HISTOLOGICAL AND BIOCHEMICAL EFFECTS OF MELATONIN AND *Azadirachta indica* ADMINISTRATION ON LIVER AND PANCREAS IN STREPTOZOTOCIN INDUCED DIABETES IN ADULT WISTAR RATS

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DECLARATION

I, GODAM, ELVIS TAMS, declare that work in this thesis entitled "**Histological and biochemical effects of melatonin and *Azadirachta indica* administration on liver and pancreas of streptozotocin induced diabetes in adult wistar rats**" has been performed by me in the department of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria, under the Supervision of Dr Samaila M.O.A, Dr Hamman, W and Dr Ibegbu A.O. The information sourced from literature in the text has been duly acknowledged and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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CERTIFICATION

This thesis entitled "**Histological and biochemical effects of melatonin and *Azadirachta indica* administration on liver and pancreas in streptozotocin induced diabetes in adult wistar rats**" by Godam, Elvis Tams meets the regulations governing the award of the degree of Master of Science of Ahmadu Bello University for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty for his mercies throughout the course of this programme and my lovely and adorable wife and partner Kufre Elvis Godam for her immense love and support throughout my course of study, my daughters Ellis Zina and Eva Lesi and to my able sister Mrs. Blessing Nwate and my Mother Mrs. Comfort Mananwa. I truly appreciate you and love you both.

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ABSTRACT

This study was aimed at evaluating the histological and biochemical effects of melatonin and *Azadirachta indica* leaves ethanolic extract administration on streptozotocin (STZ) induced diabetes in adult Wistar rats. Forty five male Wistar rats were used in the study. The animals were divided into two main groups control group A and diabetic group B. The animals in Group A were sub divided into group 1, 2, 3 and 4 and group B animals subdivided into group 5, 6, 7, 8 and 9 comprises of five rats per group respectively. Group 1 received placebo orally once daily, Group 2 (extract control) received 200mg/kgbw of extract orally daily, Group 3 (melatonin control) received 10mg/kgbw of melatonin intraperitoneally (IP) daily, Group 4 (extract and melatonin control) received 200mg/kg bw of extract orally and 10mg/kgbw of melatonin IP daily, Group 5 (diabetic control) received 10ml/kgbw normal saline daily, group 6 (extract treated) received 200mg/kgbw of extract orally daily, Group 7 (melatonin treated) received 10mg/kgbw of melatonin IP daily, group 8 (extract and melatonin treated group) received 200mk/kgbw of extract orally and 10mk/kgbw of melatonin IP daily while group 9 (metformin treated) received 500mg/kgbw of metformin orally daily. The weight and fasting blood glucose levels were determined at intervals of seven days. At the end of 21 days of treatment, the animals were sacrificed and blood samples collected from all groups and assayed spectrophotometrically for serum concentration of liver enzymes Aspartate (AST, ALT, ALP), lipid peroxidation (MDA) and intracellular antioxidant superoxide dismutase (SOD), Catalase (CAT) and Glutathione perioxidase (GPx), while the pancreatic and liver tissues were harvested and histologically processed and stained using H&E, The liver tissues were specially stained with PAS for glycogen, Gordon and Sweet for reticular fibers and HVG for collagen fibers. The study

showed that there was a significant decrease ($p<0.05$) in the blood glucose levels in STZ-induced diabetic treated groups with extract alone, melatonin alone, and when extract and melatonin were combined at ($P<0.001$). There was a significant decrease ($P<0.05$) in the liver enzymes AST, ALT and ALP in the STZ-induced diabetic extract treatment alone and when combined with melatonin, melatonin treatment alone at ($P<0.001$). For lipid peroxidation there was a significant reduction ($P<0.05$) in serum MDA for the group treated with extract alone and when combined and high significant value ($P<0.001$) for melatonin treated group only. The study also showed significant increase ($P<0.05$) in serum intracellular antioxidants of the extract and melatonin treatment groups alone for SOD and a highly significant value ($p<0.001$) when extract and melatonin were combined. CAT gave a significant values ($p<0.05$) for melatonin and extract treated groups alone and a high significant value ($p<0.001$) when extract and melatonin were combined. GPx gave significant value ($p<0.05$) of extract treatment alone and highly significant values ($p<0.001$) for melatonin treatment alone and when extract and melatonin were combined. The histological studies showed regeneration of the pancreatic islets, liver collagen and reticular fibers and improved hepatic glycogen stores in all treated diabetic groups. Results obtained in this study have shown that *A. indica* and melatonin potentiates diabetic control and regeneration of the pancreatic islets and liver tissues.

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CHAPTER ONE

1.0 INTRODUCTION

Diabetes mellitus (DM) is a disease with devastating complications. It has global distribution and all ages are affected. In 1985, an estimated 30 million people around the world were diagnosed with diabetes; in 2000, that figure rose to over 150 million, and it is projected to rise further to 380 million by 2025 (IDF, 2008). The International Diabetes Federation stated that "every ten seconds, two people are diagnosed with diabetes somewhere in this world," and given the current trend, it is estimated that more people will have diabetes by 2025 than the current populations of the United States, Canada and Australia combined (IDF, 2007). The impact of diabetes is felt in both developed and developing countries. The Canadian Journal of Diabetes (2008) classified DM into Type 1 diabetes which primarily occurs as a result of pancreatic beta cell destruction, Type 2 due to predominant insulin resistance with relative insulin deficiency to a predominant secretory defect and Gestational diabetes which results from glucose intolerance in the course of pregnancy.

A study to assess the prevalent rate of type 2 diabetes and to determine the associated risk factors of the disease in Port-Harcourt, Nigeria was done using 502 subjects above 40 years. The study result revealed that 34 subjects had diabetes giving a prevalence rate of 6.8% with a male to female rate of 7.7% and 5.7% respectively. Thus a resultant relative high prevalence of type 2 diabetes mellitus in Port Harcourt (Ebenezar *et al.,* 2003). This was related to the changing lifestyle associated with industrialization. In another study to assess the prevalence of diabetes mellitus in Dakace village in the outskirt of Zaria, Nigeria; of

199 subjects 94 was males and 105 females had their fasting blood glucose and body mass indices determined. The overall prevalence of diabetes was 2.0%. The study concluded that the prevalence of diabetes mellitus (Type 2 DM) in the semi urban community is rising as seen in other parts of Nigeria (Dahiru *et al.*, 2008).

Various hypoglycemic drugs, such as sulfonylurea, metformin are being used for the treatment of diabetes but their use is restricted by their limited action and accompanying side effects such as hypoglycaemic shock and weight gain. Insulin treatment also fails to prevent the long term complication (Shailey and Basir, 2011).

Plants and plant products continue to play a dominant role in traditional remedies against ailments from antiquity (Kelly, 1995). Conventional measures used in the management of diabetes usually aim at improving glucose homeostasis and delay the onset of complications but, these measures are not curative (Kelly, 1995).Undue weight gain, drug resistance to Insulin and hypoglycaemia are some side effects associated with conventional measures used in treating diabetes mellitus (Luna and Feinglos, 2001). Many complications of diabetes result due to an increased free radical load (Kahler, *et al*, 1993). The search for natural antioxidative agents that will ameliorate the harmful effects associated with hyperglycaemia still continues in spite of considerable progress in the management of diabetes mellitus with synthetic drugs. There is therefore an increasing preference for whole plant extract among patients and professionals as these rarely produce side effects, rather they tend to protect the patient from the usual degenerative changes (Ebong, 2006). The naturally endowed antioxidant components of plants including flavonoids, vitamins A and C and other secondary metabolites may alleviate these complications when used as whole extract. Over 400 traditional plants have been used in the treatments of diabetes (Bailey and

Day, 1989). Opinion about the mechanism of action of plants include: presence of insulin-like substances, interference with carbohydrate absorption, inhibition of insulinase activity and increase in beta cells in the pancreas (Jelodar *et al*, 2005).

Azadirachta indica of the family- Meliaceae Melioideae, is a medium-sized tree that is found throughout the South Asian region, Africa and in Northern Nigeria. It is one of the most versatile medicinal plants having a wide spectrum of biological activities. Previous studies have reported the beneficial effect of *Azadirachta indica* leaves in the management of diabetes mellitus and the amelioration of the oxidative stress associated with the disease (Chattophadhyay *et. al*., 2004). This was explained by the presence of terpenoids and saponins which have been found to be potentially useful for the treatment of hyperglycaemia. Flavonoid, a known antioxidant is present in *Azadiracta indica* leaves and its extract is currently included in the poly-herbal anti diabetic drugs being subjected to controlled clinical trials in Man.

Melatonin has been shown to be a major scavenger of both oxygen and nitrogen based radicals (Baydas *et al.*, 2003; Reiter *et al.*, 2003; Lapshina, *et al*, 2006), including peroxynitrite anion ($ONOO^-$) (Gilad *et al*., 1997; Topal *et al.*, 2005; Ucar *et al*., 2007). Melatonin may influence diabetes and associated metabolic disturbances not only by regulating insulin secretion, but also by providing protection against reactive oxygen species, since pancreatic β-cells are very susceptible to oxidative stress because they possess only low-antioxidative capacity (Javier *et al.*, 2011). Melatonin has scavenging actions at both physiologic and pharmacologic doses and it also supports several intracellular enzymatic antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Reiter *et al*, 2005; Rodriguez *et al*., 2005). It influences both antioxidant

enzyme activity and cellular mRNA levels for these enzymes under physiological conditions and during elevated oxidative stress (Reyes-Toso *et al*, 2000). Melatonin is a non-toxic indolamine which shows significant benefits in the treatment of experimental hyperglycemia. These beneficial effects are mediated by a variety of means including as an antioxidant and as an epigenetic regulator (Reiter *et al*, 2005).

Accumulated data proved DM as an alarming epidemic in virtually all ethnic groups throughout the world. Chronically-elevated blood glucose is not only important in diabetes but also several other chronic diseases such as metabolic syndromes, obesity, and cardiovascular disorders. Thus any beneficial treatment that limits hyperglycemia and its harmful effects could greatly improve public health (Korkmaz, *et al*., 2008).

1.1 STATEMENT OF RESEARCH PROBLEM

In developing countries adequate treatment measures for diabetes mellitus are often unavailable or too expensive hence the need to test for the viability of *Azadirachta indica* ethanolic leaves extract and melatonin a known potent antioxidant as alternatives to conventional antidiabetic drugs.

1.2 SIGNIFICANCE OF THE STUDY

- The result of this study can be applied in the management of diabetes by using natural, easily and readily available and cheaper products with negligible side effects thus, serving as a preferred alternative to known diabetic drugs especially in developing countries.

- To contribute to the pool of knowledge on the beneficial effects of ethanolic extract of *Azadirachta indica* leaves and Melatonin in the management of diabetes mellitus
- If proven to be efficacious, these products could provide accessible and affordable treatment alternatives for the poor in developing countries.

1.3 JUSTIFICATION

- Diabetes mellitus has assumed worldwide epidemics and the life expectancy in developing countries in particular is halved by many disabling diseases including diabetes mellitus. Thus this study becomes necessary in order to provide readily available, cheaper and safer alternative.
- However, the histopathological and biochemical effects of melatonin and ethanolic extract of *Azadiracta indica* leaves on pancreatic islets and Liver of diabetic subjects are yet to be reported.

1.4 AIM OF THE STUDY

The aim of the study is:

To evaluate the morphological and biochemical effects of melatonin and *Azadirachta indica* leaves ethanolic extract administration on liver and pancreas of streptozotocin induced diabetes in adult Wistar rats

1.5 OBJECTIVES OF THE STUDY

The objectives of the study are to:

a) Determine the histomorphological and biochemical changes on the pancreas and liver due to the administration of ethanolic extract of *Azadirachta indica* leaves and melatonin.

b) Study the effect of melatonin and *Azadirachta indica* ethanolic leaves extract on blood glucose levels in streptozotocin-induced diabetic rats.

c) Study the combined effects of *Azadirachta indica* leaves and melatonin on free radicals in streptozotocin –induced diabetic Wistar rats.

d) Study the effects of *Azadirachta indica* ethanolic leave extract and melatonin on liver enzymes AST, ALT and ALP in streptozotocin-induced diabetic Wistar rats.

1.6 STUDY HYPOTHESIS

a) *Azadirachta indica* leaves ethanolic extract mediates its hypoglycaemic effects directly on the pancreas through antioxidant effects and mechanisms related to insulin production.

b) Melatonin efficacy can be potentiated in combined therapy with *Azadirachta indica* by scavenging free radicals and reversing the complications that arises from acute and chronic hyperglycaemia.

c) Melatonin counteracts several pathophysiologic steps and displays significant beneficial effects against hyperglycemia-induced cellular toxicity.

CHAPTER TWO

2.0 DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, defective insulin action or both. The chronic hyperglycemia of diabetes is associated with significant long-term sequelae, particularly damage, dysfunction and failure of various organs – especially the kidneys, eyes, nerves, heart and blood vessels (Ehud *et al*., 2008).

2.1 CLASSIFICATION OF DIABETES

2.1.1 TYPE 1 DIABETES MELLITUS

This is a disease resulting from absolute insulin deficiency, usually caused by autoimmune destruction of pancreatic islet cells. The initial clinical presentation may be ketoacidosis with an acute illness, or a more gradual presentation with symptoms of hyperglycemia. Other autoimmune disorders may also be present such as Addison's disease, thyroiditis, and pernicious anaemia. A small subsets of patients with type 1 diabetes have a non-immune mediated disease process with a waxing and waning clinical course. This form of type 1 diabetes is strongly inherited and most commonly affects persons of African and Asian descent (Ehud *et al*., 2008).

The typical patient with type 1 diabetes: Criteria for type 1 diabetes

- Is often diagnosed as a child or young adult though any age group may be affected.
- Lean physique with BMI < 25 kg/m^2.

- Normal insulin sensitivity, i.e., insulin requirements do not exceed 0.7 units of insulin/kilogram body weight/24 hours.
- Evidence of anti-beta cell autoimmunity (i.e., anti-insulin antibodies).
- Is more "ketosis prone" and usually have history of repeated bouts of diabetic ketoacidosis (DKA).

2.1.2 TYPE 2 DIABETES

This is a disease resulting from a relative, rather than an absolute, insulin deficiency with an underlying insulin resistance. Type 2 diabetes is associated with obesity, age, and physical inactivity. Patients with type 2 diabetes are not prone to ketoacidosis, frequently do not require insulin, and may be asymptomatic, despite being hyperglycemic for many years, (Ehud *et al*., 2008).

The typical patient with type 2 diabetes:

- Is more likely to be diagnosed as an adult.
- Overweight or obese with BMI >27-29 kg/m².
- Family history of diabetes. Over 90% of those patients with type 2 diabetes will have a first degree relative with the disease.
- Requires very large doses of insulin(>0.7 units/kg/day) to control the blood glucose (e.g., >0.7 units/kg/day) due to insulin resistance. Such individuals frequently have characteristics associated with insulin resistance, including abdominal obesity, hypertension, lipid abnormalities, atherosclerosis, and hyperuricemia.

- No evidence of anti-beta cell specific antibodies. Adult onset diabetes with circulating antibodies are sometimes referred to as having latent autoimmune diabetes of the adult (LADA). Such individuals seem to have a slowly progressive beta cell destructive process much like that occurring in children with typical type 1 diabetes, but the beta cell destruction occurs more slowly.
- No history of diabetic ketoacidosis (DKA), but may have a history of hyper osmolar coma.
- Consequences of the "metabolic syndrome," e.g. hypertension, lipid abnormalities, abdominal obesities, artherosclerosis and hyperureacaemia are common (Ehud *et al*., 2008).

2.1.3 OTHER TYPES OF DIABETES MELLITUS

This group includes gestational diabetes mellitus due to glucose intolerance with onset during pregnancy. Other causes of diabetes include: Genetic defects of islet cell function; genetic defects in insulin action; endocrinopathies such as Cushing's disease or syndrome; drug or chemical-induced hyperglycemia; infections; and insults to the pancreas from a variety of causes such as pancreatic cancer, cystic fibrosis, trauma, and pancreatitis (Ehud *et al*., 2008). There is also wide variety of relatively uncommon conditions and diseases induces diabetes (Ehud *et al*., 2008)

2.2 COMPLICATIONS OF DIABETES

Diabetes is a chronic, life-long condition that requires careful monitoring and control of blood sugar level. Uncontrolled high blood sugar levels result in long term damage to various organs and tissues (IDF, 2011).

2.2.1 Cardiovascular accidents

Cardiovascular accidents affect the heart and blood vessels (arteriosclerosis) and may cause fatal complications such as coronary heart disease leading to heart attack and stroke. Cardiovascular disease is the major cause of death in people with diabetes, accounting in most populations for over 50% of all diabetes fatalities, and much disability (gangrene) often resulting in heart attacks and stroke (IDF, 2011).

2.1.2 Diabetic nephropathy, neuropathy and retinoparhy

Diabetic nephropathy can result in total kidney failure and the need for dialysis or kidney transplant. It is an increasingly important cause of renal failure, and indeed has now become the single most common cause of end stage renal disease, (IDF, 2011). Diabetic neuropathy is a condition that can ultimately lead to ulceration and amputation of the feet and lower limbs due to loss of feeling which thus allow injuries to escape unnoticed and treatment (IDF, 2011). Diabetic retinopathy is characterized by damage to the retina of the eye, thus leading to visual loss.

2.3 DIAGNOSTIC CRITERIA

The clinical diagnosis of diabetes is often prompted by symptoms such as increased thirst (polyphagia) and urine volume , (polyurea), recurrent infections, unexplained weight loss and, in severe cases, drowsiness and coma; high levels of glycosuria are usually present (WHO, 1999).

Reports have shown that single blood glucose estimation in excess of the diagnostic value establishes the diagnosis in such cases. For clinical purposes, an Oral Glucose Tolerance

Test (OGTT) to establish diagnostic status need only to be considered if casual blood glucose values lie in the uncertain range (between the levels that establish or exclude diabetes) and fasting blood glucose levels are below those which establish the diagnosis of diabetes. If an OGTT is performed, it is sufficient to measure the blood glucose values while fasting and at 2 hours after a 75g oral glucose load

Glucose concentration, mmol l–1 (mg dl–1)			
		Whole blood	**Plasma ***
	Venous	**Capillary**	**Venous**
Diabetes Mellitus: Fasting	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥126)
2-h post glucose load or both	≥ 10.0 (≥180)	≥ 11.1 (≥200)	≥11.1 (≥ 200)
Impaired Glucose Tolerance (IGT): Fasting(if measured)	< 6.1 (< 110)	< 6.1 (< 110)	< 7.0 (< 126)
2-h post glucose load	≥6.7 (≥120) and < 10.0 (< 180)	≥7.8 (≥140) and < 11.1 (< 200)	≥7.8 (≥ 140) and < 11.1 (< 200)
Impaired Fasting Glycaemia (IFG): Fasting	≥5.6 (≥100) and < 6.1 (< 110)	≥5.6 (≥100) and < 6.1 (< 110)	≥ 6.1 (≥110) and < 7.0 (< 126)
and (if measured) 2-h post glucose load	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)

Table 2.1: This table shows the diagnostic for diabetes mellitus and other categories of hyperglycaemia according to WHO (2008)

For children the oral glucose load is related to body weight: 1.75g per kg. The diagnostic criteria in children are the same as for adults. Diagnostic interpretations of the fasting blood glucose levels and a 2 hour post–load concentration in non–pregnant subjects is shown in the table 2.1 (WHO, 2008).

2.4 PATHOPHYSIOLOGY OF DIABETES MELLITUS

Diabetes mellitus (DM) represents one of the most important health problems worldwide and according to recent estimations, it is likely to worsen to critical levels in the next decades, it is of great concern that it is rising rapidly in younger population groups, especially children and adolescents (Vivian, 2006). Liebel and Berrie (2008) showed in fig 2.1 below the various factors responsible for causing diabetes mellitus.

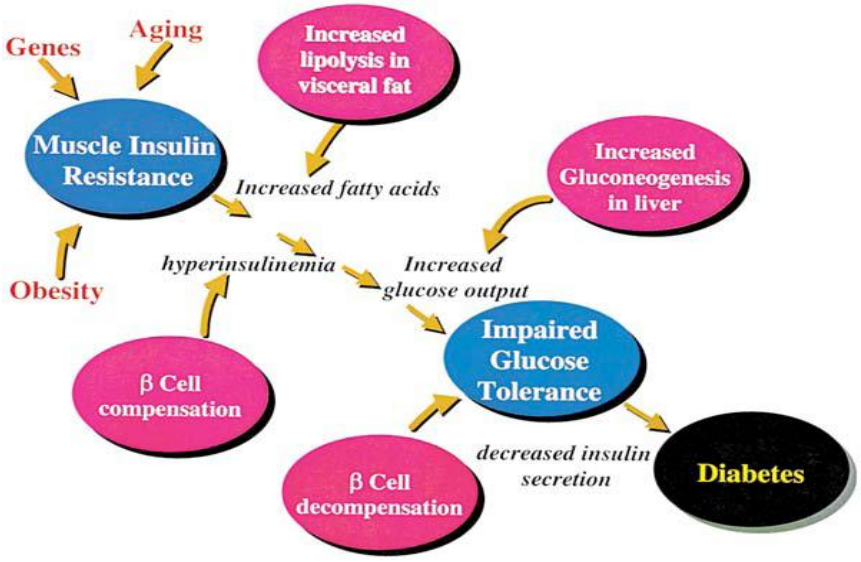


Fig.2.1: Some factors responsible for causing diabetes (Liebel and Berrie, 2008).

Diabetes has long been viewed as a disorder of carbohydrate metabolism due to its hallmark feature of hyperglycemia, indeed hyperglycemia is not only the cause of the acute symptoms such as polydypsia, polyuria, and polyphagia (Brownlee, 2011), but also the long-term complications including retinopathy, nephropathy, and neuropathy. In addition, hyperglycemia may contribute to the development of macro-vascular disease, which is associated with the development of coronary artery disease, the leading cause of death in individuals with diabetes (Wilson *et. al*, 2002). Thus, a primary goal in the prevention and the management of diabetes is the regulation of blood glucose to achieve near-normal levels. Hyperglycemia affects many tissues including vascular endothelium and pancreatic β-cells which leads to their dysfunction, (Wardle, 1999; Ding and Triggle, 2005). In this progression, the initial stage is a relatively long period of chronically elevated blood glucose, hyperglycemia persists and compromises metabolic activity leading to endothelial dysfunction (ED), β-cell dysfunction and disrupted vascular smooth muscle relaxation (Ding and Triggle, 2007).

The mechanism by which hyperglycemia is harmful remains unclear. Several works suggest that the levels of all biomarkers of oxidative stress are elevated in diabetic patients, thus this indicate an over-production of free radicals. The main source of oxidative stress in diabetes appears to be hyperglycemia and excessive generation of toxic species which play a key role in the pathogenesis of diabetic complications (Robertson *et. al.* 2007). In particular, recent studies show that a hyperglycemia-mediated process of superoxide ($O2^{-}$) formation, due to the leakage of electrons from the mitochondrial electron transport chain, is an initial and essential event in the activation of pathways involved in the pathogenesis of secondary negative events of diabetes (Kiersztan *et. al* 2004; Piconi *et. al*, 2006).

