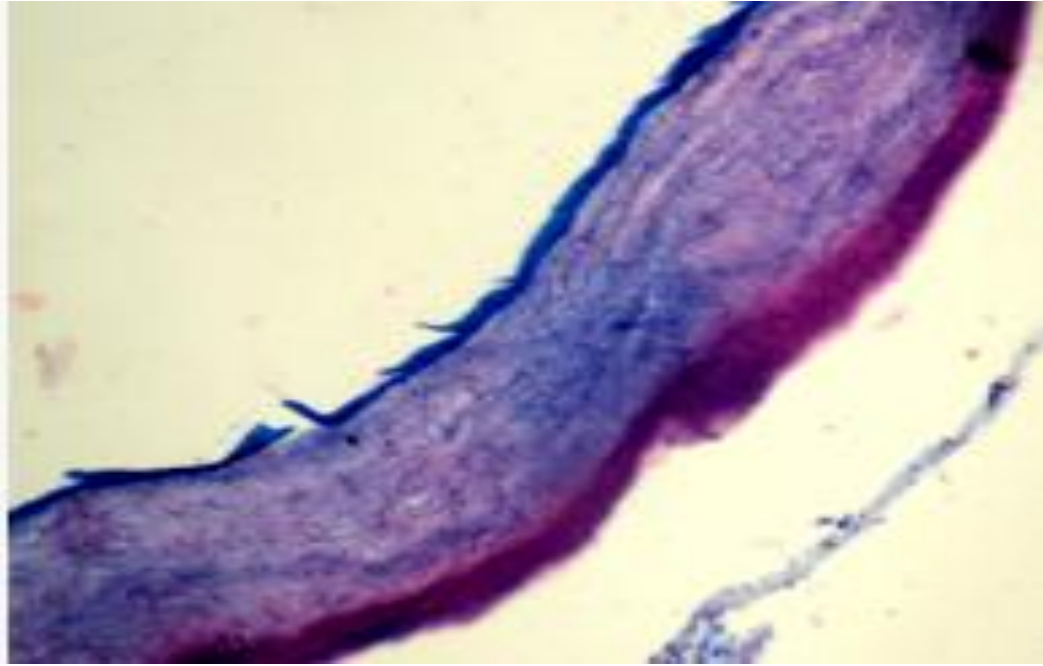
**Plate XL: Photomicrographs of a section of the retina of a Sitagliptin treated rat (LFB X 400)**



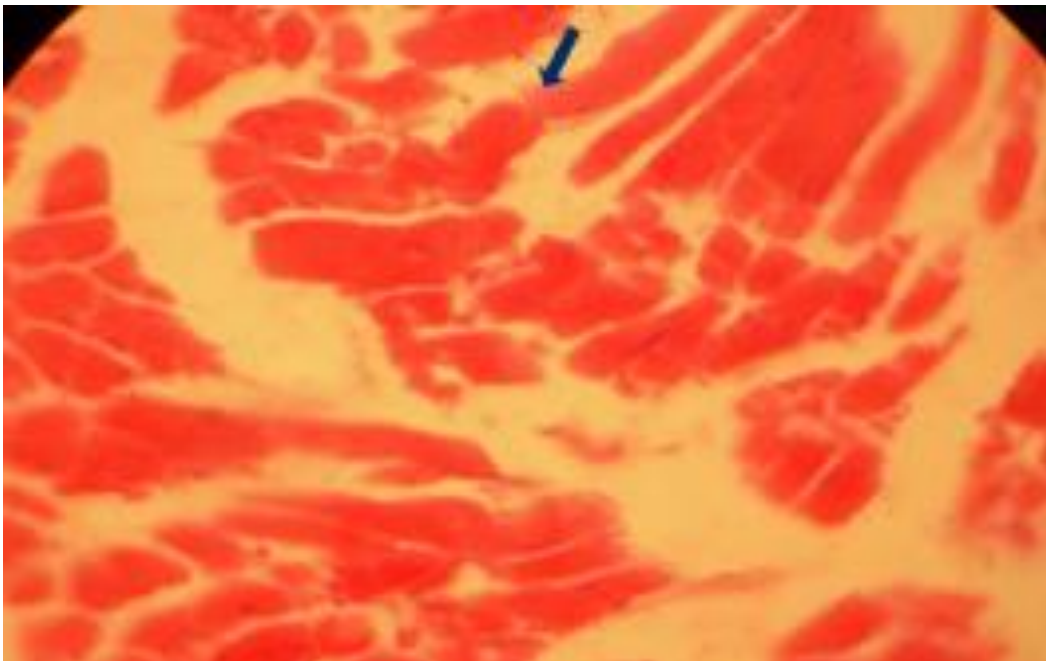
**Plate XLI: Photomicrograph of a section of the adjoining structures of the retina of a Sitagliptin treated rat (H&E X 400).**

It shows thickened capillary wall (↑), oedema between layers of myofibrils (↑) and haemorrhage (↑).