2.4.1 VASCULAR ENDOTHELIUM PANCREATIC β-CELLS AND HYPERGLYCEMIA

The endothelium, one of the largest cell populations in the body, is strategically located between the wall of blood vessels and the streaming blood. In adults approximately ten trillion (10^{13}) cells form a layer of cells in the vessel endothelium. It senses mechanical stimuli such as pressure and shear stress and response to hormonal stimuli including vasoactive substances (Tesfamariam and DeFelice, 2007). Endothelial cells are also intimately involved in the manifestation of infection, atherogenesis, hypertension, diabetes and cancer (Meigs *et. al*, 2007). These cells are known to be influenced by hyperglycemia which leads to their dysfunction earlier than other tissues in the organism.

Chronically-elevated glucose level is damaging to the structure and function of organs, including the vascular endothelium and pancreatic islets. Multiple biochemical pathways and mechanisms of action for glucose toxicity have been suggested. These include increased glucose autoxidation, augmented polyol pathway flux, increased advanced glycation end-products (AGE) formation, activation of protein kinase C (PKC) and nuclear factor kappa B (NF-κB), and increased hexosamine pathway flux, sorbitol formation, and oxidative phosphorylation (Mokini and Chiarelli, 2006).

Hyperglycemia also promotes directly the activation of NF-κB, increased expression of inducible nitric oxide synthase (iNOS), accompanied by excessive generation of nitric oxide (NO), and an over activity of NAD(P)H, which, in turn, produces an over abundance of $O2^{-}$ (Mohamed *et. al*, 1999). One potential central mechanism for glucose toxicity is the formation of excess reactive oxygen species (ROS), which are generated *via* multiple mitochondrial and non mitochondrial pathways (Newsholme, 2007). The pancreatic islets

are especially vulnerable to ROS because of their low intrinsic level of antioxidant enzymes. It has been long known that pancreatic islets contain relatively small amounts of the antioxidant enzymes Cu,Zn-superoxide dismutase (Cu,Zn-SOD; also known as SOD1), Mn-SOD (SOD2), catalase (CAT) and glutathione peroxidase (GSH-Px), and as a result, they are readily damaged by chronic oxidative stress (Robertson, 2004).

The beneficial effects of superoxide dismutase (SOD) treatment in the prevention of alloxan induced diabetes in mice are also known (Grankvist, *et al.*, 1979). These and many other observations have reinforced the notion that the intrinsically low level of antioxidant activity of the pancreatic islets renders them particularly at risk for ROS-induced damage. Moreover, chronically-elevated glucose and ROS levels can cause reduced insulin gene expression (Lupi *et al.*, 2007) and insulin resistance (Kaneto, 2005).

This pathophysiologic sequence sets the scene for considering antioxidant therapy as an adjunct in the management of insulin resistance and diabetes where oxidative stress is elevated. It seems likely that chronic oxidative damage is a major contributor to hyperglycemia-induced damaging processes (Korkmaz et. al, 2008). One means of testing this hypothesis would be to determine whether better maintenance of glucose levels in hyperglycemic patients is accompanied by lower levels of ROS and improved insulin secretion and/or insulin resistance. Another would be to add antioxidants to conventional therapy to determine whether this manoeuver would prevent continued deterioration in β-cell and endothelial functions, despite continued hyperglycemia (Korkmaz, *et. al*, 2008).

2.4.2 PHYSIOLOGIC AND PATHOLOGIC REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

Under normal circumstances, cells are able to balance the production of oxidants and antioxidants, resulting in redox equilibrium. Oxidative stress occurs when cells are subjected to excess levels of ROS or as a result of depletion of antioxidant defenses (Sies, 1991). Several environmental and biochemical changes cause elevated ROS production; these include hazardous contaminants (e.g. nitroso compounds, polycyclic hydrocarbons, alcohol, aflatoxin, heterocyclic aromatic amines, and even "over nutrition", tissue damage (e.g. mechanical, heat, acid), infectious factors (e.g., *Helicobacter pylori*, hepatitis B and C virus, Epstein-Bar virus), inflammatory reactions (e.g., pancreatitis, ulcerative colitis), environmental hazards (e.g., ultraviolet light, ionizing radiation, tobacco smoke, gas exhaust, lead, asbestos), and biochemical changes (e.g., hyperglycemia, dyslipidemia) are recognized as generating excess ROS (Sies, 2007)

During aging, the redox equilibrium between oxidants and antioxidant defenses gradually deteriorates in favor to more ROS and tissue damage occurs. ROS play important roles in regulating a variety of cellular functions and act as secondary messengers in the activation of specific transcription factors including NF-κB and activator protein-1 (AP-1) within a certain local concentration range; however, excessive production of ROS is harmful to cells *via* the same means. Once excess ROS production induces transcription actor activation (e.g., NF-κB, AP-1), the harmful effects of excess ROS are spread due to gene activation for TNF-α, IL-1β and other cytokines (Sies, 2007)

Inducible NOS is predominantly expressed in inflammatory cells such as macrophages, although epithelial cells from affected tissues also express iNOS. Intensified expression of

iNOS has been detected in virtually all cell types tested including macrophages, fibroblasts, chondrocytes, osteoclasts, and epithelial cells and results in the production of large amounts of NO in animals and patients with inflammatory diseases (Cooke, *et al*., 2002; Stockklauser-Farber, *et al*., 2002; Weidig, *et al*., 2004). The level of iNOS expression is well correlated with the degree of inflammation. The controversy arises from observations reporting both cytotoxic and cytoprotective effects of NO. In cases where NO was found cytotoxic, it was questioned whether NO, directly or indirectly, or through the formation of more reactive species such as the peroxynitrite anion ($ONOO^-$) exerted these effects. The combination of elevated NO plus excess $O2^-$ with the formation of high levels of $ONOO^-$ is the proverbial intracellular "devil's triangle".

Essentially any pathophysiologic process caused exclusively by oxygen-derived free radicals could presumably be alleviated by conventional antioxidants such as vitamin E and C and/or intracellular enzymatic antioxidants such as SOD, CAT and GSH-Px. Once iNOS is activated, however, because of NO's affinity for the $\cdot O2^-$, neither enzymatic nor pharmacologic levels of conventional antioxidants are able to compete with NO for $\cdot O2^-$; as a result, high $ONOO^-$ levels follow (Beckman, et al., 2003). In case of chronic hyperglycemia and/or dyslipidemia, highly activated iNOS could readily shift the molecular destruction from oxidative to nitro-oxidative damage leading to a situation where conventional antioxidants are less efficient in reducing damage.

2.5 ANTIDIABETIC DRUGS

2.5.1 Insulin and Oral Hypoglycemic Agents

Insulin is a polypeptide hormone consisting of two peptide chains that are connected by disulfide bonds. It is synthesized as a precursor a (pro-insulin) that undergoes proteolytic cleavage to form insulin and C peptide, both of which are secreted by the beta cells of the pancreas (Richards and Pamela, 2009). Insulin is a hormone that is central to regulating the energy and glucose metabolism in the body. Insulin causes cells in the liver, muscle and fat tissue to take up glucose from blood, storing it as glycogen in the liver and muscle (Federwisch *et al*., 2002). When control of insulin level fails, diabetes mellitus result. (Melloul *et al*., 2002).Patients with type 1 diabetes mellitus depend on external insulin (most commonly injected subcutaneously) for survival because the hormone is no longer produced internally (William *et al*., 1998).

2.5.2 Mechanism of Insulin Secretion

Insulin is regulated not only by blood glucose levels but also by certain amino acids, other hormones (gastrointestinal hormones), and autonomic mediators. Secretion is most commonly triggered by high blood glucose, which is taken up by the glucose transporter into the beta cells of the pancreas; there it is phosphorylation by glucokinase, which acts as a glucose sensor. The products of glucose metabolism enter the mitochondrial respiratory chain and generate adenosine triphosphate (ATP). The rise in ATP levels causes a block of K^+ channels, leading to membrane depolarization and an influx of Ca^{2+}, which results in pulsatile insulin exocytosis (Richards and Pamela, 2009). Glucose given by injection has a weaker effect on insulin secretion than does glucose taken orally, because when given

orally, glucose stimulates production of digestive hormones by the gut, which in turn stimulates insulin secretion by the pancreas, (Richards and Pamela, 2009).

2.5.3 Mechanism of Insulin Action

Insulin receptors are found in different cells of the body, including cells in which insulin does not increase glucose uptake. The insulin receptor, which has a molecular weight of approximately 340,000, is a tetramer made up of two α and β glycoprotein subunits.The α subunit bind insulin and is extracellular, whereas the β subunits span the membrane. The intracellular portions of the β subunits have tyrosine kinase activity. The α and β subunits are both glycosylated, with sugar residues extending into the interstitial fluid. Binding of insulin triggers tyrosine kinase activity of the β subunits, producing auto-phosphorylation of the β subunit on the tyrosine residues. The auto phosphorylation which necessary for insulin to exert its biologic effects triggers phosphorylation of some cytoplasmic proteins and dephosphorylation of others, mostly on serine and theorine residues, (Williams *et al*., 1998). Four related insulin receptor substrate (IRS) proteins in cells have been described: IRS-1, IRS-

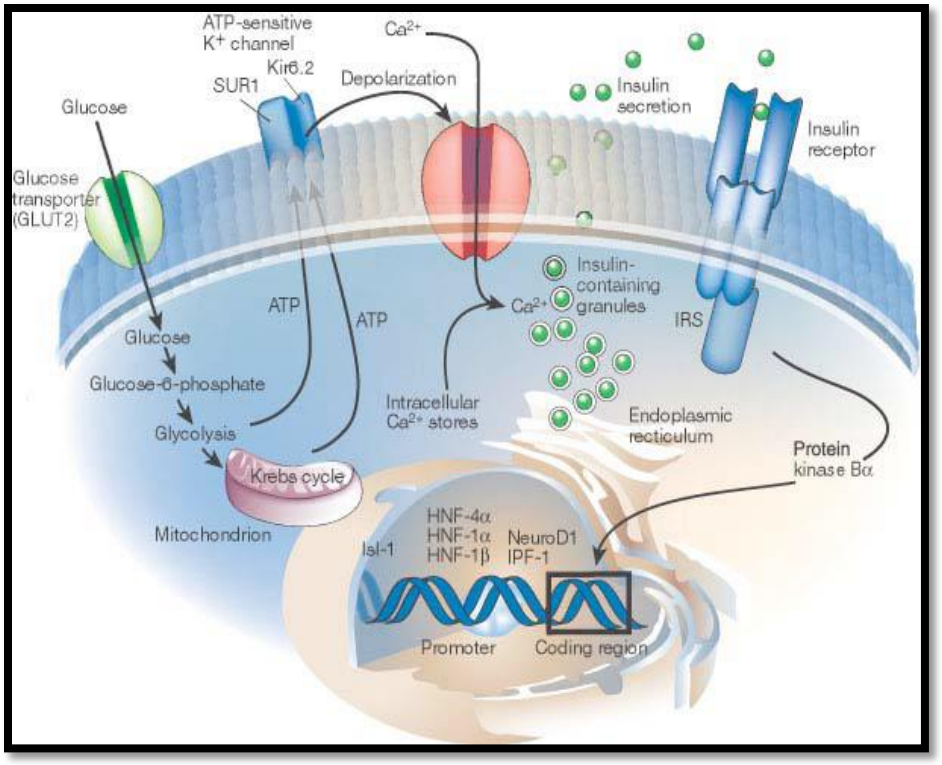


Fig. 2:2: Mechanism of glucose transport and insulin receptors (William et al., 2008).

2, IRS-3 and IRS-4. IRS-1 has received the most attention (Williams *et al*., 1998). When insulin binds to its receptor (Fig 2.2) they aggregate in patches and are taken into the cell by receptor mediated endocytosis. Eventually, the insulin-receptor complexes enter the lysosomes, where the receptors are broken down or recycled (Williams *et al*., 1998). The number or the affinity, or both of insulin receptors is affected by insulin and other hormones, exercise, food, and other factors. Exposure to increased amounts of insulin decreases receptor concentration (down regulation), and exposure to decreased insulin levels increases the number of receptors (Hellman *et al*., 2007).

2.5.4 Oral Hypoglycemic Agents/ Insulin Secretagogues

Anti-diabetic drugs treats diabetes mellitus by lowering glucose levels in the blood. With the exception of insulin, exenatide and pramlintide, all other antidiabetic drugs are administered orally and are thus, called oral antihyperglycemic agents (Rendell, 2004). Insulin secretagogous agents are only useful in type 2 diabetes mellitus but who cannot be managed by diet alone, as they work by stimulating endogenous release of insulin. They work best in patients who develop diabetes after age 40 has had diabetes less than 5 years (Richard and Pamela, 2009).

2.5.5 Sulfonylureas

Sulfonylureas were first widely used oral anti-hyperglycemic medications. They are classified as insulin secretagogues, because they produce insulin release from beta cells of the pancreas. They are insulin secretagogues, triggering insulin release from the β cells of the pancreas by blocking the ATP-sensitive channels, resulting in depolarization and ca^{2+} influx, reduction in hepatic glucose production and increase in peripheral insulin sensitivity (Fimognari *et al.*, 2006). The primary drugs used today are tolbutamide and second-generation derivatives, glyburide, glipizide and glimepiride. They cannot be used with type 1 diabetes, or diabetes of pregnancy. The primary side effect is hypoglycemia (Fimognari *et al*., 2006).

2.5.6 Meglitinides

This class of agents includes repaglinide and nateglinide. Meglitinide help the pancreas produce insulin and are often called “short acting secretagogues”. Although they are not sulfonylureas, they have common actions. They act on the same potassium channels as

sulfonylureas, but at a different binding site, like the sulfonylureas, their action is dependent on functioning pancreatic β cells. By closing the potassium channels of the pancreatic beta cells, they open the calcium channels, hence enhancing insulin secretion. They are taken with or shortly before meals to boost the insulin response to each meal. Adverse reactions include weight gain and hypoglycemia (Fimognari *et al.*, 2006)

2.5.7 Insulin Sensitizers, Biguanides and Thiazolidinediones

Two classes of oral agents; the biguanides and thiazolinedinediones improve insulin action. These agents lower blood sugar by improving target–cell response to insulin without increasing insulin secretion (Richard and Pamela, 2009).

Metformin, the currently available biguanide, is classed as an insulin sensitizer; that is increases glucose uptake and utilization by target tissues, thereby decreasing insulin resistance. Like the sulfonylureas, metformin requires insulin for its action, but differs from the sulfonylureas in that it does not promote insulin secretion (Richard and Pamela, 2009). Metformin reduce hepatic glucose output largely by inhibiting hepatic gluconeogenesis, slow intestinal absorption of sugars and increase uptake of glucose by the periphery, including skeletal muscle. Metformin, a biguanide, has become the most commonly used agent for type 2 diabetes in children and teenagers. Among common diabetic drugs, metformin is the only widely used oral drug that does not cause weight gain (Cvetko and Plosker, 2007).

Another group of agents that are insulin sensitizers are the thiazolidinediones (TZDs) or, more familiarly the glitazones. Although insulin is required for their action, these drugs do not promote its release from the pancreatic β cells; thus hyperinsulinemia does not occur.

Presently, two members of this class are available, pioglitazone and rosiglitazone (Richard and Pamela, 2009).

2.5.8 α-Glucosidase Inhibitors, Peptide Analogs and Incretin Mimetics

Acarbose and miglitol are orally active drugs used for the treatment of type 2 diabetes mellitus. These drugs are taken at the beginning of meals. They act by delaying the digestion of carbohydrate, thereby resulting in lower postprandial glucose levels. Both drugs exert their effects by reversibly inhibiting membrane-bound α-glucosidase in the intestinal brush border, (Richard and Pamela, 2009).

Peptide analogs include: Incretin mimetics, Glucagon-like peptide (GLP) analogs and Agonists, Amylin Analogues and Dipeptidyl peptidase-4 Inhibitors.

Incretins are insulin secretagogues. Oral glucose results in a higher secretion of insulin than occurs when equal load of glucose is given intravenously. This effect is referred to as the "incretin effect" and is apparently reduced in type 2 diabetes mellitus. It demonstrates the important role of gastrointestinal hormones, notably GLP-1 and gastric inhibitory peptide in the digestion and absorption of nutrients (Richard and Pamela, 2009).

2.5.9 Glucagon-like peptide (GLP) Analog and Agonists, Amylin Analog and Dipeptidyl peptidase-iv Inhibitors

Glucagon-like peptide agonists bind to a membrane GLP receptor. As a consequence of this, insulin release from the pancreatic beta cells is increased. Endogenous GLP has a half life of only a few minutes (Khan *et al*., 2003).

Pramlintide is a synthetic amylin analog that is indicated as an adjunct to meal time insulin therapy in type1 or type2 diabetes. By acting as an amylinomimetic, pramlitide delays gastric emptying, decreases postprandial glucagon secretion, and improves satiety. Like, insulin, it is administered by subcutaneous injection (Lee and Reasner, 1994).

Sitagliptin is an orally active Dipeptidyl peptidase-IV (DPP-IV) inhibitors used for the treatment of type 2 diabetes mellitus. Other agents in this category are currently in development. Sitagliptin inhibits the enzyme dipeptidyl peptidase-IV, which is responsible for the inactivation of incretin hormones, e.g. glucagon-like peptide-1 (GLP-1) (Richard and Pamela, 2009).

2.6 STREPTOZOTOCIN

Streptozotocin (STZ; *N*-nitro derivative of glucosamine) is a naturally occurring, broad spectrum antibiotic and cyto-toxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals (Weiss, 1982 and Szkudelski, 2001).

Streptozotocin is a mixture of α- and β-stereoisomers that appear as a pale yellow or off-white crystalline powder. STZ is very soluble in water, ketones, and lower alcohols and only slightly soluble in polar organic solvents. Streptozotocin functions as a DNA synthesis inhibitor in both bacterial and mammalian cells (Bolzan and Bianchi, 2002). In bacterial cells, a specific interaction with cytosine moieties leads to the degradation of the bacterial DNA (Reusser, 1971). In mammalian cells, the mechanism of action that results in cell death has not been fully identified, but is thought to be a result of DNA and chromosomal damage brought forth by mechanisms involving free radical generation during STZ metabolism (Bolzan and Bianchi, 2002). In many animal species, STZ induces diabetes that

resembles human hyperglycemic non ketotic diabetes mellitus (Weis *et al*., 1982). This effect has been extensively studied and appears to be mediated through a lowering of beta cell nicotinamide adenine dinucleotide (NAD^+) and results in histopathologic alteration of pancreatic islet beta cells. When administered intravenously, plasma levels of STZ rapidly decrease within 15 minutes and concentrate in the liver and kidneys. Induction of experimental diabetes in the rat using streptozotocin is very convenient and simple to use (Weiss, 1982). Streptozotocin injection leads to the degeneration of the Langerhans islets beta cells. Clinically, symptoms of diabetes are clearly seen in rats within 2-4 days following single intravenous or intraperitoneal injection of 45- 60mg/kg STZ.

During metabolism of streptozotocin, a variety of toxic intermediates are produced. Besides alkylating agents like methyl cations and methyl radicals, it has been shown that ROS are produced by streptozotocin as well (Takasu, *et al*., 1991**)**. Moreover, streptozotocin liberates NO which has been proposed to be one of the key intermediates of its toxicity (Rao, et al., 2002). Taken together, streptozotocin-induced diabetes increases oxidative stress through generation of free radicals (Aksoy, *et al*., 2003), lipid peroxidation, superoxide dismutase, protein glycosylation (Montilla, *et al*., 1999), decreased levels of catalase and glutathione peroxidase (Armagan, *et al*, 2006), as well as DNA single-strand breaks (Takasu, *et al*., 1991).

2.6.1 Streptozotocin dosage

Selection of an appropriate dosage of STZ is a very important issue. Owing to strain differences (Rodrigues *et al*. 1997), the diabetogenic doses of STZ range from 45 to 70 mg/kg (Rakieten, *et al*., 1963; Ar'Rajab and Ahrén 1993). In an experiment, STZ dose of 70

mg/kg was lethal for all animals tested. In groups given STZ doses of 50 or 60 mg/kg, there was stable diabetes with persistent hyperglycaemia over 20 mmol/1 of plasma glucose was observed during the 4-month study period. Non-homogenous glycaemia values below 20 mmol/L were observed in group dosed with 40 mg/kg STZ. Spontaneous recovery of STZ induced diabetes was observed at STZ dose levels 30-40 mg/kg (Ar'Rajab and Ahrén 1993). After administration of streptozotocin, a characteristic triphasic response in blood glucose was described (Junod *et al*. 1967).

In the first two hours blood glucose rises. This transient hyperglycaemia is due to sudden breakdown of liver glycogen. The second phase, starting at about 6 hours after STZ dosing, is a hypoglycaemic one, which may be severe enough to lead to death. Hypoglycaemia is more pronounced in fasted animals; therefore STZ should be administered to feed animals to avoid mortalities (Bell and Hye, 1983). Administration of 5% glucose solution during the first 24 hours following STZ injection prevented early mortalities. The third phase is that of permanent hyperglycemia, begins at about 10 to 12 hours after STZ administration. In keeping with the findings of Ramanadham *et* al. (1989), groups given STZ doses of 50 or 60 mg/kg manifested major clinical signs of diabetes mellitus, such as polyphagia, polydipsia, polyuria and body weight reduction. In agreement with literary data (Leuenberger, *et al*., 1971), cataract formation was observed between the days 90 and 100. Significant albuminuria, a marker of starting kidney damage, occurred at the end of the 4-month test period. Starting renal dysfunction was corroborated by significantly increased activity of the lysosomal cathepsin D in kidney tissue which was in agreement with literary data (Chouinard and Viau 1992).

Day 2 and 3 after STZ administration proved to be most critical for the animals. The animals found dead were cachectic with indigestion, bloody effusion from nasal and genital openings and with strong hyperaemia of all parenchymatous organs. This state seems likely to be due to urea, ammonia and nitrate poisoning. These ions are formed during decomposition of the N-nitrosomethyl urea moiety of streptozotocin (Wilson *et al*. 1984).

2.7 MELATONIN: A MULTIFUNCTIONAL MOLECULE

Melatonin is secreted from pineal gland during night. This indolamine has a variety of means by which it influences the physiology of the organism; some of these actions are receptor-mediated while others are receptor-independent (Tan, *et al*., 2003; Reiter, *et al*., 2007). Melatonin is a highly lipophilic indole which easily enters all cells. Melatonin has been administered in both physiological and pharmacological amounts to humans and animals, and there is widespread agreement that it is a highly non-toxic molecule, (Reiter, *et al*., 2004).

2.7.1 Melatonin as a versatile antioxidant

Researchers have shown evidences that melatonin is a major scavenger of both oxygen and nitrogen based radicals (Baydas, et al., 2003), including $ONOO^{-}$ (Topal, *et al*., 2005; Ucar, *et al*., 2007). Melatonin has scavenging actions at both physiologic and pharmacologic doses. Melatonin also supports several intracellular enzymatic antioxidant enzymes including SOD and GSH-Px (Reiter, *et al*., 2005; Winiarska, *et al*., 2006). Moreover, melatonin induces the activity of gamma-glutamyl cysteine synthase thereby stimulating the production of another intracellular antioxidant, glutathione (Winiarska, *et al*., 2006). Some

researchers have demonstrated the antioxidative effects of melatonin on hyperglycemic and/or diabetic conditions (Baydas, *et al*., 2002; Winiarska, *et al*., 2006) and melatonin is significantly better than other antioxidants in this regard e.g., more effective than vitamin E.

Several antioxidants reportedly exhibit SOD and/or GSH-Px preservation properties. These effects are, however, indirect due to their ability to scavenge free radicals and protect the protein from damage. Melatonin, on the other hand, possesses genomic actions and regulates the expression of several genes including those for SOD and GSH-Px. Melatonin influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes under both physiological conditions and during elevated oxidative stress (Reyes-Toso, *et al*., 2004). These two features in a single molecule are unique for an antioxidant and both actions protect against pathologically-produced free radicals during hyperglycaemia.

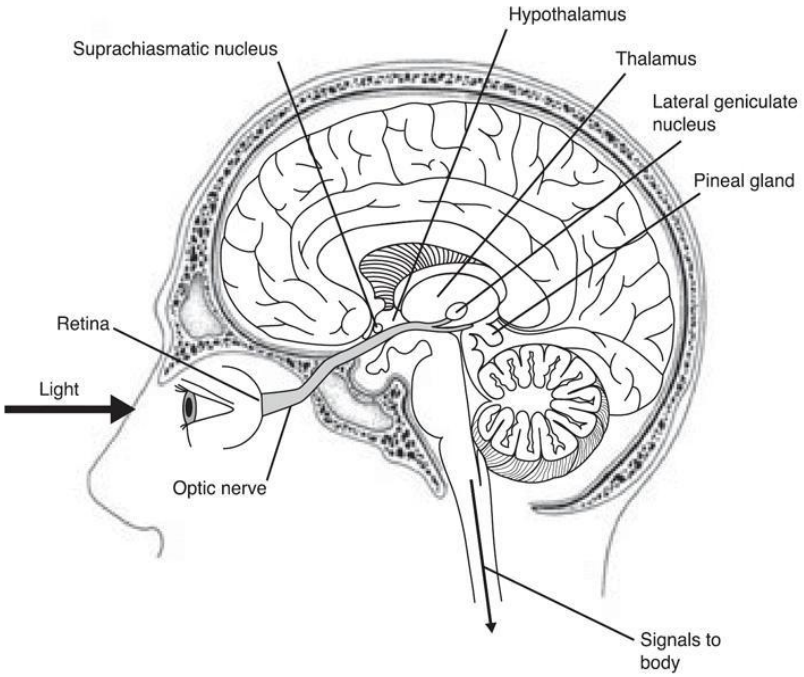


Fig.2.3 the Pineal Gland and the Suprachiasmatic nucleus.

Pancreatic β-cells have naturally lower antioxidant enzyme levels (Robertson and Harmon, 2007), thus, melatonin supports these cells in two ways, i.e., by scavenging the free radicals produced and by inducing the enzymes involved in metabolizing toxic reactants to innocuous products.

In the serum of animals with streptozotocin-induced diabetes, melatonin remarkably reduces the degree of both lipid peroxidation and protein glycosylation (Montilla, *et al.*, 1998), decreases the levels of cholesterol, triglyceride, low-density lipoprotein (Baydas**,** *et al.*, 2002), sialic acid and glucose, as well as possibly regulating the activities of antioxidant enzymes (Armagan, *et al.*, 2006).

2.7.2 Melatonin counteracts inhibitory nitric oxide synthetase (inos) and perioxynitrite anion (onoo^{-})

In many inflammatory processes including hyperglycemic conditions $ONOO^{-}$ rather than oxygen-based radicals is the predominant molecule which decides the fate of cells. Once formed by the coupling NO and $O2^{-}$, $ONOO^{-}$ cannot be removed or scavenged by vitamin E or C or by other conventional antioxidants. As a multifunctional antioxidant, however, melatonin and its metabolites have unique features over the usual antioxidants including iNOS inhibitory (Gilad, *et al.*, 1998; Dong, *et al.*, 2003) and $ONOO^{-}$ scavenging, properties. These features of melatonin, apart from direct antioxidative effects, have been documented in STZ-induced hyperglycemia (Winiarska, *et al.*, 2006) and other circumstances such as colitis (Dong, *et al.*, 2003), liver and lung damage, and alkylating agent toxicity (Sadir, *et al.*, 2007). Thus, melatonin is the only medically suitable molecule

which has the ability of blocking all sides of the "devil's triangle". Melatonin has been shown to ameliorate inflammation by blocking transcriptional factors (Sasaki, *et al*., 2002), TNF-α and IL-1 (Wang, *et al*., 2004), *via* several mechanisms (Li, *et al*., 2005). Researched evidence confirms that these cytokines are capable of inducing formation of free radicals and promoting iNOS activity and transcriptional factor activation within cells. These events inevitably induce a vicious cycle of cellular damage. In the case of $ONOO^-$-induced DNA damage, PARP over-activates in an attempt to repair the genome, consumes NAD+ as a substrate which causes an energy crisis within cells leading to their eventual necrosis. Preservation of NAD+ and cellular energetics may be helpful for PARP to repair the DNA damage rather than blocking PARP. Melatonin preserves cellular energy production, *via* different means including inhibiting iNOS and scavenging $ONOO^-$ and other oxidizing/nitrosating species (Baydas, *et al*., 2002).

2.7.3 Synthesis and function of melatonin

Melatonin is an integral part of the homeostatic mechanism in the body. It signals whether light or dark prevails. Melatonin, like the neurotransmitter serotonin, is an indoleamine. It is converted in two steps from the amino acid tryptophan into serotonin (5-hydroxytryptamine, 5-HT), and then acetylated by arylalkylamine *N*-acetyltransferase (AA-NAT), the rate-limiting step in melatonin biosynthesis, before finally being converted into melatonin by hydroxyindole-*O*-methyltransferase (HIOMT) (Axelrod and Weissbach , 1960).

The indoleamine is mainly secreted by endocrine cells (pinealocytes) in the pineal gland, which is located in the midline of the brain, just above the posterior commissure at the dorsal edge of the third ventricle. Melatonin remains detectable after pinealectomy in some

species (Ozaki and, Lynch, 1976), and subsequent investigations have revealed that the hormone is also produced by neuro-endocrine cells in the retina, Harderian glands, gastrointestinal tract and pancreas (Kvetnoy, 1999). Melatonin is also produced by numerous non-endocrine cells, e.g., immune cells. Hence, while the pineal gland quantitatively accounts for the circulating pool of the hormone, substantial local synthesis also occurs in retinal and peripheral tissues such as the gastrointestinal tract.

From a physiological perspective, the most well-known role of melatonin is that as a chronobiotic factor or *zeitgeber*, adjusting the timing or reinforcing oscillations of the biological clock, i.e., entrainment (Arendt, 1994). As such, it is thought to participate in the control of seasonal as well as circadian rhythms. This is based on the fact that the secretion of melatonin reflects ambient light and normally exhibits a tightly regulated diurnal pattern. For this reason, melatonin is sometimes called 'the hormone of darkness'. Disruptions may occur in individuals deprived of light, e.g., shift workers or travellers across time zones. On a daily basis, melatonin has a small modulatory effect on the pacemaker activity of the circadian clock in the suprachiasmatic nucleus. On a seasonal basis, the varying lengths of the peaks and troughs of the circulating levels of melatonin follow the changes in the duration of daylight. The seasonal regulation of the nocturnal secretory duration is the primary cue regulating the reproductive function in mammals that breed seasonally. Melatonin also affects the cardiovascular system (Krause**,** 1999**)** and interacts with the immune system (Reiter, 2000). It has also been implicated in metabolic control (Peschke, 2008). Given that the sites of melatonin production are widespread, its effects may be both endocrine, *via* melatonin released from the pineal gland, and paracrine/autocrine, *via* melatonin released in the vicinity of its target tissues (Kvetnoy, 1997**)**. An interesting

feature of melatonin is its capacity to act as an antioxidant, owing to its chemical structure. However, melatonin does not undergo redox cycling, i.e., repeated oxidation and reduction, but is a terminal or suicidal antioxidant instead (Tan, 2000).

2.7.4 Chronobiology, metabolic control and disease

As far back as the eighteenth century it has been observed that organisms, ranging from unicellular to multicellular, exhibit inherent rhythms. Such rhythmicity plays an important role in the temporal control of a wide range of biological processes in the body, the most notable of which is metabolism (Karlsson *et al.*, 1998). The most important and well-known biological rhythm is the circadian rhythm, which is defined as the roughly 24 h cycle that characterizes virtually all organisms on Earth. It is an adaptation to the periodicity at which our planet moves around its axis, which determines day length. In addition to circadian rhythms, there are ultradian rhythms, which are shorter than 24 h, and infradian rhythms, which extend beyond 24 h. To be considered a circadian rhythm, three major criteria must be fulfilled (King et al., 1995):

(1) It should persist under constant external conditions, i.e. be endogenously generated (Green et al., 2008) ;

(2) It should be temperature-insensitive; and

(3) It can be reset by an external stimulus, i.e. entrainment.

Given the intimate relationship between circadian rhythms and metabolism, a link between the disruption of circadian rhythm and metabolic perturbation has been considered (Green, 2008). Indeed, the metabolic syndrome is more prevalent in shift workers (Karlsson, 2001), known to exhibit disturbances of the circadian rhythm, and sleep-deprivation has been

associated with both obesity and type 2 diabetes (Knutson, 2006). Moreover, when the circadian rhythm is experimentally misaligned in humans, a profound effect on plasma insulin and glucose levels, promoting glucose intolerance, is observed (Scheer, 2009).

2.7.5 Melatonin rhythm and insulin secretion

Studies in rat has proved that the synthesis of melatonin declines with increasing age, whereas the synthesis of insulin and leptin increases (Rasmussen *et al*., 1999) and that melatonin is able to stop the age related insulin increase (Rasmussen *et al*., 2001).

Complementary to these findings are publications reporting that melatonin levels are reduced in diabetic hamsters (Champney *et al*., 1986). On the other hand, there is evidence for a diabetes preventing effect of melatonin, whereas pinealectomy increases the risk (Conti and Maestroni, 1998; Conti and Maestroni, 1996). Likewise, further data demonstrates that melatonin directly influences both glucose metabolism and insulin secretion from the β-cell. Peschke *et al*, (2002) reported reduced circulating melatonin levels and elevated insulin levels in type 2 diabetic patients, with a statistically significant negative correlation between both molecules. Similarly, nocturnal melatonin levels are reduced in the Goto-Kakizaki (GK) rat, a model of type 2 diabetes (Peschke *et al*., 2006).

2.7.6 Insulin secretion in pancreatic β-cells is organized by a circadian rhythm

Insulin and melatonin plasma concentrations change in an opposing manner during the 24-h period, i.e., melatonin peaks when insulin is at a low level, and *vice versa*. Further information on the circadian rhythms of insulin secretion was obtained from isolated rat pancreatic islets, maintained in an in-vitro perfusion system (Peschke *et al*., 2006). In this case, a circadian pattern was also observed, with periods between 22 and 26 hours. Adding

melatonin as *zeitgeber* during analysis of the phase responses in insulin secretion resulted in circadian phase shifts.

After melatonin application, the circadian period was maintained, but the amplitude was enhanced. From this experiment, it was concluded that an endogenous oscillator is located within the pancreatic islets of the rat which regulates the insulin secretion of β-cells in a circadian fashion

2.7.7 Melatonin receptors in β-cells

Melatonin has been shown to have direct effects on insulin secretion and that its receptors are present in islets of Langerhans, preferably β-cells. This indeed appears to be the case, as inferred from studies using the non-hydrolysable guanosine-5'-trisphosphate (GTP) analogue guanosine 5'-*O*-(3-thiotrisphosphate) and the melatonin antagonist luzindole (Peschke *et al*, 2007), both of which block the effects of melatonin on insulin secretion from neonatal rat islets. Likewise, using molecular techniques, it was demonstrated that a melatonin receptor mRNA identical to that cloned from the rat brain is expressed in pancreas tissue of newborn rats (Reppert *et al*, 1994). The specificity of the single amplification product was confirmed by restriction analysis and nested PCR, indicating that it corresponds to the predicted MT1 receptor. A possible co-expression of the MT2 receptor in the pancreatic tissue was initially excluded (Reppert *et al*., 1994), Thus the results indicate that a melatonin receptor, most likely the MT1 receptor, was located in the pancreatic islets of neonate rats and that the pancreatic islets are targets for receptor-mediated melatonin influences (Picinato, *et al*., 2002). Recently, molecular and immunocytochemical investigations have established the presence of the melatonin

membrane receptors MT1 and MT2 in human pancreatic tissue and notably, also in the islets of Langerhans (Mühlbauer and Peschke, 2007). On the other hand, an up regulation of the expression of melatonin receptors in type 2 diabetic patients was also observed in immunocytochemical investigations (Pescheke *et al*., 2007).

Melatonin plays an important role in many aspects of mammalian physiology by acting via two types of G protein coupled receptors (melatonin receptor type 1 MT1 and MT2) (Dubocovich and Markowska, 2005). Previous studies have suggested a role for the pineal gland and melatonin in the regulation of carbohydrate metabolism (Peschke, 2008). Additional investigations have also indicated that melatonin receptors are present on pancreatic islets and insulin secretion from isolated islets can be regulated by melatonin (Mulder *et al*., 2009). Finally, a study using melatonin receptor knock-out mice has indicated an active role of these receptors in the regulation of blood glucose (Mühlbauer *et al*., 2009). A recent article has also reported that melatonin treatment can improve glucose metabolism in an insulin resistant mouse model by restoring the action of insulin on the vasculature (Sartori *et al*., 2009).

Melatonin is a pleitropic, nocturnally peaking and systemically acting chronobiotic. Several generalizations can be proposed regarding melatonin. Since it readily passes many biological membranes to reach intracellular organelles, many cells can synthesize melatonin, presumably to scavenge the oxygen and nitrogen based reactants produced in these cells, moreover, its membrane receptors are widespread in mammals and mediate some of the melatonin's actions. It has been determined that the effects of melatonin on insulin secretion are mediated through the melatonin receptors (MT1 and MT2). By inhibiting cAMP and/or cGMP pathways, melatonin reduces insulin secretion. However, it has been shown that

melatonin activates the PLC/IP3 pathway, which mobilises Ca2+ from intracellular stores and, subsequently, increases insulin secretion (Espino, *et al*., 2011).

Further studies published in the World Journal of Diabetes, (2011) shows that, insulin secretion, both *in vivo* and *in vitro*, exhibits a circadian rhythm, apparently generated within the islets, which is influenced by melatonin by inducing a phase shift in insulin secretion. The observation that clock genes exhibit circadian expression in pancreatic tissue could be an indicator of the generation of circadian rhythms in the pancreatic islets themselves. Also, plasma melatonin levels and AA-NAT are decreased in type 2 diabetes patients. Taken together, these results indicate a close interrelationship between insulin and melatonin, which may be significant for the genesis of diabetes. This has been recently supported by genome-wide association studies revealing a close link between SNPs of the MT2 receptor (*MTNR1B*) locus and an increased prognostic risk of type 2 diabetes (Espino *et al*., 2011).

2.8.0 MEDICINAL PLANTS

From the inception of the existence of the earth, plant has been of great importance to the animal kingdom. It contributes either as a source of food or shelter. In the primitive ages, plants were also used for clothing therefore provides the basic needs of humans and animals as well. Out of these plants medicinal plants are common and are source of much attention in Africa (Akenova, *et al*., 1996).

Uniformity of the quality of drug is also of great importance as far as the therapeutically active constituent of raw material is concern. This makes it possible for pharmacist to

prescribe Numerical values through which commodities are assessed and are used to ensure uniformity of standards (Ames *et al*., 1992).

Historically, plants have been used in folk medicine to treat various diseases and these have been shown to be rich in natural antioxidants. Thus Researchers have examined the effects of plants used traditionally by people to support her function and treat diseases, including diabetes mellitus and have confirmed traditional experience by discovering mechanisms and mode of actions of these plants ((Frease and Evans, 1985).

Medical plant (Akinmoladun, *et al*., 2007) is defined as one, which contains substance that can be used for therapeutic purposes and its precursor for the synthesis of useful drugs. They contain nutrients that can heal the body (Frease and Evans, 1985). Material like these plants that has cellular structure is referred to as organized drug in pharmacy and those with Non-cellular structure as unorganized or a acellular drug (Akinpelu *et al*., 2006). Medicinal plants are termed as crude drugs of natural or biological origin by pharmacists to describe whole plant or plant parts having medicinal properties (Okwu *et al*., 2006).

2.8.1 THE PLANT *AZADIRACHTA INDICA* (NEEM)

Azadirachta indica (Neem tree) (Meliaceae) is a native of Asia but has now naturalized in West Africa. The plant is commonly known as Dalbejia or Dogon-yaro in Hausa language, it is widely cultivated throughout Nigeria as an ornamental plant. The plant is drought resistant and therefore grows well even in the arid parts of Nigeria; growing up to 25 m high, but it occurs mostly as a medium sized tree. Various parts of this plant are employed in Nigerian traditional medicine for the treatment of variety of ailments. Dogon-yaro is used extensively in Nigeria for the treatment of malaria using aqueous infusion, decoction or

alcoholic extracts of the leaves (fig 2.4) and stem bark. The effect of Azadirachta extract on methaemoglobin generation and the conversion of glutathione to its oxidized counterpart have also been investigated (Iwu, *et al.*, 1986).

During last five decades, considerable progress has been achieved regarding the biological activity and medicinal application of *Azadirachta indica* A Juss (Neem). Each part of Neem tree is being used in the traditional systems of medicine for the treatment of a variety of human ailments (Biswas, *et al*., 2002). Neem oil, bark and leaf extracts have been therapeutically used as folk medicine for leprosy, intestinal helminthiasis, respiratory disorders, constipation and also as a general health promoter (Shukla, *et al*., 2000 and Biswas, *et al*., 2002). *A. indica* has been commonly used to treat diabetes in Indian system of medicine from the time immemorial and several reports which suggest the hypoglycemic potential of *Azadiracht. indica* (Chattopadhyaya, *et al*., 1990; El-Hawary *et al*., 1990).

2.8.1.0 Effects of unprocessed neem on animals

2.8.1.1 Acute effects

Acute toxicity of unprocessed material in animals was reported only for a sheep that ate neem leaves. Ingestion resulted in nervous symptoms (head movements, walking in circles) with dyspnoea, an increase in body temperature, hepatic failure and tympanites. The symptoms lasted for 12 h and were followed by the death of the animal (Ali and Salih, 1982).

More positively, administration of leaf sap caused an anti-anxiety effect in rats at low doses, while high doses did not cause such an effect (Jaiswal et al., 1994). In sheep, kernel powder

caused a decrease in the number of nematode eggs in their faeces, and an increase in body weight (Ahmed et al., 1994).



Fig 2.4: ***Azadirachta indica leaves***

2.8.1.2 Sub acute effects

An important effect upon subacute exposure to leaf powder is that on reproductive ability in male rats. Leaf powder caused a decrease in the weight of the seminal vesicle and the ventral prostate, (Kasturi *et al*., 1997), a reduction in the sperm count and sperm motility as well as an increased percentage of malformed sperm, (Parveen *et al*., 1993). Moreover, at a dose of 100 mg/rat, a reduction in the diameters of the seminiferous tubule was observed. Gradual recovery in histological and biochemical parameters was found after termination of the treatment, (Joshi *et al*., 1996).

At slightly lower doses, the height of the epithelium in caput and cauda epididymis was reduced dose-dependently. The lumen of the caput was packed with lymphocytes and the serum testosterone concentration was decreased, (Kasturi *et al*., 1995). Biochemically, the leaf powder caused decreases in protein content and acid phosphatase activity, and increases in activities of alkaline phosphatase and lactate dehydrogenase, (Kasturi *et, al*., 1997), and in total free sugar, glycogen and cholesterol contents, (Joshi *et al*., 1996).

2.8.1.3 Subchronic effects

In a semi-chronic study, Aladakatti *et al*. (2001) found that leaf powder in rats caused a decrease in total sperm-count and in sperm motility. The relative percentage of abnormal sperm increased. Since the effects of the powder were annihilated when testosterone was administered simultaneously, the authors suggested that the effects were due to an androgen deficiency, thereby affecting the physiological maturation of sperm.

A positive effect against intestinal nematodes was found for cattle upon neem leaf feeding without any effect on the weight gain, (Pietrosemoli *et al*., 1999). In rabbits, neem fruits caused decreased serum activities of acid phosphatase, alkaline phosphatase and glucose and an improvement of glutamic oxaloacetate transaminase (SGOT), glutamic pyruvate transaminase (SGPT), cholesterol, total protein and bilirubin values (Tanveer *et al*., 1998).

2.8.1.4 Aqueous extracts

A simple way to prepare a plant extract is the soaking of plant material in water. This provides aqueous neem extracts.

2.8.1.5 Effects on humans and animals

Kroes et al. (1993) reported that in Sri Lankan medicine a fermented decoction of neem bark is taken as a drug with immunomodulatory activity. An in vitro haemolytic assay proved that the human complement system and the activity of polymorphonuclear leukocytes from healthy volunteers were inhibited. Investigations on effects of aqueous neem extracts on animals are numerous, and reveal in most cases beneficial rather than harmful effects.

2.8.1.6 Acute effects

Leaf extract caused a moderate decrease of the blood glucose levels in mice (Mossa, 1985). It produced hypoglycaemia in normal rats. The clotting time of blood was higher than normal. Serum cholesterol level increased with a concomitant decrease in liver fat and a dose-related drop in liver proteins (El Hawary and Kholief, 1990). The extract had toxic effects, as reflected by body weight loss and high percentage mortality. Leaf extract was effective against *Plasmodium yoelii nigeriensis* in mice (Obaseki and Fadunsin, 1986).

The tail flick reaction time increased and a reduction in induced writhing was observed in rats that were administered leaf extract. Naloxone pre-treatment partially reversed the effects. The effects of the leaf extract were more pronounced than those of the seed oil (Khosla *et al*., 2000). Leaf extract reduced gastric ulcer severity in rats and decreased gastric mucosal damage (Garg *et al*., 1993). Chemically-induced carcinogenesis with accompanying high levels of lipid peroxidation and low levels of glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and gamma glutamyl transpeptidase (GGT) in rats could be effectively reduced with leaf extract. A five-day pre-treatment with leaf extract decreased the formation of lipid peroxides and enhanced the

levels of antioxidants and detoxifying enzymes in the stomach, the liver and circulation (Arivazhagan *et al.*, 2000).