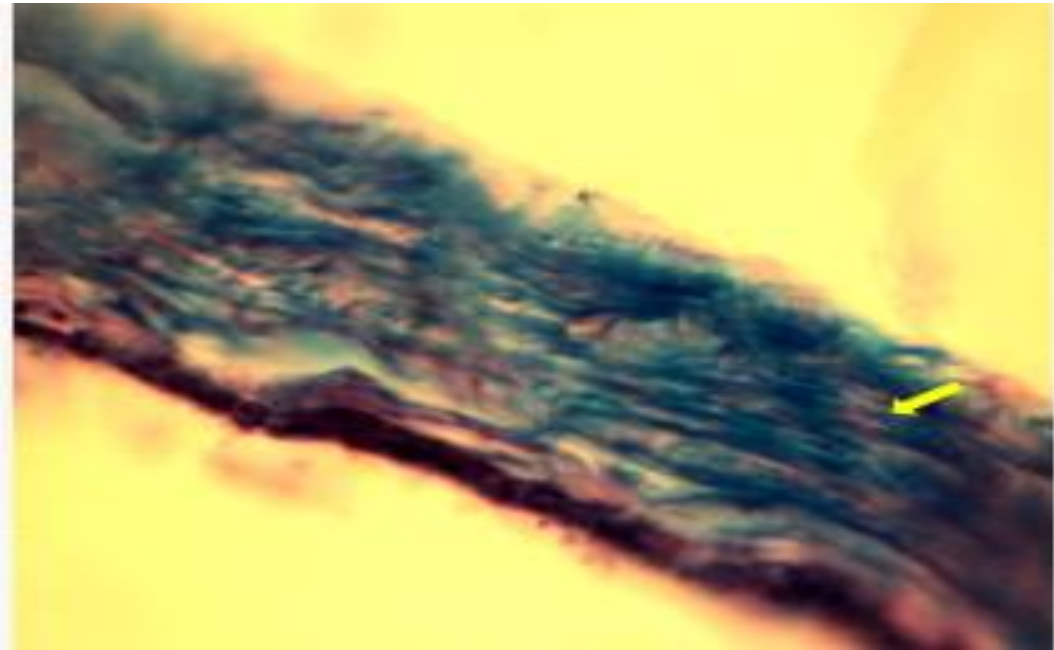


**Plate XLII:** Photomicrograph of a section of the retina of an *M. oleifera* treated rat (LFB X 400).

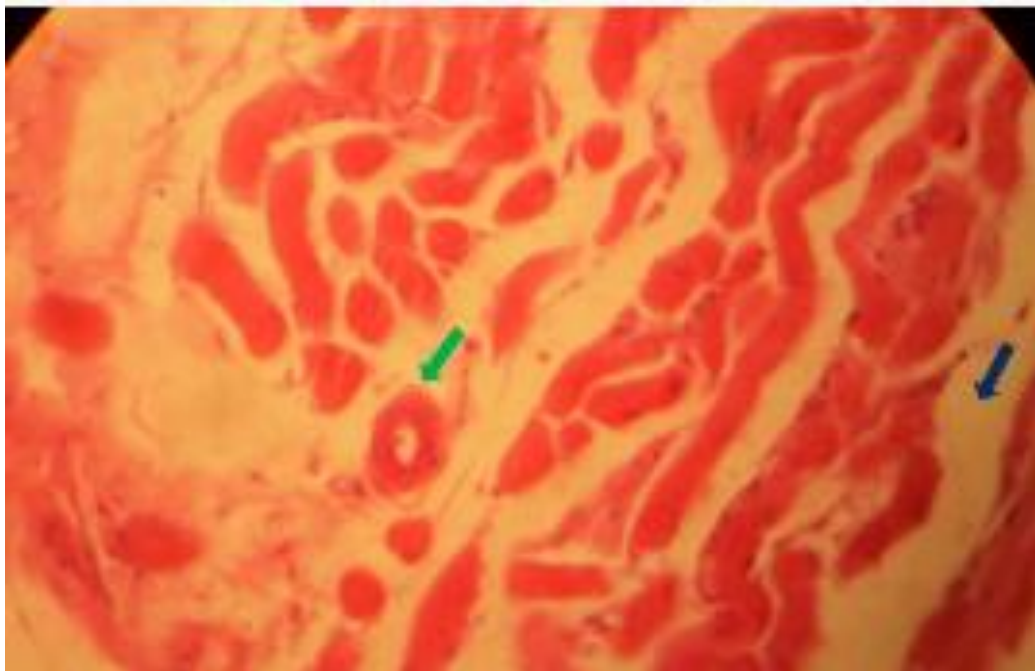


**Plate XLIII:** Photomicrograph of a section of the adjoining structures of the retina of an *M. oleifera* treated rat (H&E X 400).