2.9 Diabetes treatment and antioxidant potential

Neem reduced diabetic symptoms in non-insulin dependent diabetics. Ninety participants in three groups were given tulsi (*Ocimum sanctum*), neem leaf powder, or a tulsi-neem leaf powder mixture. Diabetic symptoms were significantly reduced in all groups—commonly polydypsia (excessive thirst), polyuria (increased frequency of urination), polyphagia (excessive hunger) and fatigue, and less commonly sweating, burning feet, itching, and headaches. Extracts from young flowers and leaves have strong antioxidant potential. An indicator of oxidative stress, malondialdehyde (MDA), was reduced by 46.0% and 50.6% for flower- and leaf-based extracts, respectively, prompting the recommendation to use neem as a vegetable bitter tonic to promote good health (Sithisarn *et al.*2005)**.**

2.10.0 THE PANCREAS

The pancreas is an elongated accessory digestive gland that lies retroperitoneally and transversely across the posterior abdominal wall, posterior to the stomach between the duodenum on the right and the spleen on the left. The transverse mesocolon attaches to its anterior margin. It produces an exocrine secretion the pancreatic juice from the acinar cells that enters the duodenum through the main and accessory pancreatic ducts and endocrine secretions glucagon and insulin from the pancreatic islets of Langerhans that enter the blood (Moore, 2006).

The pancreas is salmon pink in colour with a firm, lobulated smooth surface. The main portion of the pancreas is divided into four parts; head, neck, body and tail and it possess

one accessory lobe the uncinate process which is an anatomically and embryologically distinct portion of the pancreas. In adults the pancreas measures between 12 and 15 cm long and is shaped as a flattened 'tongue' of tissue, thicker at its medial end (head) and thinner towards the lateral end, the tail (Standrig, 2005).

The head is bound laterally by the curved duodenum and the tail extends to the hilum of the spleen in the lieno-renal ligament. The superior mesenteric vessels pass behind the pancreas, then anteriorly, over the uncinate process and third part of the duodenum into the root of the small bowel mesentery. The inferior vena cava, aorta, coeliac plexus, left kidney (and its vessels) and the left adrenal gland are posterior pancreatic relations. In addition, the portal vein is formed behind the pancreatic neck by the confluence of the splenic and superior mesenteric veins. The lesser sac and stomach are anterior pancreatic relations (Standrig, 2005).

2.10.1 Structure

Pancreatic duct of Wirsung courses the length of the gland, ultimately draining pancreatic secretions into the ampulla of vater, together with the common bile duct, and hence into the second part of the duodenum. An accessory duct of Santorini drains the uncinate process of the pancreas, opening slightly proximal to the ampulla into the second part of the duodenum (Standrig, 2005).

2.10.2 Blood supply of the Pancreas

The pancreatic arteries derive mainly from the branches of the markedly tortuous splenic artery, which form several arcades with pancreatic branches of the gastro-duodenal and

superior mesenteric arteries. Up to 10 branches of the splenic artery supply the body and tail of the pancreas. The anterior and posterior superior pancreatico-duodenal arteries, branches of the gastro-duodenal artery, and the anterior and posterior inferior pancreatico-duodenal arteries, branches of the SMA, supply the head. The corresponding pancreatic veins are tributaries of the splenic and superior mesenteric parts of the portal vein; however, most of them empty into the splenic vein (Moore, 2006).

2.10.3 Functions of the Pancreas

The Pancreas is a lobulated structure which performs both exocrine and endocrine functions. The exocrine secretory glands drain pancreatic juice into the pancreatic ducts and, from there, ultimately into the duodenum. The secretion is essential for the digestion and absorption of proteins, fats and carbohydrates. The endocrine pancreas is responsible for the production and secretion of glucagon and insulin, which take place in specialized cells of the islets of Langerhans. The pancreatic islet of Langerhans is a diffuse endocrine component of the pancreas. It is of endodermal origin and is most numerous in the tail. These cells of the pancreas secrete three amino acid derived hormones, insulin, glucagon and somastostatin (Standrig, 2006).

2.10.4 Histology of the Pancreas

In H and E stained section, islets cells appear as small, irregularly shaped light staining areas sparsely scattered among the brightly stained acini, the Gomori method demonstrates large pink A (α) and small blue staining B (β) cells (Cormack, 2009). The entire endocrine portion of the pancreas consists of Langerhans islets (*insulae pancreaticae*). The islets consist of cords of cells, which form an irregular network. This network is extensively

vascularized so that virtually every islet cell is connected to the bloodstream. Five cell types can be defined for the cell cords: α-, β-, δ-, PP- and D1-cells. α-, β- and δ-cells produce the following polypeptide hormones: α-cells secrete glucagon, β-cells secrete insulin and δ-cells secrete somatostatin. About 5–7% of the cells are δ-cells (Wolfgang, 2003). Many fenestrated capillaries lie between the anastomosing cords of secreting cells, but they are inconspicuous in light microscope sections. From the surrounding sheath of loose connective tissue, reticular fibers extend into the islet and support its secretory cells and capillaries. Also, some of these cells are provided with autonomic efferent nerve endings. Parasympathetic stimulation of the pancreas augments secretion of insulin and glucagon by the islets. There are also another group of cells called F cells that secretes pancreatic polypeptides (Cormack, 2009).

2.10.5 THE LIVER

This is the second largest organ in the body. It is related by its domed upper surface to the diaphragm, which separates it from pleura, lungs, pericardium and heart. Its postero-inferior or visceral surface abuts against the abdominal oesophagus, the stomach, duodenum; hepatic flexure of colon and the right kidney and suprarenal, as well as carrying the gall-bladder (Ellis, 2006). The liver is divided into a larger right and small left lobe, separated superiorly by the falciform ligament and postero-inferiorly by an H-shaped arrangement of fossae

- anteriorly and to the right—the fossa for the gall-bladder
- posteriorly and to the right—the groove in which the inferior vena cava lies embedded
- anteriorly and to the left—the fissure containing the ligamentum teres;

- posteriorly and to the left—the fissure for the ligamentum venosum.

The cross-bar of the H is the *porta hepatis*. Two subsidiary lobes are marked out on the visceral aspect of the liver between the limbs of this H—the *quadrate lobe* in front and the *caudate lobe* behind (Ellis, 2006).

The *ligamentum teres* is the obliterated remains of the left umbilical vein which, in utero, brings blood from the placenta back into the fetus. The *ligamentus venosum* is the fibrous remnant of the fetal *ductus venosus* which shunts oxygenated blood from this left umbilical vein to the inferior vena cava, short-circuiting the liver. The grooves for the ligamentum teres, ligamentum venosum and inferior vena cava, representing the pathway of a fetal venous trunk, are continuous in the adult (Ellis, 2006).

Lying in the porta hepatis are:

1 the common hepatic duct—anteriorly;

2 the hepatic artery—in the middle;

3 the portal vein—posteriorly.

2.10.6 Structure

The liver is made up of lobules, each with a solitary central vein which is a tributary of the hepatic vein which, in turn, drains into the inferior vena cava. In spaces between the lobules termed *portal canals*, lie branches of the hepatic artery bringing systemic blood and the portal vein, both of which drain into the central vein by means of sinusoids traversing the lobule.

Branches of the *hepatic duct* also lie in the portal canals and receive fine bile capillaries from the liver lobules (Ellis, 2006).

2.10.7 Functional subdivision of the Liver

The liver is not distinctly demarcated internally, though it has functionally independent right and left lobes that are much more equal in size than the anatomical lobes; however, the right liver is still somewhat larger. Each part receives its own primary branch of the hepatic artery and portal vein and is drained by its own hepatic duct. The caudate lobe may in fact be considered a third liver; its vascularization is independent of the bifurcation of the portal triad as it receives vessels from both bundles and is drained by one or two small hepatic veins, which enter directly into the IVC distal to the main hepatic veins. The liver can be further subdivided into four divisions and then into eight surgically resectable hepatic segments, each served independently by a secondary or tertiary branch of the portal triad (Moore, 2005).

2.10.8 Blood vessels of the Liver

The liver, like the lungs, has a dual blood supply, a dominant venous source and a lesser arterial one. The portal vein brings 75-80% of the blood to the liver. Portal blood, containing about 40% more oxygen than blood returning to the heart from the systemic circuit, sustains the liver parenchyma. The portal vein carries virtually all of the nutrients absorbed by the alimentary tract except lipids, which bypass the liver in the lymphatic system to the sinusoids of the liver. Arterial blood from the hepatic artery, accounting for only 20-25% of blood received by the liver, is distributed initially to non-parenchymal structures, particularly the intrahepatic bile ducts (Moore, 2005).

The portal vein is formed by the superior mesenteric and splenic veins posterior to the neck of the pancreas and ascends anterior to the IVC as part of the portal triad in the hepato-duodenal ligament. The hepatic artery, a branch of the celiac trunk, divides into the common hepatic artery, from the coeliac trunk to the origin of the gastro-duodenal artery, and the hepatic artery proper, from the origin of the gastro-duodenal artery to the bifurcation of the hepatic artery. At or close to the porta hepatis, the hepatic artery and portal vein terminate by dividing into right and left branches; these primary branches supply the right and left livers, respectively. Within each part, the simultaneous secondary branching of the portal vein and hepatic artery portal pedicles are consistent enough to supply the medial and lateral divisions of the right and left liver, with three of the four secondary branches undergoing further branching to supply independently seven of the eight hepatic segments (Moore, 2005). Between the divisions are the right, intermediate (middle), and left hepatic veins, which are inter-segmental in their distribution and function, draining parts of adjacent segments. The hepatic veins, formed by the union of collecting veins that in turn drain the central veins of the hepatic parenchyma open into the IVC just inferior to the diaphragm. The attachment of these veins to the IVC helps hold the liver in positions (Moore, 2006).

2.10.9 The hepatic veins

These veins are massive and their distribution is somewhat different from that of the portal, hepatic arterial and bile duct systems. There are three major hepatic veins, comprising a right, a central and a left. These pass upwards and backwards to drain into the inferior vena cava at the superior margin of the liver (Ellis, 2006). Their terminations are variable but usually the central hepatic vein enters the left hepatic vein near its termination. In other specimens it may drain directly into the cava. In addition, small hepatic venous tributaries

run directly backwards from the substance of the liver to enter the vena cava more distally to the main hepatic veins. Although these are not of great functional importance they obtrude upon the surgeon during the course of a right hepatic lobectomy (Ellis, 2006).

2.10.10 Histology of the Liver

The liver is essentially an epithelial-mesenchymal outgrowth of the caudal part of the forgut, with which it retains its connection via the biliary tree. The surface of the liver facing the peritoneal cavity is covered by a typical serosa, the visceral peritoneum. Beneath this, and enclosing the whole structure, is a thin (50-100 μm) layer of connective tissue from which extensions pass into the liver as connective tissue septa and trabeculae. Branches of the hepatic artery and hepatic portal vein, together with bile ductules and ducts, run within these connective tissue trabeculae which are termed portal tracts (portal canals). The combination of the two types of vessel and a bile duct is termed a portal triad; these structures are usually accompanied by one or more lymphatic vessels (Standrig, 2006).

The liver parenchyma consists of a complex network of epithelial cells, supported by connective tissue, and perfused by a rich blood supply from the hepatic portal vein and hepatic artery. The epithelial cells, hepatocytes, carry out the major metabolic activities of this organ, but additional cell types possess storage, phagocytic and mechanically supportive functions. In the mature liver, hepatocytes are arranged mainly in plates or cords, as seen in two-dimensional sections - usually only one cell thick. Until about seven years of age, plates are normally two cells thick. Between the plates are venous sinusoids, which anastomose with each other via gaps in the hepatocyte plates (Standrig, 2006). Bile secreted by the hepatocytes is collected in a network of minute tubes (canaliculi). The hepatocytes can

therefore be regarded as exocrine cells, secreting bile to the alimentary tract ultimately via the hepatic ducts and bile duct. However, their other metabolic orientation is towards the blood, with which hepatocytes carry out complex biochemical exchanges. The fetal liver is a major haemopoietic organ; erythrocytes, leukocytes and platelets develop from the mesenchyme covering the sinusoidal endothelium (Standrig, 2006).

2.10.10.1 Histology of cells of the Liver

Cells of the liver include hepatocytes, hepatic stellate cells - also known as perisinusoidal lipocytes, or Ito cells - sinusoidal endothelial cells, macrophages (Kupffer cells), the cells of the biliary tree - cuboidal to columnar epithelium - and connective tissue cells of the capsule and portal tract (Standrig, 2006).

2.10.10.2. Ito cells

Hepatic stellate cells are also known as perisinusoidal lipocytes or Ito cells and are much less numerous than hepatocytes (Standrig, 2006). They are irregular in outline and lie within the hepatic plates, between the bases of hepatocytes. They are thought to be mesenchymal in origin and are characterized by numerous cytoplasmic lipid droplets. These cells secrete most of the intralobular matrix components, including collagen type III (reticular) fibres (Standrig, 2006). They store the fat-soluble vitamin A in their lipid droplets and are a significant source of growth factors active in liver homeostasis and regeneration (Standrig, 2006). Hepatic stellate cells also play a major role in pathological processes. In response to liver damage, they become activated and predominantly myofibroblast-like. The cells are responsible for the replacement of toxically damaged hepatocytes with collagenous scar tissue - hepatic fibrosis, seen initially in zone 3, around central veins. This can progress to

cirrhosis, where the parenchymal architecture and pattern of blood flow are destroyed, with major systemic consequences (Standrig, 2006).

2.10.10.2. Sinusoidal endothelial cells

Hepatic venous sinusoids are generally wider than blood capillaries and are lined by a thin but highly fenestrated endothelium which lacks a basal lamina. The endothelial cells are typically flattened, each with a central nucleus and joined to each other by junctional complexes. The fenestrae are grouped in clusters with a mean diameter of 100nm, allowing plasma direct access to the basal plasma membranes of hepatocytes. Their cytoplasm contains numerous typical transcytotic vesicles (Standrig, 2006).

2.10.10.3 Kupffer cells

Kupffer cells are hepatic macrophages derived from circulating blood monocytes. The cells are long-term hepatic residents, lying within the sinusoidal lumen, attached to the endothelial surface. These cells originated in the bone marrow, and form a major part of the mononuclear phagocyte system responsible for removing cellular and microbial debris from the circulation, and secreting cytokines involved in defence. Kupffer cells remove aged and damaged red cells from the hepatic circulation, a function normally shared with the spleen, but fulfilled entirely by the liver after splenectomy. Kupffer cells are irregular in shape, with long processes extending into the sinusoidal lumen (Standrig, 2006).

2.10.10.4 Hepatic plates (cords)

The endothelial linings of the sinusoids are separated from hepatocytes of the hepatic plates by a narrow gap, the perisinusoidal space of Disse which is normally about 0.2-0.5 μm wide,

but distends in anoxic conditions. It contains fine collagen fibres - chiefly type III, with some types 1 and IV - the microvilli of adjacent hepatocytes, and occasional non-myelinated nerve terminals. There is no basal lamina within the space of Disse (Standrig, 2006).

Minute bile canaliculi form nets with polygonal meshes in the hepatic plates. Each polygonal hepatocyte is surrounded by canaliculi except on the surfaces - at least two - facing sinusoids. Hepatic plates thus enclose a network of canaliculi which pass to the lobular periphery, where they join to form narrow intralobular ductules (terminal ductules or the canals of Hering) lined by squamous or cuboidal epithelium. These enter bile ductules in the portal canals, lined by cuboidal or columnar cells. The flow of bile is thus towards the periphery of lobules, in the opposite direction to the blood flow, which is centripetal (Standrig, 2006).

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CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 EXPERIMENTAL ANIMALS

Fifty three young adult male Wistar rats, weighing approximately 140g each were obtained from the Faculty of Pharmaceutical Sciences of Ahmadu Bello University, Zaria. They were kept in plastic cages and allowed to acclimatize for 2 weeks after which their weight were > 150g in the Faculty of Pharmaceutical Sciences Animal house before the experiment, and maintained under laboratory conditions of temperature, humidity and light. They were allowed free access to water and standard pellet diet obtained from Grand Cereals Ltd, Jos Plateau State. The rats were divided into nine groups of five each.

3.2 PLANT MATERIAL

Leaves of fresh *Azadirachta indica* were harvested from Ahmadu Bello University Faculty of Medicine Zaria in the month of April 2012 and authenticated at the Department of Biological Sciences, Ahmadu Bello University Zaria Herbarium by Mr. U.S. Gallah with a voucher specimen number 900151.

3.3 CHEMICALS

Melatonin M5250-1G (Sigma Aldrich USA), Streptozocin SP0130 (Sigma Aldrich, USA)

SOD Assay kit Gluthathione Peroxidase Assay kit (North West life Science Specialties LLC, assay kit) and Malonaldehyde (MDA), AST, ALP and ALT (Randox assay kit) were used during the study.

3.4 PREPARATION OF EXTRACT

The Fresh leaves of *A. indica* were air dried, minced and powdered using laboratory mortar, after which 1000g of the powder was extracted in 1.5 liters of 80% ethanol using a soxhlet extractor. This was filtered using a Whatman filter paper (24mm). The filtrate was dried in a laboratory water bath set at 67°C and total yield of 46.8g was obtained per 1000g of the leaves.

3.5 EXPERIMENTAL DESIGN

3.5.1 Diabetes induction

A baseline blood glucose levels was taken for all the control and test rats before grouping them. This was done to ensure that the rats were all normoglycaemic. Thirty three Wistar rats were randomly selected and were given a single dose of intra peritoneal injection of streptozotocin, (STZ) (Sigma, Aldrich, USA), at 55mg/kg body weight in citrate buffer (0.1M, pH 4.5). The solution (STZ in citrate buffer) was used within 5 minutes to induce chemical diabetes in the wistar rats after overnight fasting of twelve hours.

Blood samples were collected at 72 hours after STZ treatment from the dorsal vein of the tail and the blood glucose levels detected using a One Touch Ultra 2 Glucometer, (Lifescan, CA, USA). Streptozotocin treated adult Wistar rats with fasting blood glucose level at 11mmol/L and above was considered diabetic. Twenty eight Wistar rats in this group were found to be chemically diabetic giving a 84 % diabetic induction. These animals were further grouped into five groups of five Wistar rats each (Group 5, 6, 7, 8, 9) called the diabetic group while group 1, 2, 3, and 4 were the normal control groups with five rats per group as shown in Table 3.1.

Streptozotocin was selected to chemically induce diabetes since it potentially destroys the β-cells of the Pancreas to produce diabetes signified by sustained hyperglycaemia above 200mg/dl, (11mmol/L), (Atangwo *et al.*, 2010).

The extract (200mg/kgbw) (Atangwo, *et al*., 2010) was administered by orogastric intubation once daily for three weeks while melatonin (10mg/kgbw) was administered intraperitoneally once daily for three weeks in all treated groups and metformin was the standard drug (500mg/kgbw).

Table 3.1: Different experimental groups and their treatments

CONTROL GROUP		
Group	**No. of animals**	**Treatment**
1. NC (Normal control)	5	Normal saline orally daily for 3 weeks
2. NAI (Normal + *A. indica*)	5	*A. indica* extract (200mg/kg b.w.) orally daily for 3 weeks
3. NM (Normal + melatonin)	5	Melatonin (10mg/kg b.w.) IP daily for 3 weeks
4. NM/AI (Normal + melatonin + *A . indica*)	5	Melatonin 10mg/kg bw IP and *A. indica* 200mg/kg bw Orally daily for 3 weeks
DIABETIC GROUP		
Group	**No. of animals**	**Treatment**
5. DC (Diabetic control)	5	Normal saline orally daily for 3 weeks
6. DAI(Diabetic + *A. indica*)	5	*A.indica* extract,(200mg/kg b.w.) orally daily for 3nweeks
7. DM (Diabetic + Melatonin)	5	Melatoni*n* (10mg/kg b.w.) IP orally daily for 3 weeks
8. DAI /M (Diabetic *A. indica* + Melatonin)	5	*A. indica* extract (200mg/kg b.w.) orally and Melatonin (10mg/kg bw) IP daily for 3 weeks
9 DMF (Diabetic + metformin)	5	Diabetic metformin (500mg/kg b.w) orally daily for 3 weeks

3.6 PHYSICAL STUDIES

The experimental animals were weighed weekly, at the start and during the experiment. On day 22, the animals were humanely sacrificed using chloroform anaesthesia. Blood samples were collected through cardiac puncture into plain bottles. Liver and pancreatic tissues were harvested and processed for histological analysis using paraffin wax processing method.

3.7 BIOCHEMICAL STUDIES

Blood was collected at sacrifice through cardiac puncture into plain sterilized centrifuged bottles and allowed to clot. The clotted blood samples were centrifuged and the serum harvested for analysis to quantify the liver enzymes alanine aminotransferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), and the ratio of ALT: AST determined.

3.7.1 Alanine aminotransferase (ALT)

ALT was assayed using Randox Alanine aminotransferase assay kit according to the manufacturer's instructions.

3.7.1.1 *Preparation of reagents*

Reagents 1 (R1) was prepared by adding 100 mmol/l phosphate buffer (pH 7.4), 200mmol/l L-alanine and 2.0 mmol/l α-oxoglutarate in a test tube while reagent 2 (R2) comprised of 2.0 mmol/l 2,4-dinitrophenylhydrazine.

3.7.1.2 *Procedure*

Five milliliters of R1 was put in test tube 1, blank Sample while to the Serum test tube 2 was added 0.5 ml R1 and 0.1 ml of serum. This was incubated at 37°C for 30 minutes.

After this, 0.5 ml of R2 was added to both the test tubes, in addition to 0.1ml of serum to the blank sample tube only. This was allowed to stand for 20 minutes at 25°C. Finally, 5.0ml of sodium Hydroxide was added to both test tubes, content mixed and the absorbance of the test tube 2 was read at (546nm wavelength) against the sample blank after 5 minutes. This procedure was repeated for all experimental samples.

3.7.2 *Aspartate aminotranferase (AST)*

AST was assayed using Randox Aspartate aminotransferase assay kit according to manufacturer's instructions.

3.7.2.1 *Preparation of reagents*

Reagent 1(R1) was prepared by adding 100ml/l phosphate buffer, (pH 7.4), 100 mmol/l L-Aspartate and 2.0 mmol/l α-oxaloacetate in a test tube while reagent 2 (R2) contains 2.0 mmol/l 2.4-dinitrophenylhydrazine.

3.7.2.2 *Procedure*

Five milliliters of R1 was pipetted into test tube 1 blank sample while to Serum test tube 2 were added 0.5ml of R1 and 0.1 ml of serum. This was incubated at 37°C for 30 minutes. After this, 0.5 ml of R2 was added to all test tubes in addition to 0.1 ml of serum to sample blank test tube only. This was allowed to stand to stand for 20 minutes at 25°C.

Finally, 0.5 ml of Hydroxide was added to both test tubes, the contents were mixed and the absorbance of R2 samples read at Hg 546 nm against the sample blank after 5 minutes. This procedure was repeated for all experimental samples.

3.8 DETECTION OF OXIDATIVE STRESS MARKERS

3.8.1 *Malondialdehyde assay (MDA)*

Malondialdehyde was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

3.8.1.1 *Assay Preparation*

Spectrophotometer was set at Wavelength of 514nm with a Spectral bandwidth: ≤ 2 nm, Resolution: ≤ 1 nm and Heat block or water bath preheated to 60°C.