Intact musculature (↑) and no significant histopathological findings.

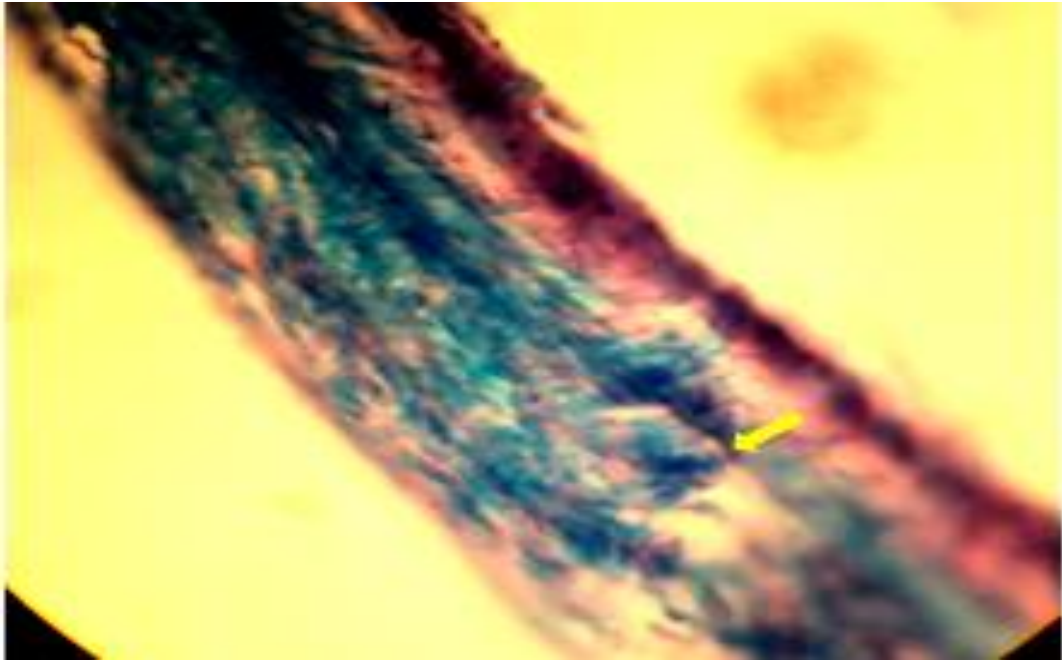


**Plate XLIV: Photomicrograph of a section of the retina of a Sitagliptin & *M. oleifera* treated rat (LFB X 400). Showing moderate distortion in retinal cell layer arrangement (↑).**