3.8.1.2 *Reagent Preparation*:

3.8.1.2.1 TBA Reagent (2-Thiobarbituric Acid)

10.5 mL deionized water was added to the TBA bottle. Magnetic stir bar was inserted and mixed until TBA had dissolved for 10 minutes approximately. This was stored at room temperature.

3.8.1.2.2 Other Reagents

The Acid Reagent, (Butylated hydroxytoluene, 2, 6-di-tert-butyl-4-methylphenol, BHT, CAS 128-37-0) Reagent, Assay Buffer and Calibrators were supplied ready-to-use.

3.8.1.2.3 Assay Protocol:

10 µL BHT Reagent was added to micro-centrifuge vials. 250 µL Calibrator (sample) was then added to the vials with further addition of 250 µL Acid Reagent to vials. 250 µL TBA Reagent was later added to the vials. They were vortex vigorously (5-count). These were incubated for 60 minutes at 60°C and centrifuged at 10,000 x*g* for 3 minutes. The reaction mixtures were then transferred to cuvettes and spectras recorded from 400-700 nm.

3.8.2 Glutathione Peroxidase Assay

Glutathione Peroxidase was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

3.8.2.1 *Assay Preparation*

Plate Reader Setup was set as recommended at a Wavelength of 340 nm, duration of 5 minutes interval with a 30 seconds data reduction and linear regression temperature of 25 ^{o}C.

*3.8.2.1.2 NADPH, (*β-Nicotinamide adenine dinucleotide phosphate) *Diluent*

This reagent was supplied ready to use.

3.8.2.1.3 NADPH Reagents

The entire contents of one NADPH diluents were added to one NADPH Reagent bottle and the caps on the vials secured. This was mixed briefly by inverting the bottle. The Working *NADPH* solution were kept on ice to prolong the useful life of the reagent during the procedure.

3.8.2.1.4 H_2O_2 Reagent

3% hydrogen peroxide diluted as supplied 1:100 i.e, .02 mL 3% H_2O_2 to 2 mL Assay Buffer was marked as Diltution 1, Dilution 1 was further diluted 3:100 (e.g. 0.3 mL Dilution 1 to 10 mL Assay Buffer), this was marked as Working H_2O_2.

3.8.2.2 *Assay Protocol: Standard Procedure for Microplate Assay*

All reagents were brought to room temperature and the microplates were removed from plastic bag. 50 µL of diluted sample and controls were added to wells. 50 µL of Working NADPH was added to each well. 50 µL of Working H_2O_2 were further added to each well and observed for 1 minute, A_{340} for 5 minutes with a recording interval of every 30 seconds. Glutathione perioxidase, (GPx) activities were calculated from the net rate.

3.8.3 Superoxide Dismutase Activity Assay

Superoxide Dismutase activity was assayed using North West life Science Specialties LLC, assay kit according to manufacturer's instructions.

3.8.3.1 *Assay/Instrument Preparation: Spectrophotometer (Cuvette Assay) Setup*

Temperature was set at 25°C. The spectrophotometer Zero absorbance was set at 560 nm using distilled H_2O

3.8.3.1.2 *Plate Reader (Microplate Assay) Setup*

Microplate Reader temperature was set at room temperature and experiment carried out.

3.8.3.1.3 *Reagent Preparation:*

The kit was allowed to warm up to room temperature completely before use.

3.8.3.1.4 Assay Buffer and Sample Dilution Buffer

This was supplied ready to use. However, the reagent bottles were shaken and opened to air and repeated four (4) more times before use to saturate with O_2. Sample dilution buffer was supplied ready to use.

3.8.3.1.5 Hematoxylin Solution:

Reconstituted hematoxylin was used as supplied with 1.2 mL (1200 µL) of dH_2O at room temperature and was used within 6 hours.

3.8.3.2.1 *Assay Protocol: Cuvette Assay*

920 µL of Assay Buffer was added to each cuvette for assay. 40 µL of Assay Buffer were added for blank and 40µL of Sample. These were mixed and incubated for two (2) minutes. 40µL haematoxylin reagent was further added to start the auto-oxidation reaction. The assays were mixed quickly and immediately the absorbance recorded at 560 nm every 10 seconds for 5 minutes.

3.8.3.2.2 *Microplate Assay*

Sample and standard lay out were recorded. 230 µL of Assay Buffer was added to each well used for testing and 10 µL of Assay Buffer to the blank and 10 µL Sample. These was shaken to mix and incubated for 2 minutes. 10 µL of hematoxylin reagent was further added

with a multichannel pipette to begin reaction. The assayed containers were mixed quickly and immediately the absorbance was taken at 560 nm every 10 seconds for 5 minutes.

3.8.3.2.3 *Employing a Control*

The control samples were prepared, aliquoted and frozen at –20°C. These Control samples were assayed each time along with other samples and the final results normalized with the control sample.

3.9 HISTOLOGICAL STUDIES

The tissues excised after the animal had been sacrificed were fixed in buffered 10% Neutral formal saline in plastic containers and embedded in paraffin wax. They were sectioned with Leica rotary Microtome to produce serial sections of 5μ thickness. The liver sections were stained for reticular fibers using Gordon and Sweet while Heamatoxylin Van Gieson Stain was used to demonstrate collagen fibers to study the micro-cytoarchitecture of the liver sections. Periodic acid Schiff (PAS) with and without Diastase digestion was used to stain glycogen in the liver while Haematoxylin and Eosin stains were used to stain the pancreas to demonstrate the pancreatic islets. All the tissues were stained for general histological studies with Haematoxylin and Eosin. Photomicrographs were obtained using an Olympus light microscope eye piece attached to a computer monitor and observation made.

3.10 STATISTICAL ANALYSIS

Data generated were presented as Mean±SD and analysis performed using student's T-test and a prevalence less than or equal to 0.005 ($p \leq 0.05$) was considered to be significant. One way analysis of varience (ANOVA) was used to compare the level of difference between

and within the groups, while Duncan Multiple Range Test was used as a Post hock Test. The analysis was done using Statistical Packages for Social Sciences (SPSS) 19 statistical packages

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CHAPTER FOUR

4.0 RESULTS

4.1 Physical Observations

Table 4.1 showed that the weights of the animals were affected by STZ induction in the diabetic control group and metformin treated groups. There was significant weight decrease ($p \leq 0.05$) on day 21 in the diabetic control group (DC), while there was significant weight increase ($p \leq 0.05$) in the metformin treated group (DMF). Control treated group showed no significant weight change except for normal melatonin (NML) treated group where slight increase in weight was observed. The extract (DAI, NAI), Melatonin (DML, NML) treated groups showed results similar to the normal control group (NC).

4.2 Blood Glucose Level

Table 4.2 showed results obtained for blood glucose levels in STZ induced diabetic Wistar rats in control groups and diabetic groups. The table showed that there was significant reduction ($p \leq 0.05$) in blood glucose levels in *Azadirachta indica* leaves extract (DAI, NAI), melatonin treatment (NML, DML) and the combined treatment groups (NAI/NML and DAI/DML) in both the diabetic and control groups while diabetic control group (DC) and diabetic metformin (DMF) treated groups showed no significant decrease in blood glucose levels during the treatment period.

Table 4.1 Changes in weight, following STZ diabetes induction, extract and melatonin treatments.

GROUPS	DAY 1	DAY 8	DAY 15	DAY 22
NC	159.00 ± 6.542[a]	165.80 ± 7.459[a]	172.80 ± 10.307[a]	181.00 ± 11.417[a]
NAI	159.60 ± 6.377[a]	164.20 ± 6.216[a]	167.80 ± 3.747[a]	172.20 ± 3.720[a]
NML	164.40 ± 9.108[a]	175.60 ± 6.683[a]	188.20 ± 8.874[a]	195.20 ± 8.714[b]
NAI/NML	155.20 ± 6.763[a]	160.60 ± 9.892[a]	168.20 ± 17.611[a]	167.60 ± 20.96a[a]
DC	147.60 ± 17.626[a]	166.20 ± 6.530[a]	164.40 ± 6.185[a]	162.00 ± 6.189[b]
DAI	164.40 ± 7.096[a]	165.40 ± 6.038[a]	168.20 ± 4.620[a]	173.20 ± 5.024[a]
DML	159.40 ± 4.285[a]	159.60 ± 4.411[a]	163.20 ± 4.800a	171.00 ± 5.941[a]
DAI/DML	151.00 ± 4.980[a]	156.80 ± 4.934[a]	164.00 ± 4.000[a]	167.20 ± 3.20[a]
DMF	167.80 ± 2.905[a]	174.40 ± 3.027[a]	182.00 ± 1.342[a]	191.00 ± 2.550[b]

Values are expressed as mean ±SD of five determinations. Values with different superscripts in a column are significantly different ($p<0.05$)

N = Normal

C = Control

D = Diabetic (Normal saline)

AI= *Azadirachta indica* (200 mg/kg bw)

ML = Melatonin (10 mg/kg bw)

MF = Metformin (500 mg/kg bw)

Table 4.2: Changes in blood glucose levels of controls, *Azadirachta indica* and melatonin treated

Treated Group	DAY 1	DAY 8	DAY 15	DAY 22
NC	**71.40 ±2.015^{b}**	**70.0±2.345^{b}**	**82.40 ± 8.078^{c}**	**96.20 ±3.734^{b}**
NAI	**82.40 ±3.982^{b}**	**72.40±4.261^{b}**	**67.80 ±4.17^{c}**	**63.80 ± 3.760^{e}**
NML	**105.20 ±2.059^{b}**	**93.60±2.713^{b}**	**84.80 ±3.105^{c}**	**79.20 ± 3.527^{c}**
NAI/NML	**107.40 ±5.016^{b}**	**94.00±2.713^{b}**	**90.00 ±2.025^{c}**	**87.00 ± 1.844^{c}**
DC	**272.60 ±20.488^{a}**	**279.80 ±23.731^{a}**	**298.00 ±27.120^{a}**	**320.80 ±33.466^{a}**
DAI	**345.80 ±60.728^{a}**	**265.80 ±39.434**	**219.40 ±30.615^{b}**	**153.20 ± 8.680^{c}**
DML	**336.20 ±54.369^{a}**	**259.60 ±44.715^{a}**	**189.80 ±18.359^{c}**	**141.60 ± 4.331^{c}**
DAI/DML	**337.20 ±42.677^{a}**	**278.60 ±38.281^{a}**	**187.20 ±17.758^{c}**	**138.80 ± 5.544^{c}**
DMF	**353.40 ±42.646^{a}**	**337.20 ±43.718^{a}**	**321.40 ±44.667^{a}**	**299.40 ± 48.264^{a}**

Values are expressed as mean ±SD of five determinations. Values with different superscripts in a column are significantly different ($p<0.05$)

N = Normal

C = Control

D = Diabetic (Normal saline)

AI= *Azadirachta indica* (200 mg/kg bw)

ML = Melatonin (10 mg/kg bw)

MF = Metformin (500 mg/kg bw)

4.3 BIOCHEMICAL ANALYSIS

4.3.1 Liver enzymes

The diabetic control group (DC) given normal saline expressed high levels of AST and ALT (Table 4.3). Diabetic melatonin treated group and *Azadirachta indica* leaves extract treated group caused significant decrease in AST and ALT levels in the serum therapeutically in both groups and when combined (DAI/DML) group had similar effects. Effect of *Azadirachta indica* leaves extract and melatonin administration on ALP showed a decrease in the treated group while there was an elevation in the Diabetic control (DC) and diabetic metformin (DMF) treated group.

4.3.2 Oxidative stress markers

There were significant increases in superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) due to the administration of melatonin and *Azadirachta indica* extract as compared to low levels obtained in the Diabetic control (DC) group (Table 4.4). The level of Malonaldehyde (MDA) showed a significant decrease in the treated groups as compared with the Diabetic control group (DC) where there was a significant increase in MDA.

Table 4.3 Biochemical analysis of liver enzymes AST, ALT and ALP

GROUPS	AST	ALT	ALP
NC	**17.60±1.288^{a}**	**30.80±1.463^{a}**	**59.20 ± 1.463^{a}**
NAI	**17.68±1.414^{a}**	**39.40±2.502^{b}**	**69.00 ± 1.703^{b}**
NML	**18.40±1.166^{a}**	**42.00±1.304^{b}**	**68.60 ± 2.909^{b}**
NAI/NML	**18.20±1.625^{a}**	**41.00±2.098^{b}**	**71.20 ± 2.990^{b}**
DC	**27.20± 0.490^{e}**	**48.20±0.490^{c}**	**79.40 ± 2.441^{c}**
DAI	**23.20±1.020^{c}**	**42.80±1.744^{b}**	**68.80 ± 2.267^{b}**
DML	**21.20±1.200^{a}**	**39.20±2.059^{b}**	**67.60 ± 1.400^{b}**
DAI/DML	**23.80±1.356^{c}**	**37.80±1.241^{b}**	**68.20 ± 3.362^{b}**
DMF	**25.00±0.837^{c}**	**42.20±0.970^{b}**	**69.20 ± 0.583^{b}**

Mean values were separated using Duncan's multiple range test:

Mean values with different superscripts in a column are significantly different (at P<0.05)

C = Control

N - Normal

D = Diabetic (Normal saline)

AI= *Azadirachta indica* (200 mg/kg bw)

ML = Melatonin (10 mg/kg bw)

MF = Metformin (500 mg/kg bw

Table 4.4 Serum levels of Malonaldehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT), and Gluthathione Peroxidase (GPx).

GROUP	MDA	SOD	CAT	GPx
NC	1.340 ± 0.812^{a}	1.640 ± 0.0927^{a}	47.20 ± 4.554^{ab}	50.40 ± 2.926^{a}
NAI	1.440 ± 0.0927^{ab}	1.740 ± 0.1122^{bc}	63.80 ± 4.769^{cd}	59.40 ± 2.676^{bc}
NML	1.450 ± 0.0812^{a}	1.940 ± 0.1536^{bc}	55.40 ± 3.429^{bc}	68.00 ± 1.225^{d}
NAI/NML	1.540 ± 0.0245^{a}	1.720 ± 0.0583^{bc}	66.60 ± 4.155^{cd}	58.20 ± 2.437^{bc}
DC	2.180 ± 0.1655^{d}	1.380 ± 0.0583^{a}	38.60 ± 2.112^{a}	45.80 ± 1.241^{a}
DAI	1.540 ± 0.0678^{ab}	1.720 ± 0.0663^{bc}	64.80 ± 2.853^{cd}	57.40 ± 2.272^{b}
DML	1.520 ± 0.0583^{ab}	1.740 ± 0.1166^{bc}	64.40 ± 3.370^{cd}	65.80 ± 1.393^{d}
DAI/DML	1.560 ± 0.0812^{ab}	1.820 ± 0.1158^{bc}	67.40 ± 2.159^{d}	64.40 ± 2.421^{cd}
DMF	1.820 ± 0.0860^{c}	1.520 ± 0.0860^{ab}	44.80 ± 3.826^{ab}	49.00 ± 1.049^{a}

Mean values were separated using Duncan's multiple range test:

Mean values with different superscripts in a column are significantly different (at P<0.05)

C = Control

N= Normal

D = Diabetic (Normal saline)

AI= *Azadirachta indica* (200 mg/kg bw)

ML = Melatonin (10 mg/kg bw)

MF = Metformin (500 mg/kg bw

4.5 HISTOLOGY

4.5.1 Histology of Pancreas observed in the study

Results of histological observations of the Pancreatic section of normal saline control (NC) group (Plate I) showed a normal section of pancreas with normal pancreatic islet. There was no observable pancreatic lesion of pancreatic islets. Pancreatic section of STZ induced diabetic normal saline control group (Plate II) showed areas of islet necrosis and degenerated islet cell mass as compared with normal saline control group. Pancreas section of *A. indica* extract treated (Plate III), melatonin treated (Plate IV), extract and melatonin treated group (Plate V) all showed restoration of pancreatic islet cells. When these groups (Plates III and IV) were compared with the normal control group (Plate I) showed histological features similar to normal saline control group and extract and melatonin treated control groups (VII, VIII, and IX).

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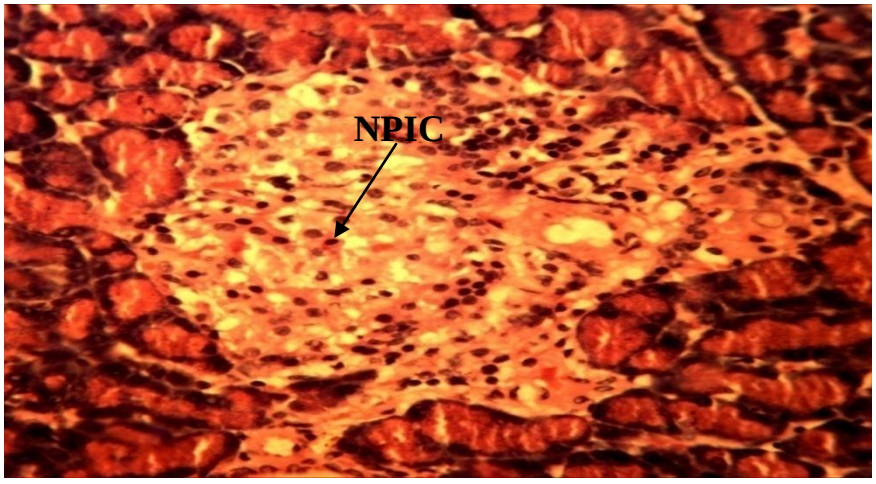


Plate I: Photomicrograph of Pancreas from normal control group (NC). Note the normal pancreatic islet cells (NPIC) of langerhans (H&E, x 250)

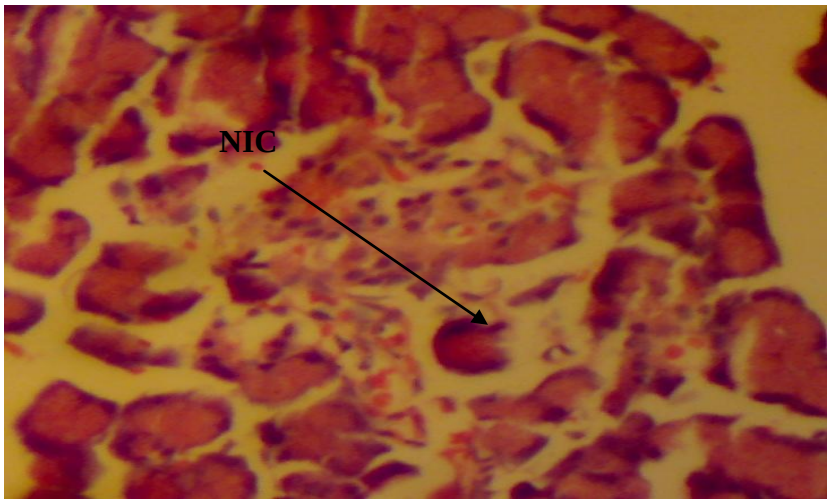


Plate II: Photomicrograph of Pancreas from STZ induced diabetic control group. Note the necrosis of islet cells (NIC). (H&E Stain, x250)

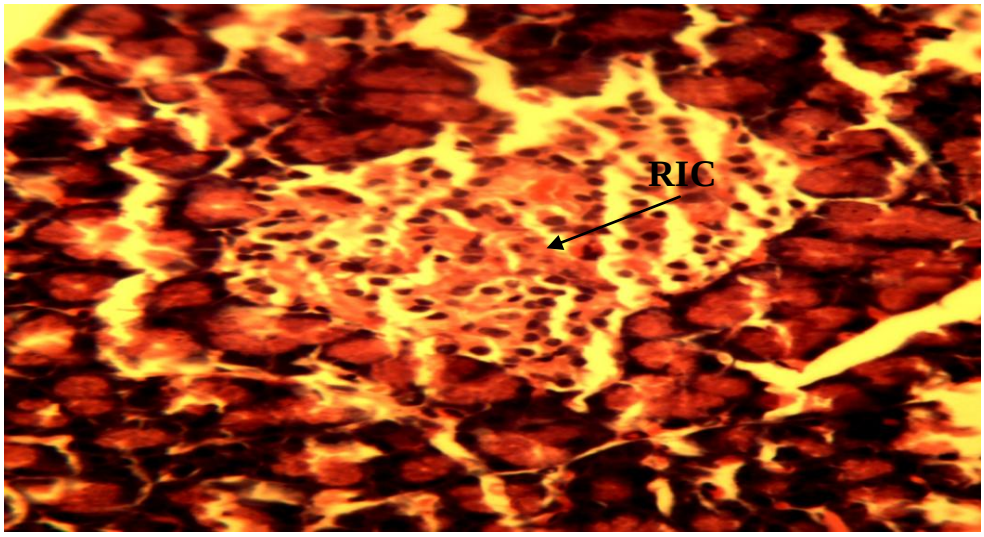


Plate III: Photomicrograph of Pancreas from STZ induced diabetic rat treated with *Azadirachta indica* (200mg/kgbw) (DAI). Note the regenerated islet cells (H&E Stain, x250)

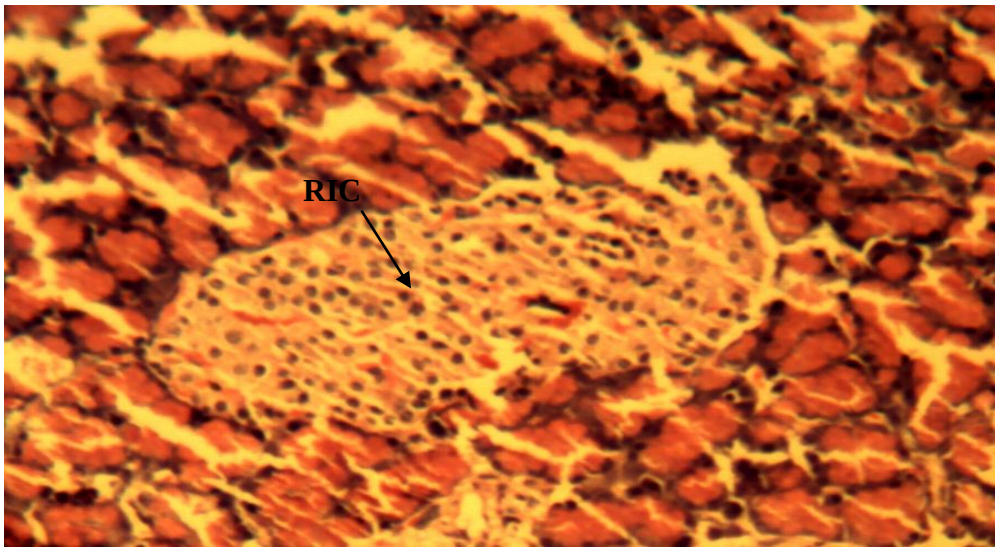


Plate IV: Photomicrograph of Pancreas from STZ induced diabetic rat (DML) treated with melatonin (10 mg/kgbw) with regenerated islet cells (RIC), (H&E, x250).

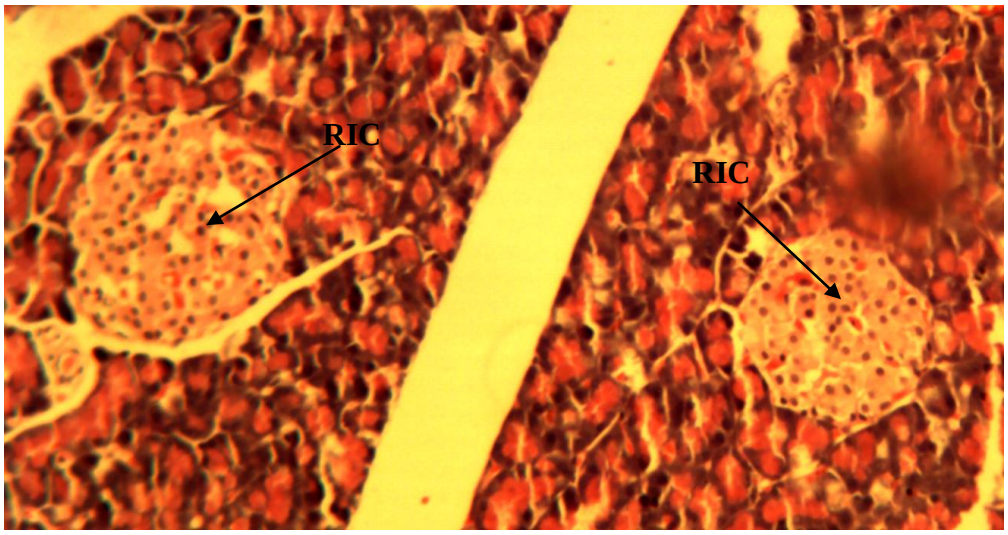


Plate V: Photomicrograph of Pancreas from STZ induced diabetic rats treated with *Azadirachta indica* and melatonin combined group (DAI/DML) showing well regenerated islet cell (RIC) mass and number (H & E, x250).

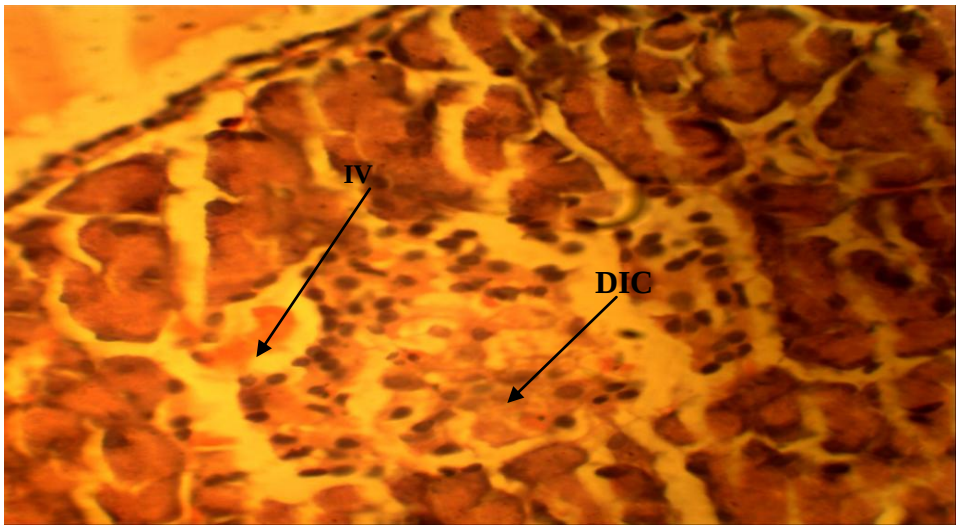


Plate VI: Photomicrograph of Pancreas from STZ induced diabetic rats treated with metformin (DMF) group showing poorly regenerated islet cells and islet veins (IV). Areas of islet necrosis are observed, DIC (Degenerated islet cells) (H&E Stain, x250).

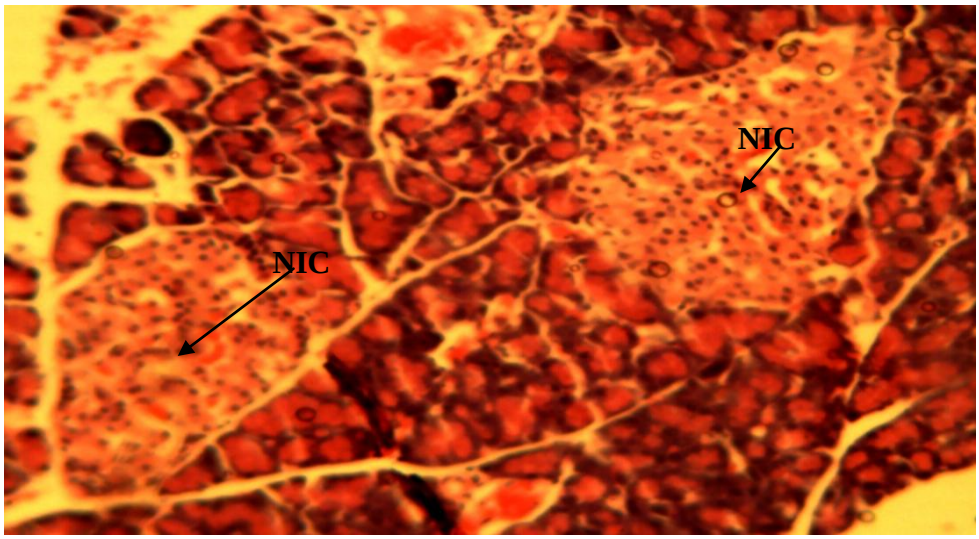


Plate VII: Photomicrograph of Pancreas of extract treated Normal control group (NAI) showing well preserved pancreatic islets. The plate showed no necrotic changes (H&E Stain, x250).

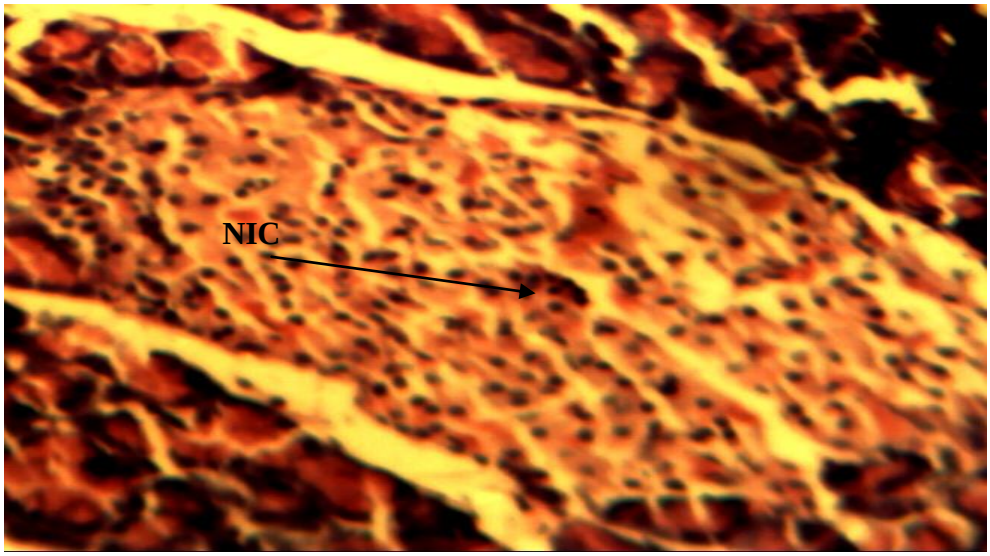


Plate VIII: Photomicrograph of melatonin treated normal control group (NML). Note the normal islet cells (NIC), (H&E Stains, 250)

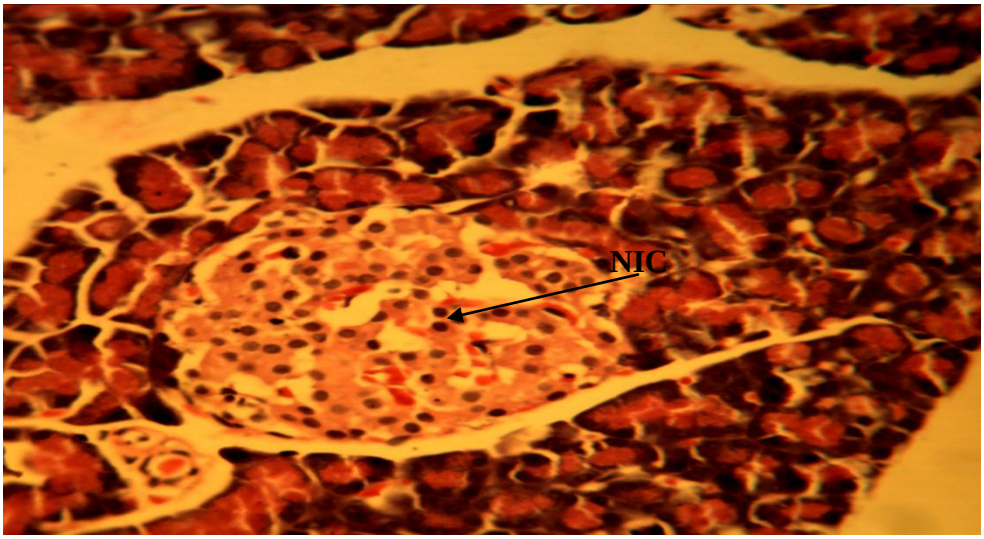


Plate IX: Photomicrograph of Pancreas of *Azadirachta indica* and melatonin treated normal control group (NAI/NML) showed preservation of the pancreas and islet cells, (NIC). (H&E Stains, x 250)

4.5.2 Histology of the Liver observed during the study

Liver section of normal saline control (NC) group (Plate X) showed normal cyto-architecture of liver tissue of hepatocytes and sinusoids. There was no observable pathological change in the liver section. The liver sections of STZ induced diabetic group (DC) (Plate XI) showed necrotic changes including nucleus and cytoplasmic vacoulation, hepatocytes and sinusoids fragmentation and fatty change. On the other hand *A. indica* treated group (DAI), melatonin treated group (DML), combined treatment (DAI/DML) and metformin (Plate XII-XV) showed that both the extract and melatonin restored the liver therapeutically, the degree of restoration of the liver cyto-architecture was least observed in the metformin treated group. Plate XVI-XVIII are non diabetic control treated control groups showing normal liver hepatocytes and sinusoids indicating that the extract and melatonin on their own are not causing any cellular damage.

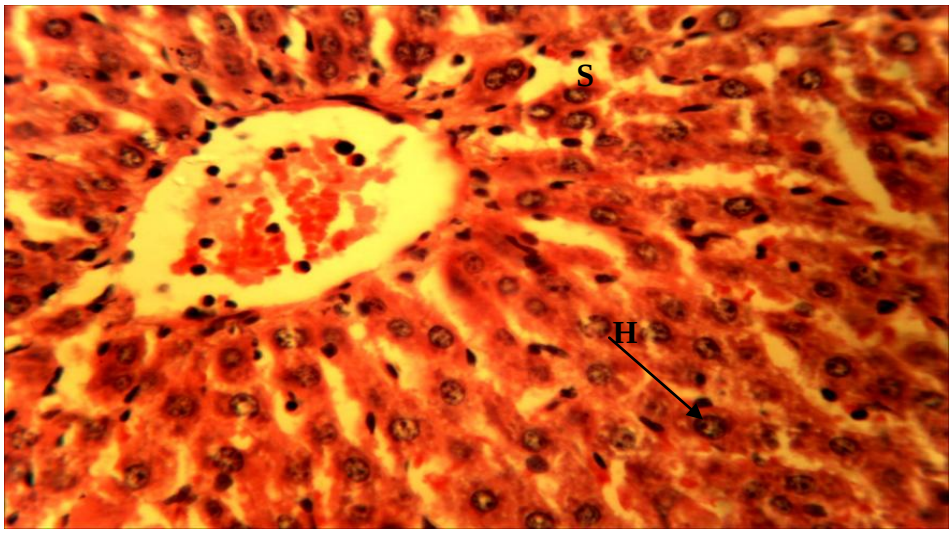


Plate X: Photomicrograph of liver section in normal Control group (NC) showing normal hepatocytes (H) and sinosuids (S) (H&E X250)

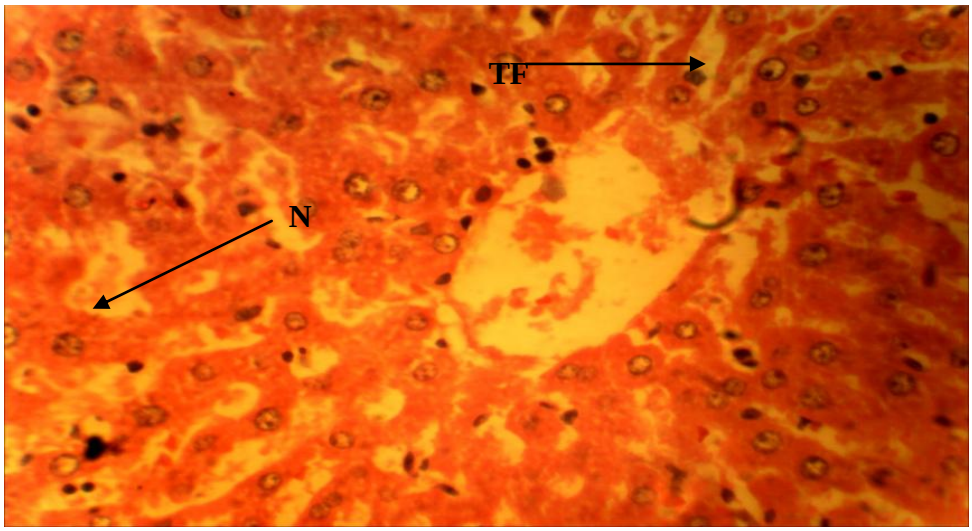


Plate XI: Photomicrograph of liver from Diabetic control group (DC) showing necrosis (N), vacoulation (V) and tissue fragmentations (TF) (H&E x250)

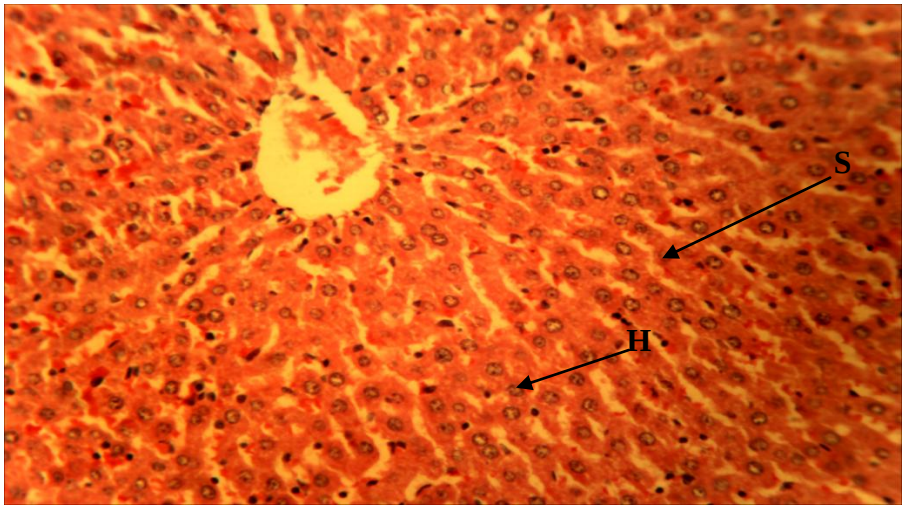


Plate XII: Photomicrograph of liver from *Azadrachta indica* treated diabetic group (DAI) showing restored micro-cytoarchitecture indicating normal hepatocytes and sinusoids (H&E 250)

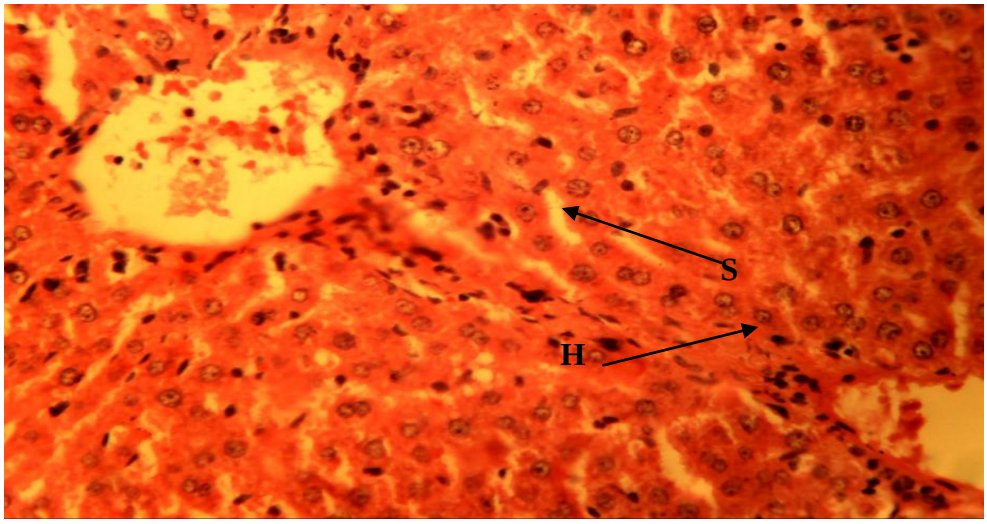


Plate XIII: Section of liver from melatonin treated diabetic group (DML) showing restoration of liver cyto-architecture indicating restored hepatocyes (H) and sinusoids (S) and no fatty change (H&E Stains, x250)

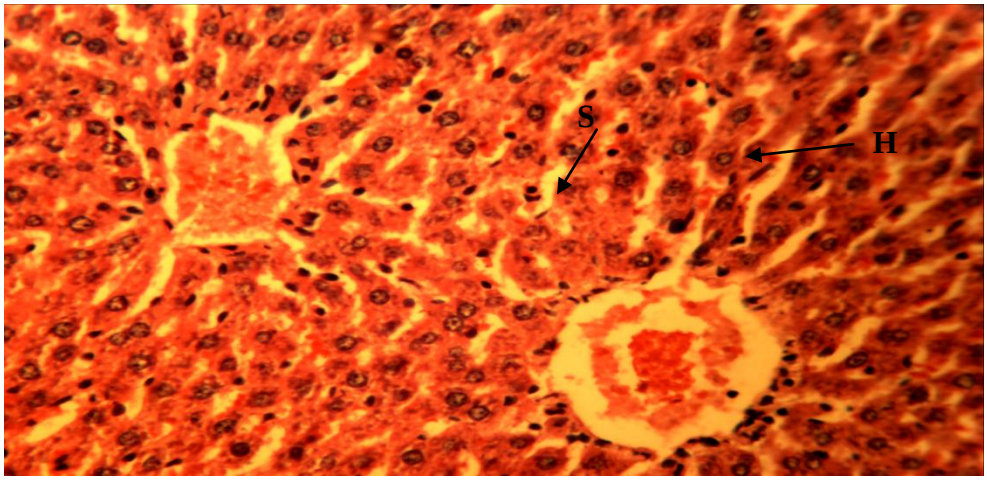


Plate XIV: Photomicrograph of liver from *Azadirachta indica* and melatonin treated diabetic group showing restored hepatocytes and sinusoids, no observable fatty change (H&E Stains, 250)

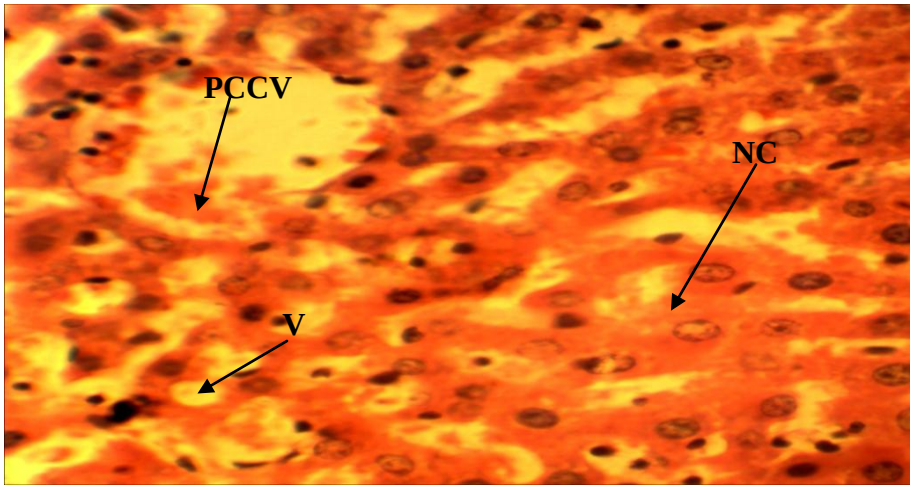
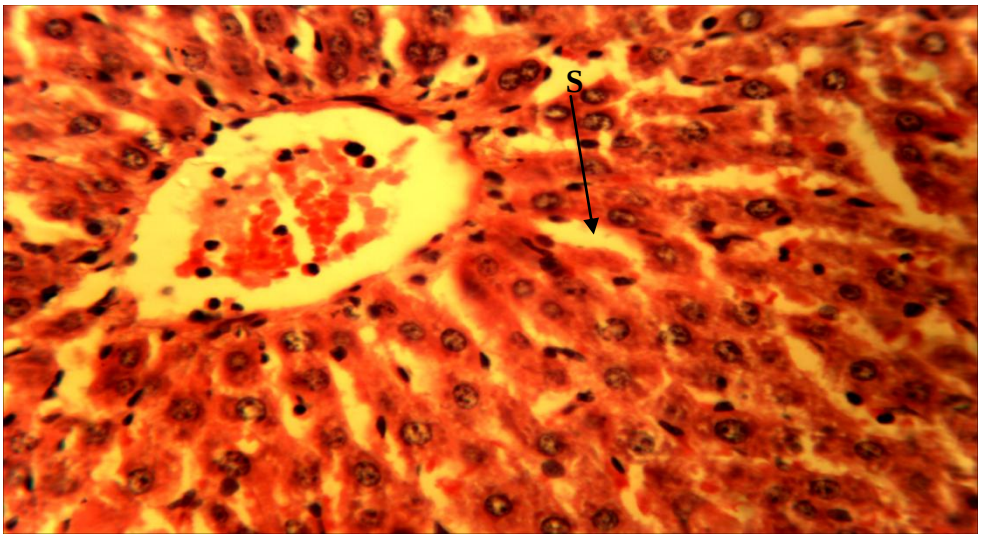
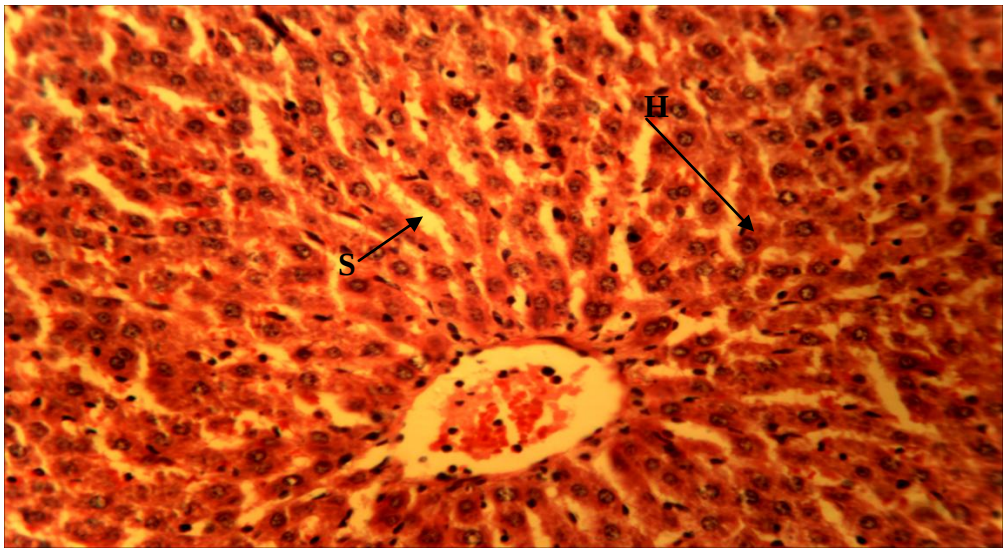


Plate XV: Photomicrograph of liver from metformin treated diabetic DMF) group showing necrosis of hepatocytes and sinusoids, vacoulation (V) and partial congestion of central vein (PCCV). There is poor restoration of liver cyto-architechture (H&E x250)



PlateXVI: Photomicrograph of liver from *Azadirachta indica* treated control group (NAI) showing normal hepatocytes (H) and sinusoids (S) (H&E, x250)



PlateXVII: Photomicrograph of liver tissue from *Azadirachta. indica* and melatonin treated control group (NAI/NML) showing normal hepatocytes and sinusoids (H&E, x100)

4.5.3: Histology of Liver showing Glycogen depletion

Liver section stained with Perodic acid Schiff (PAS) in Plate XVIII (normal control group), Plate XX, XXI, XXIII diabetic treated groups were positive for glycogen in hepatocytes cytoplasm. There was glycogen restoration in the extract and melatonin treated diabetic groups. Plate XIX from diabetic control and Plate XXII from metformin treated group demonstrates depleted glycogen stores with vacoulation. The metformin treated group showed the least amount of glycogen restoration.