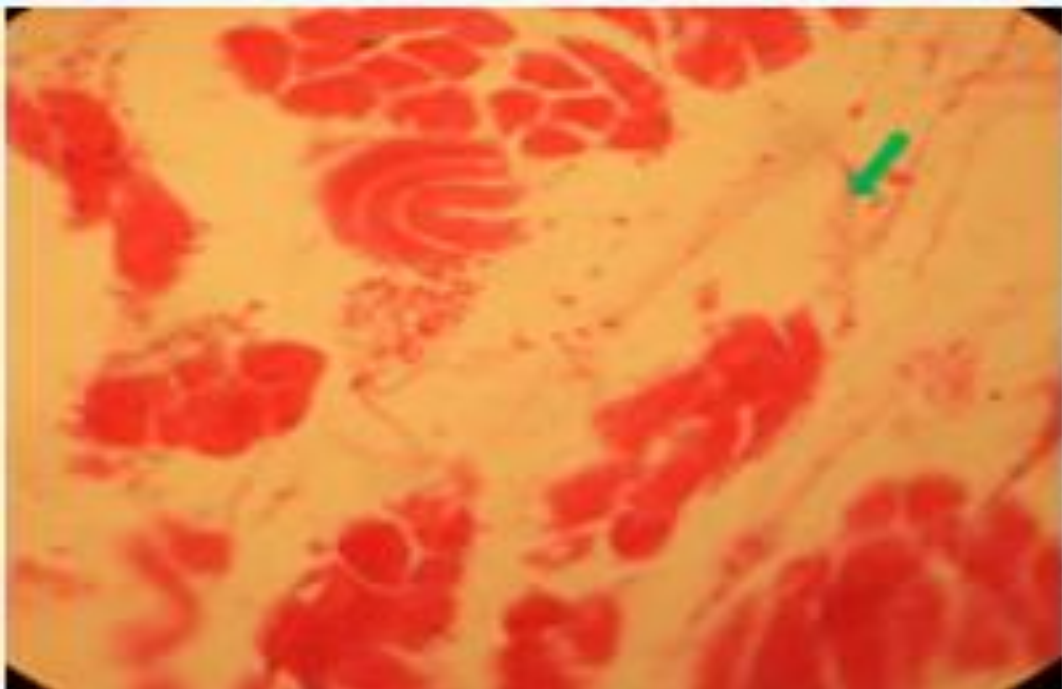


**Plate XLV: Photomicrograph of a section of the adjoining structures of the retina of a Sitagliptin & *M. oleifera* treated rat (H&E X 400).**

It shows thickened capillary wall (↑), oedema between layers of myofibrils (↑).

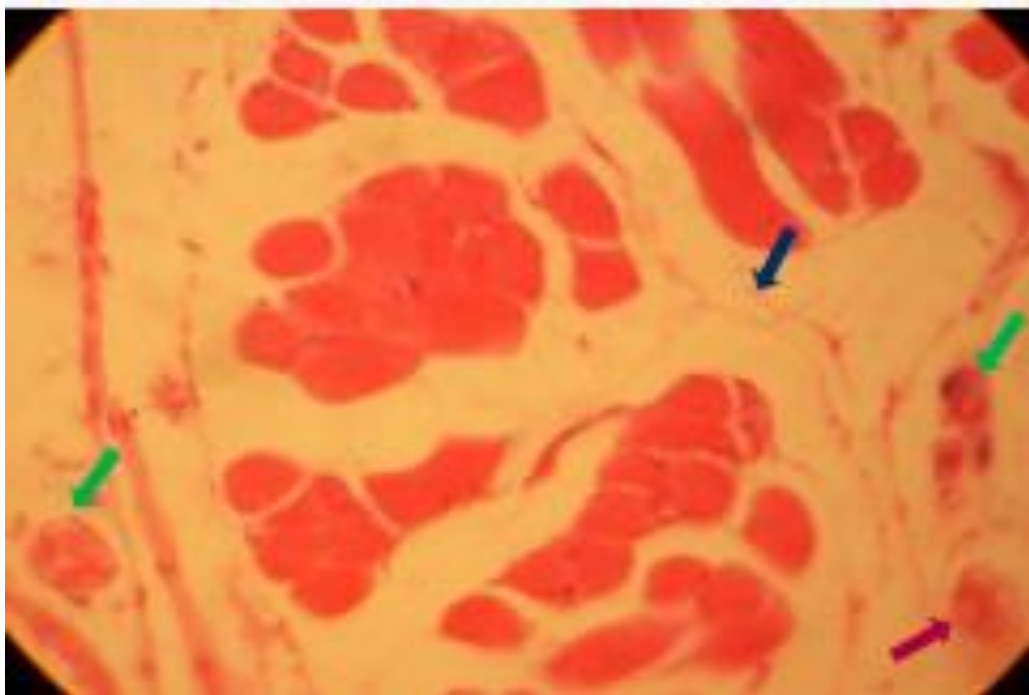


**Plate XLVI: Photomicrograph of a section of the retina of a post prandial control rat (LFB X 400). Showing distortion in retinal cell layer arrangement (↑).**



**Plate XLVII: Photomicrograph of a section of the adjoining structures of the retina of a post prandial control rat (H&E X 400).**

Oedema between layers of myofibrils (↑).



**Plate XLVIII: Photomicrograph of a section of the adjoining structures of the retina of a rat in the Ameliorative group treated with (Sitagliptin & *M. oleifera*) (H&E X 400).**

It shows thickened capillary wall (↑), oedema between layers of myofibrils (↑) and congestion (↑).