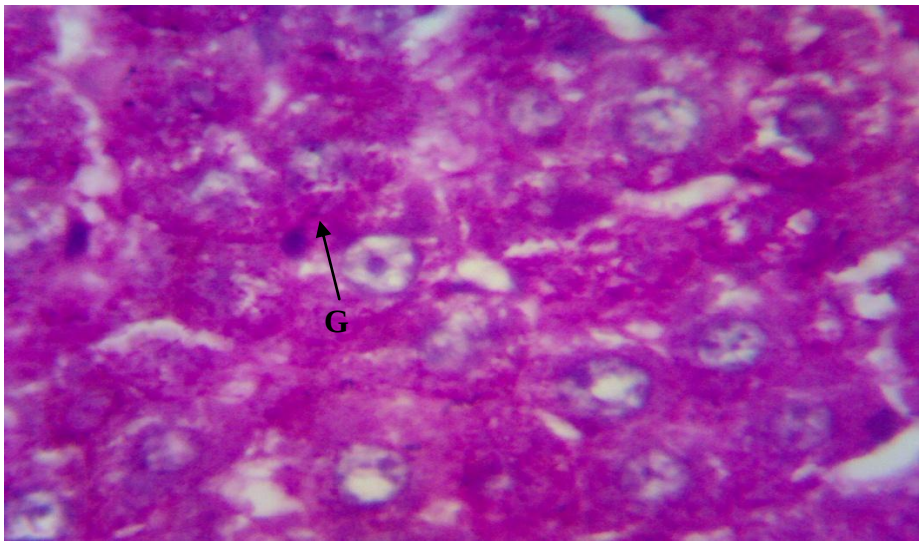


Plate XVIII: Photomicrograph of liver from normal control group demonstrating glycogen (G) hepatocytes cytoplasms (PAS Stains, x400)

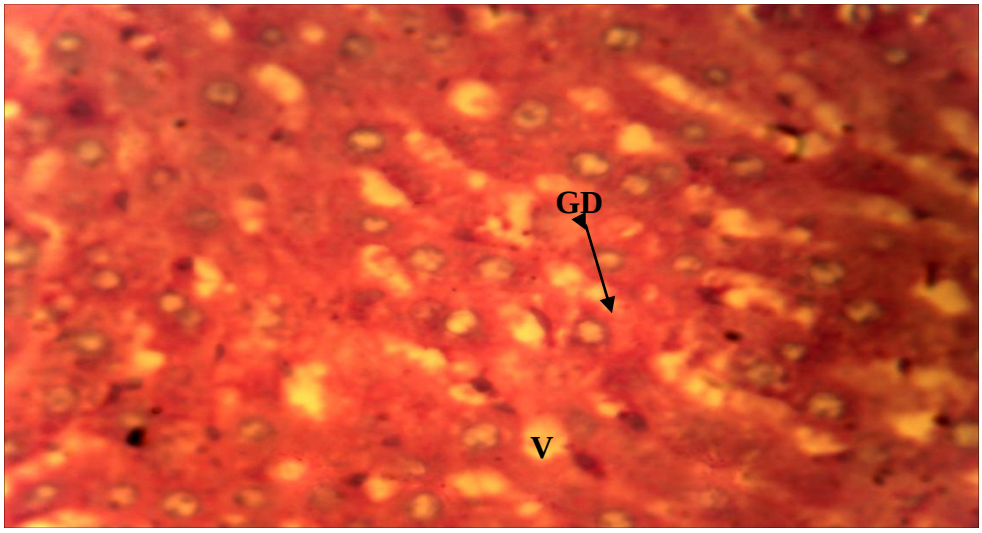


Plate XIX: Photomicrograph of Liver from diabetic control group (DC) treated with normal saline showing near total depletion of glycogen (GD), nuclear and cytoplasmic vacoulation (V) (PAS Stain, x400)

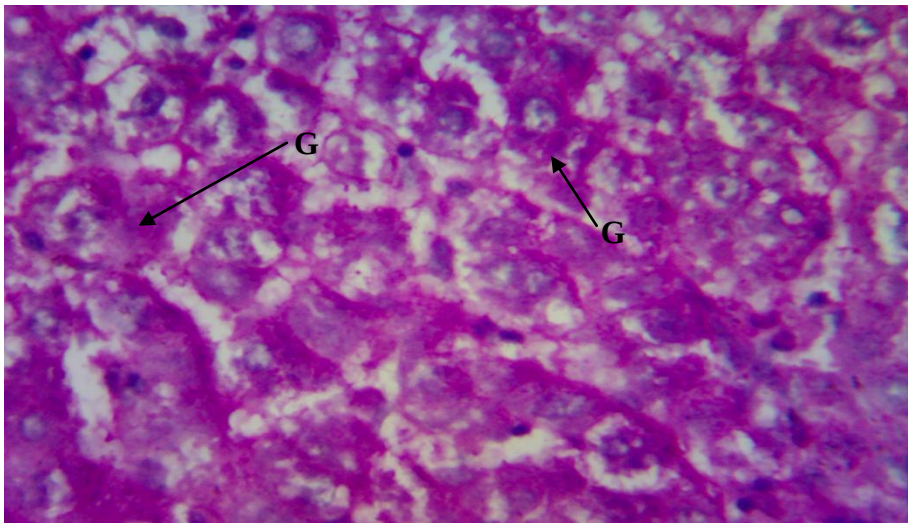


Plate XX: Photomicrograph of liver from *Azadiracta indica* treated diabetic group (DAI) showing improved glycogen (G) stores in hepatocytes, (PAS Stain, x400)

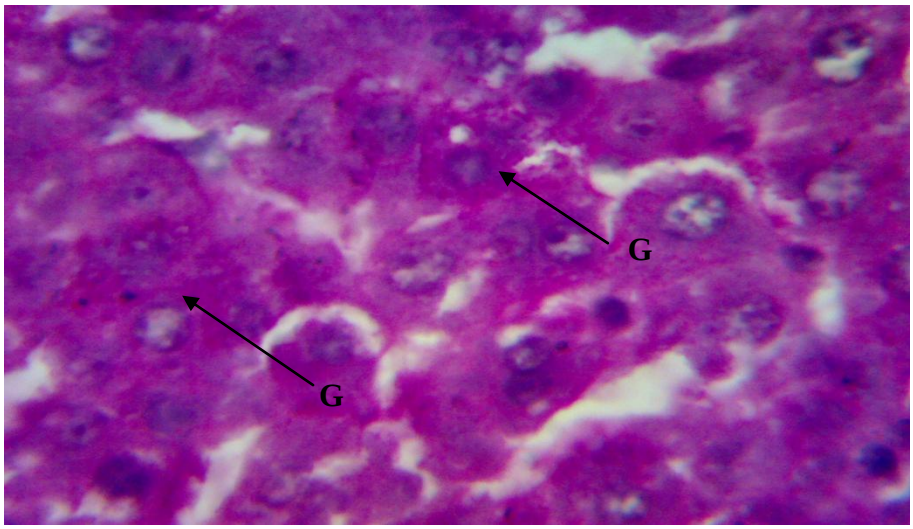


Plate XXI: Photomicrograph of liver from *Azadirachta indica* and melatonin treated diabetic group (DAI/DML) showing normal hepatocytes and sinusoids. There is an improved cytoplasmic glycogen (G) store, (PAS x400)

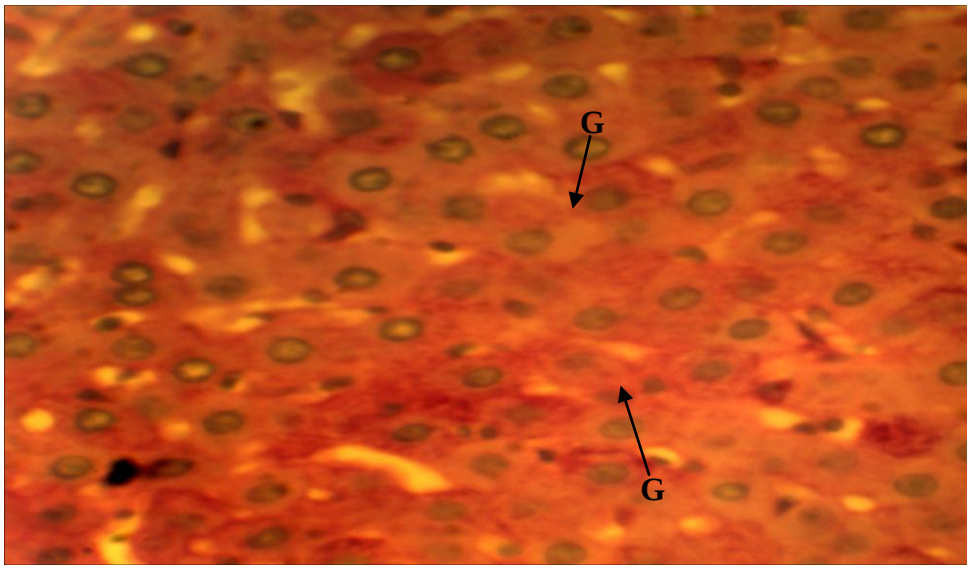


Plate XXII: Photomicrograph of Liver section from metformin treated diabetic group (DMF) showing reduced cytoplamic glycogen (G) stores (PAS x400).

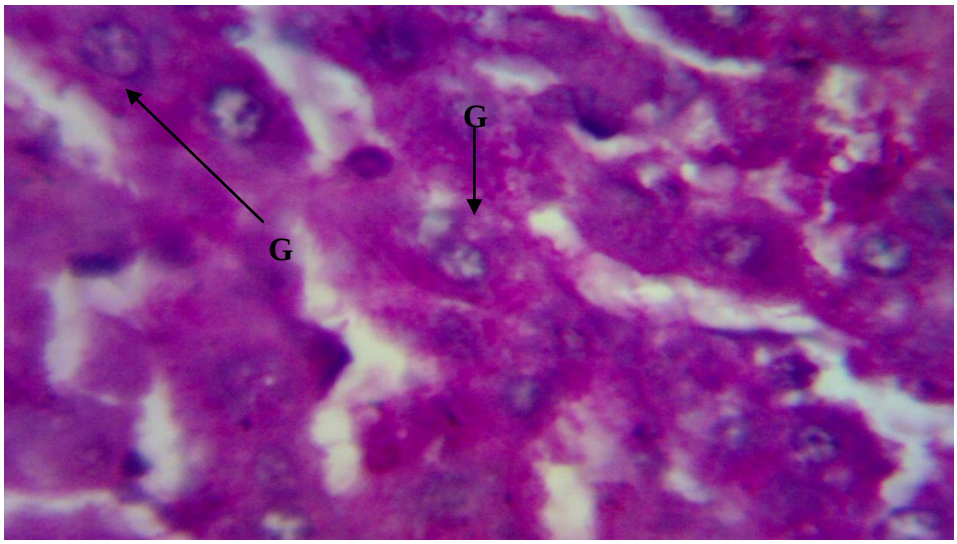


Plate XXIII: Photomicrograph of liver from melatonin treated diabetic group (DML) showing improved glycogen (G) stores in hepatocytes cytoplasm (PAS Stain, x400).

4.5.4: Collagen fibers of Liver from rats observed during the study.

The liver sections stained with Heamatoxylin Van Gieson (HVG) stain demonstrated that in the diabetic control group Plate XXV there was fragmentation of collagen fibers causing congestion of the central vein. Plate XXIV-XXVIII of the normal control and treated diabetic groups with melatonin and extract showed total and partial restoration of these fibers. Diabetic metformin (Plate XXIX) treated group showed the least collagen fibers restoration.

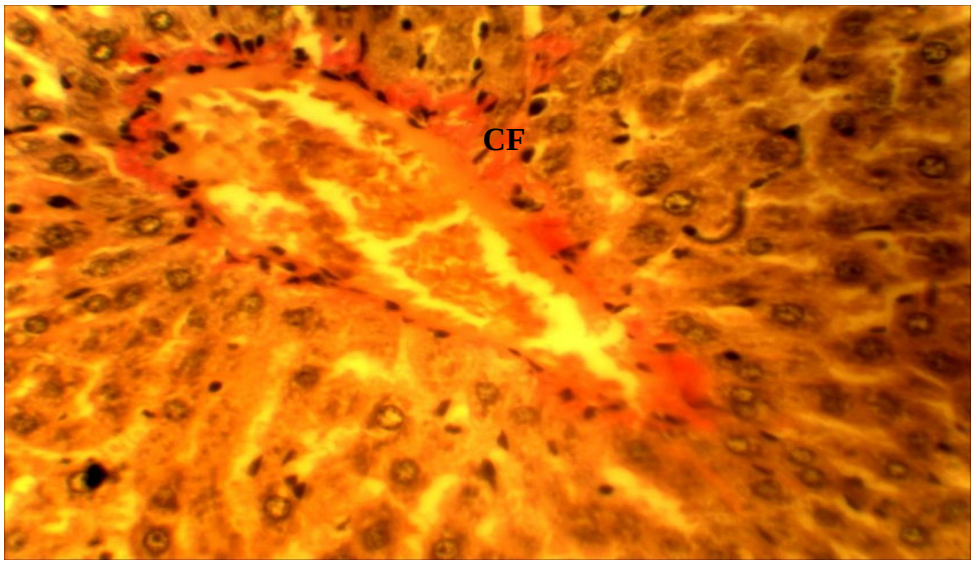


Plate XXIV: Photomicrograph of liver from normal control group stained with HVG, showing collagen fibers (CF), (HVG Stain x250).

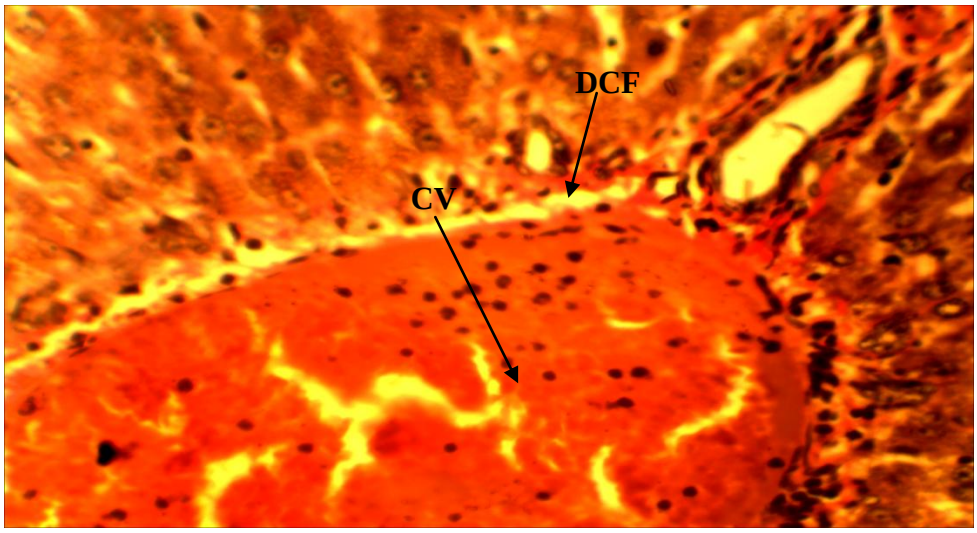


Plate XXV: Photomicrograph of liver from diabetic control group (DC) showing degeneration of collagen (DCF) fibers and congestion of central vein (CV) (HVG x250)

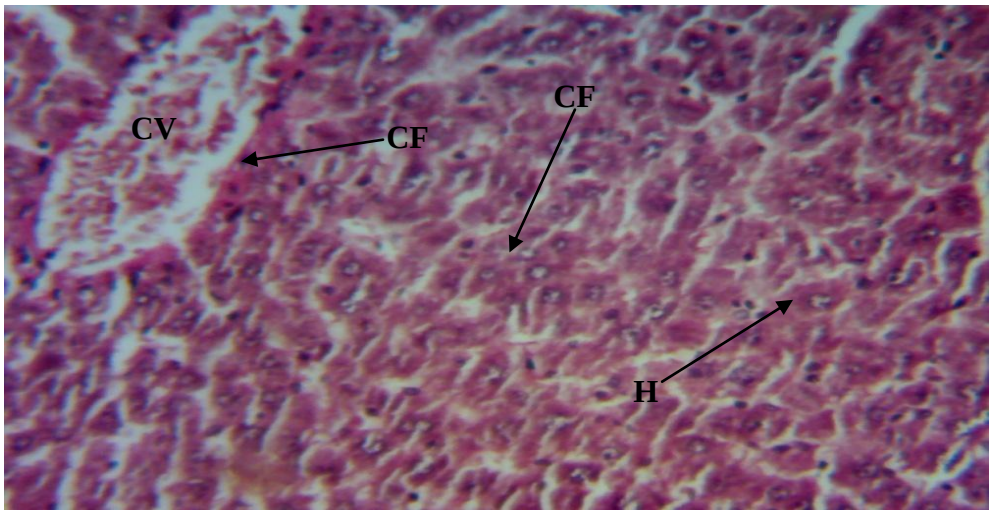


Plate XXVI: Photomicrograph of liver from melatonin treated diabetic group (DML) showing restored collagen fibers (CF) around hepatocytes, and central vein (CV). (HVG x250)

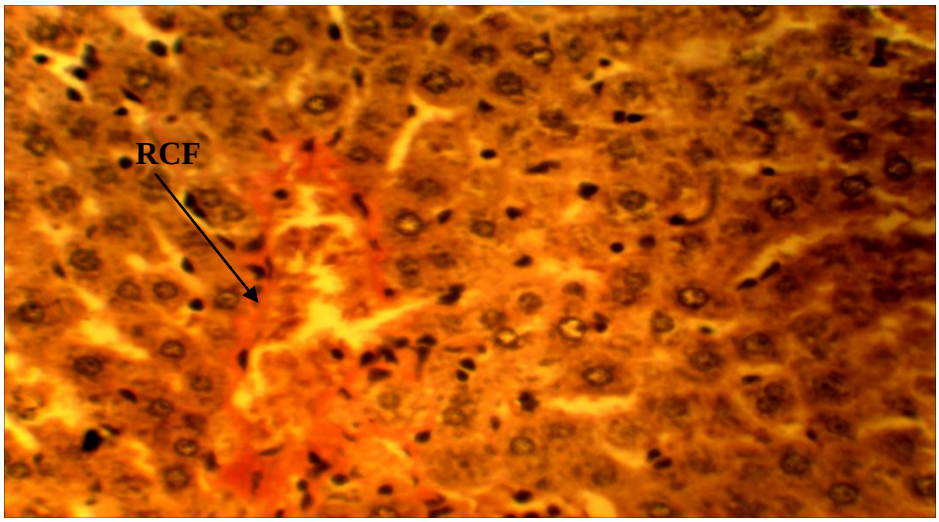


Plate XXVII: Photomicrograph of liver from *Azadiracta indica* treated diabetic group (DAI) stained with HVG showing regeneratedr collagen fibers (CF-Arrowed) around liver hepatocytes, sinusoids and around the veins. (HVG x250)

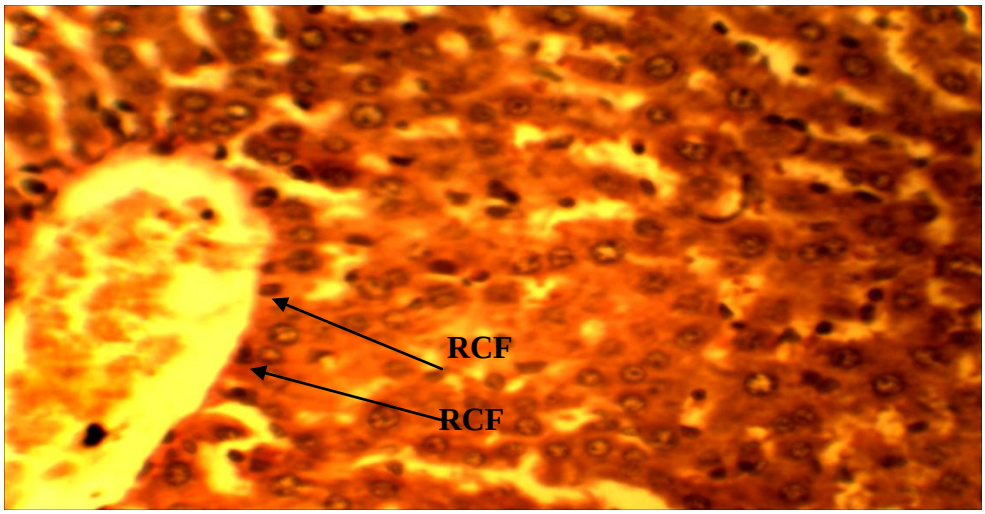


Plate XXVIII: Photomicrograph of from Liver of diabetic *A. indica* and melatonin treated group showing regenerated collagen fibers (RCF), no necrotic change, no fatty degeneration and restored hepatocytes and sinusoids (HVG X250)

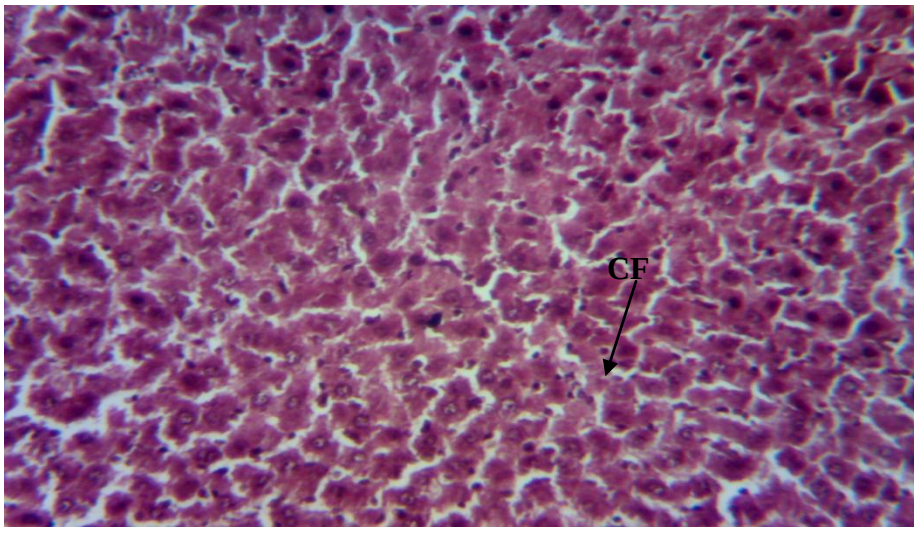


Plate XXIX: Phoromicrograph of liver from Metformin treated diabetic group (DMF) stained with HVG showing poorly restored collagen fibers (CF) around the hepatocytes, sinusoids and vessels. (HVG, x250)

4.5.5 Reticular fibers of liver from rats observed during the Study

Photomicrographs of liver tissues stained with Gordon and Sweet stain for reticular fibers showed that there was a positive result for reticular fibers in Plate XXX, XXXI, XXXIII and XXXIV in the normal and diabetic treated groups while there was negative result for Plate XXXII diabetic control group and fragmentation of reticular fibers in Plate XXXV metformin treated diabetic group.

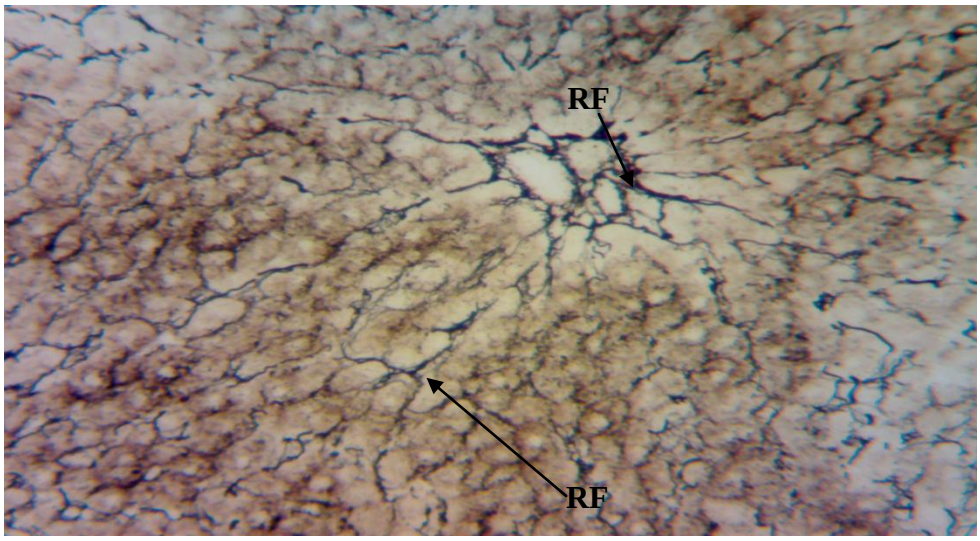


Plate XXX: Photomicrograph of liver from Normal Control group (NC) showing reticular fibers (RF) stained with Gordon and Sweet. (Gordon and Sweet, x250)

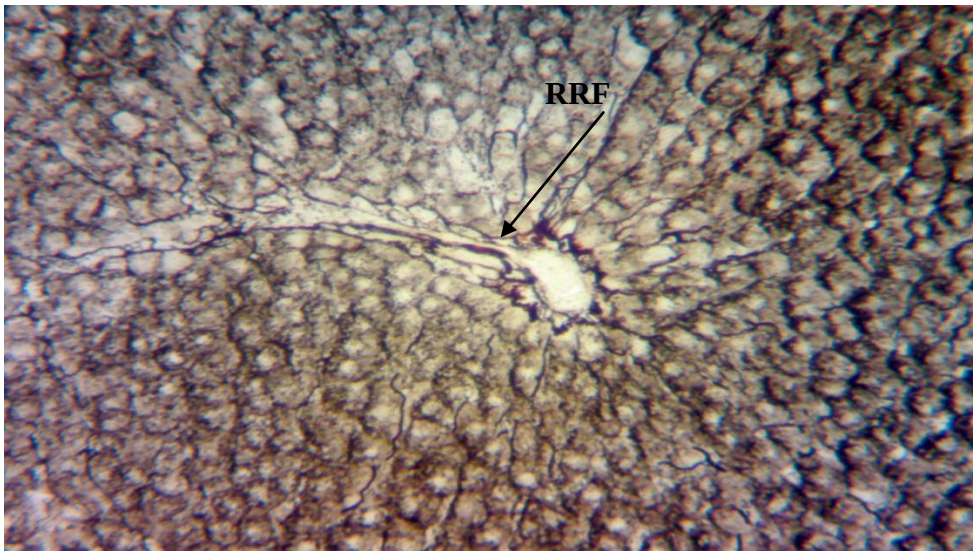


Plate XXXI: Photomicrograph of liver from *Azadiracta indica* treated diabetic group showing regenerated reticular fiber (RRF) (Gordon and sweet, x250)

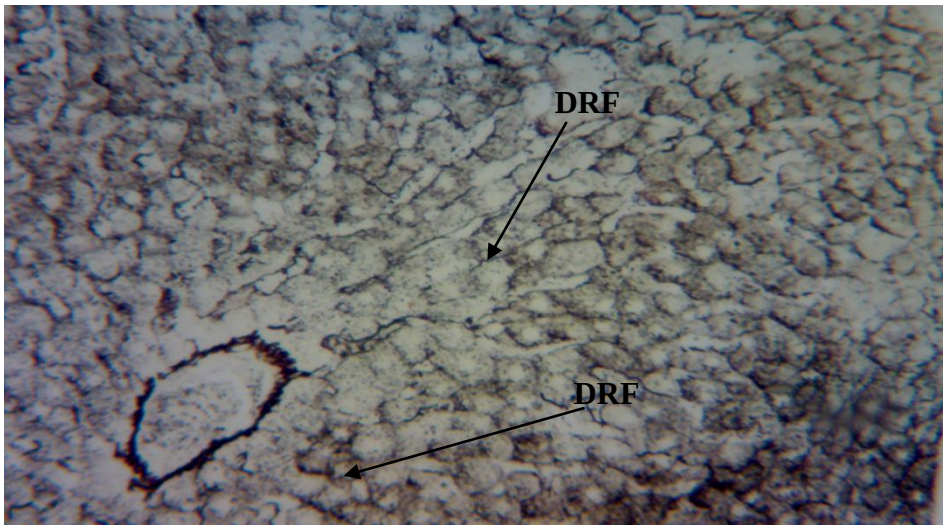


Plate XXXII: Photomicrograph of liver from Diabetic control group (DC) stained with Gordon and Sweet demonstrating degenerated reticular fibers (DRF). (Gordon and Sweet x250)

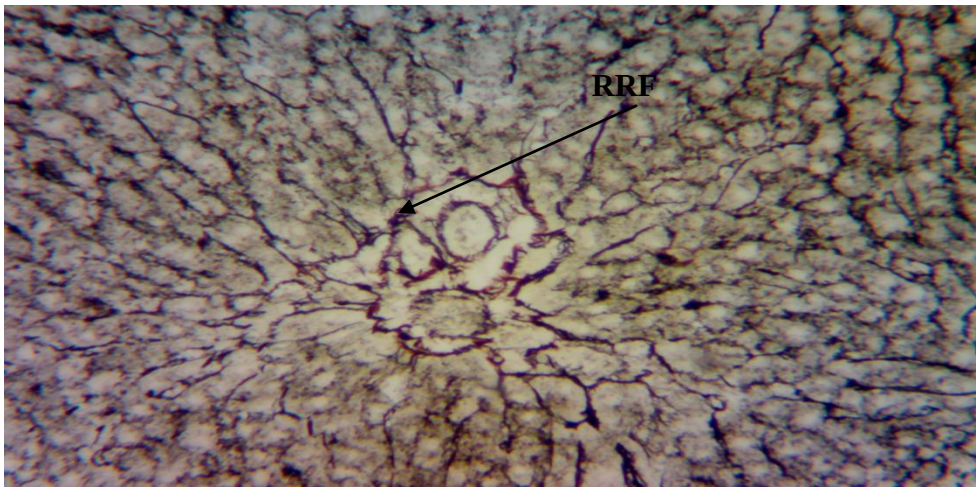


Plate XXXIII: Photomicrograph of liver from Melatonin treated diabetic group (DML) stained with Gordon and sweet to demonstrate restored reticular fibers (RRF). There is regeneration and restoration of reticular fibers in the liver hepatocytes, sinusoids and around blood vessels. (Gordon and Sweet, x250)

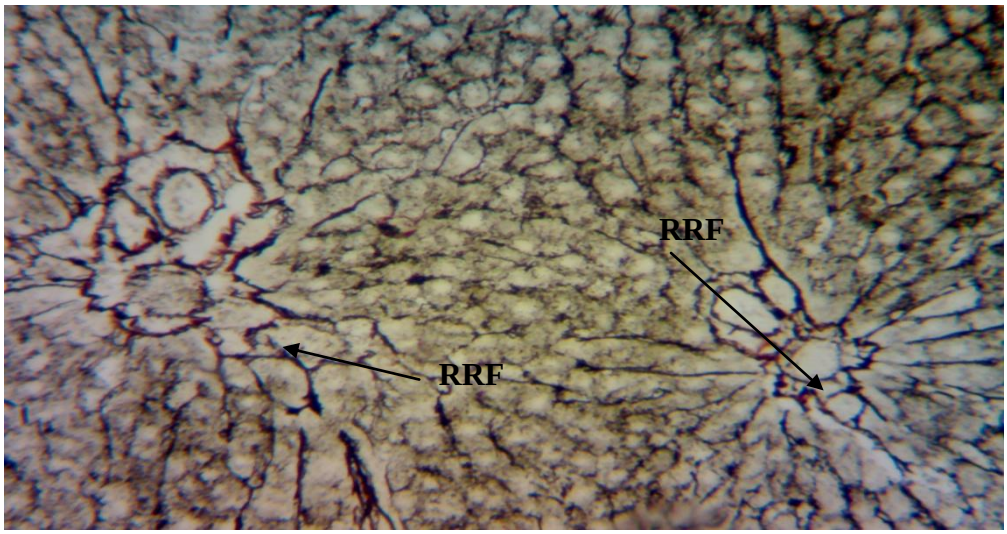


Plate XXXIV: Photomicrograph of liver from *Azadirachta indica* and melatonin treated diabetic group with Gordon and Sweet stain showing regenerated reticular fibers around the hepatocytes,sinusoids and blood vessels. (Gordon and Sweet x250)

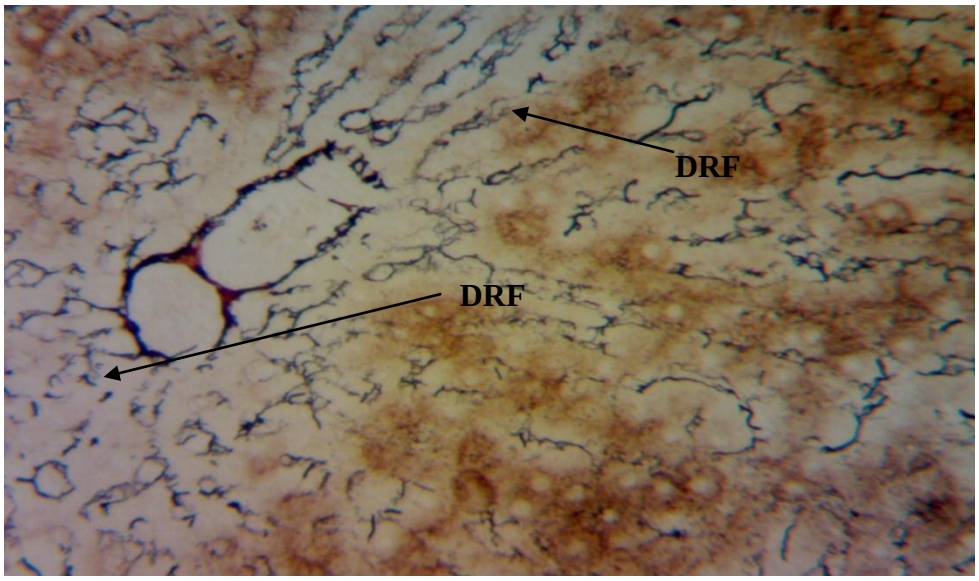


Plate XXXV: Photomicrograph of liver from Metformin treated diabetic group (DMF) stained with Gordon showing fragmented reticular fibers (DRF) around the hepatocytes, sinusoids and blood vessels. Reticular fibers are not fully regenerated. (Gordon and Sweet, x x250)

CHAPTER FIVE

5.0 DISCUSSION

5.1 Physical Observation

The present study was aimed at evaluating the physical, histomorphological and biochemical effects of melatonin and *A. indica* ethanolic leaves extract administration on streptozotocin induced diabetes in adult Wistar rats. It explored the effects *A. indica* ethanolic leaves extract and melatonin on different parameters which included physical observations, biochemistry and histology of the liver and pancreas.

Weight increase was observed in normal saline treated control group, extract, and melatonin treated groups. This suggests that *Azadirachta indica* and melatonin treatment groups did not affect food intake while the diabetic control normal saline treated group showed slight decrease in weight. The diabetic metformin treated group showed slight weight increase. This was consistent with previous findings (Das *et al* 2010; Shailey and Basir 2011), that *A. indica* increases body weight of STZ induced diabetic rats significantly while the diabetic control rats showed significant body weight decrease suggesting that diabetes itself results to weight loss. *Azadirachta indica* treatment combined with melatonin gave a better therapeutic result compared to other treated groups. On the other hand metformin treated group showed significant weight increase this complications tend to exacerbate the underlying disorder associated with diabetes mellitus according to Deckert *et al*., (1985).

5.2 Blood Glucose Studies

In this study, decrease in blood glucose levels were observed in diabetic extract treated group, melatonin treated group and melatonin and extract combined treated group in both the STZ induced diabetic and control treated groups. *Azadiracta indica* leaves extract treated group only, showed significant reduction in blood glucose levels at the end of the 21 days of treatment which is similar to the work of Das *et al* (2010).

Chronic hyperglycaemia is the cause of many debilitating changes associated with diabetes mellitus (Shailey and Basir2011), The pathophysiological changes of which can be alleviated by blood glucose control.

Melatonin treatment alone also reduced blood glucose level significantly. When the extract and melatonin were used in treating the animals, a highly potent and significant value was observed, showing that melatonin potentiates the efficacy of the extract in reducing the blood glucose levels and that melatonin on its own has hypoglycaemic effects. According to Meshchyshen *et al* (2010), melatonin caused a decrease and normalization in blood glucose levels in alloxan induced diabetic rats, probably due to its inhibitory effects on catecholamines by inhibiting ACTH-stimulating cortisol production. The metformin treated diabetic group showed reduction in blood glucose but was not significant as compared with the extract and melatonin treated groups; which is similar to observations by Das and Mostofa (2010).

5.3 Liver Function Studies

Liver enzymes Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are present in high concentration in the normal hepatocytes of the liver and the enzymes leaked into the circulation as a result of damage to cell membrane of hepatocytes (Das and Vasudevan, 2005; Ahsan *et al*., 2009), had reported that ALT is more specific for liver damage as compared to AST, and that AST activities persist longer than ALT. There were some levels of liver damage as seen in the results of the present study, where elevated levels of these enzymes were observed in the diabetic control group as compared to the experimental treated groups. The reduction in these enzymes in groups treated with *A. indica* and melatonin seem to suggest that *Azadirachta indica* leaves extract and melatonin possess some therapeutic effects against STZ induced diabetes mellitus

The decrease in the levels of these enzymes (AST, ALT, ALP), when the treated rats were with *Azadirachta indica* (AI) alone, melatonin (ML) alone and when AI and ML were combined is suggestive that the extract and melatonin have some therapeutic effects, maybe by way of membrane stabilization which could prevent further leakages of intracellular enzymes. This is consistent with findings by Atangwo *et al* (2010) that A. *indica* and *Vernonia* Amygdalina alone and when combined caused decrease in liver enzymes and values obtained were relative to the diabetic control group. The diabetic control gave high significant values in the increase of AST and ALT and ALP. Treatment with *Azadirachta indica* only and when combined with melatonin showed a significant reduction in ALT and AST while melatonin treatment alone had a high significant reduction in serum enzymes. Metformin treatment showed the least reduction in liver enzymes when compared with diabetic control. The control groups showed results similar to the extract

treatment except for normal control group, which had the lowest values for liver enzymes similar with results demonstrated by Atangwo *et al* (2010).

5.4 Lipid Peroxidation (MDA) Studies

The level of malondialdehyde (MDA) significantly increased in the animals of diabetic control group compared to the control group, the *Azadirachta indica*, melatonin, and melatonin and *A. indica* combined treated groups. In the treated groups, melatonin group, showed the least values followed by *Azdirachta indica* while the combined group gives similar results. The normal control for extract and melatonin gave similar results to the treated diabetic group, except for metformin treated diabetic group. The results obtained were similar to those obtained by Meshchyshen *et al*., (2010) which showed that treatment with melatonin decreases and normalised the MDA levels and increased and normalised GPx levels in alloxan induced diabetic rats when compared with untreated rats

Increased oxidative stress and impaired antioxidant defense have been widely accepted and suggested as participant in the development and progression of diabetes and their complications (Wild *et al*., 2004; Meshchyshen *et al*., 2010). Streptozotocin is a classical chemical diabetogenic agent which is used in β cells destruction and type 1 diabetes, due to its selective cytotoxic effects on pancreatic β-cells. Taken together, streptozotocin-induced diabetes increases oxidative stress through generation of free radical (Aksoy *et al.*, 2003), increase levels of lipid peroxidation, decreased levels of superoxide dismutase, catalase and gluthathione peroxidase (Amagan *et al*., 2006), as well as DNA single strand break.

Malondialhyde is produced by peroxidative decomposition of poly unsaturated lipid. It is used as a marker of free radicals in tissue damage and oxidative stress. The restoration of

antioxidant status is an important parameter to evaluate the effect of anti-diabetic compounds. In diabetes, the persistence of hyperglycaemia has been reported to cause increased production of free radicals. Under normal physiological condition there is a critical balance in the generation of oxygen free radicals and its antioxidant defense systems used by organisms to deactivate and protect themselves against free radical's toxicity (Sies, 1991). Impairment in the oxidant/antioxidant equilibrium in favour of the former provokes a situation of oxidative stress which is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases, and contributes substantially to the pathogenesis of diabetic complications (Halliwel, 1994; Evans et al., 2003).

5.5 Serum Antioxidant Studies (SOD, CAT, GPx)

Result from the study for SOD, CAT and GPx from the *A. indica* and melatonin treated diabetic groups resulted in significant increase in serum levels in the enzymes in diabetic treated rats. SOD levels increased significantly in combined melatonin and extract treated group, melatonin treated alone and *A. indica* extract alone when compared with diabetic control group which showed decreased enzymes values. A similar result for the normal control treatment groups was obtained when compared with the diabetic treated groups. The results showed that melatonin and *A. indica* ethanolic extract have antioxidant potentials with normalization of these enzymes when used therapeutically.

When the extract was combined, the highest values in the elevations of SOD, CAT and GPx in the serums of the treated rats showed that they complemented each other and potentiated removal of free radicals better. The SOD may have destroyed superoxide by converting it to

hydrogen peroxide that is further decomposed by CAT and GPx, thereby improving antioxidant levels in the serum and detoxifying generated free radicals released by STZ induced hyperglycaemia in the diabetic rats.

5.6 Histological Studies

Histological sections showed that *A. indica* and melatonin when given therapeutically to STZ induced diabetic Wistar rats, alleviate the degenerative changes associated with hyperglycaemia and diabetes mellitus by reducing fatty change, necrosis, hepatocyte vacoulation, glycogen depletion, connective tissues derangement and restoration of the cyto-architecture of the liver. The reticular tissue fibers restoration in the liver parenchyma of STZ induced diabetic treated groups with melatonin and A. *indica* is different from the necrotic changes in the STZ induced diabetes diabetic control group. This finding is in accordance with previous studies showing that *A. indica* has hepatoprotective properties over paracetamol induced hepatoxicity in rats, where hepatic toxicity reduced (Bhanwra et al., 2000).

The Report of Ebong *et al*., (2006) had demonstrated the presence of phytochemicals with known antioxidant properties like flavanoids, polyphenols and micronutrients (antioxidant vitamins and micronutrient) in the leaves of A. *indica* extract and suggested their actions might have inhibited the free radical generation process and mopping up of circulating free radicals causing pancreatic islet lesions and complications of diabetes, thereby resulting in the regeneration of liver and pancreatic cells. The administration of A. *indica* and melatonin restored depleted glycogen stores in treated groups as compared with untreated diabetic group where there was depletion in cytoplasmic glycogen stores.

The histology of pancreatic islet cells was normal in treated and untreated normal control groups while degenerative changes and shrunken islets of langerhan cell mass was observed in STZ induced untreated diabetic group. The Pancreas of the treated diabetic group showed regenerated pancreatic cell mass of islets of langerhans. These are comparable to the findings of Vijayanand and Wesely (2011) and Maisaa and AL-Rawi (2007) where A. *indica* reversed degenerative changes associated with liver and pancreas in alloxan induced diabetic Wistar rats, and that melatonin participated in hepatocytes regeneration in thiocetamide-induced liver injury which was due to its ability to scavenge free radicals as an antioxidant by stimulating messenger ribonucleic acid (mRNA) levels and activities of SOD and GPx.

The results of the treated group with melatonin and A. *indica* extract showed synergistic interaction between and among components of the extract and melatonin with respect to pancreatic islets of langerhans cell recovery. The results further demonstrated that regeneration and restoration is one of its anti-diabetic mechanisms including normal liver function.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

i) The extract of *Azadirachta indica* leaves showed hypoglycaemic effect on STZ induced diabetes in adult Wistar rats, therapeutically ameliorating the effects of hyperglycaemic pathophysiological changes in diabetes mellitus.

ii) The extract of *A. indica* leaves showed hepato-protection and regeneration of pancreatic islet cells of langerhans, in STZ induced diabetic Wistar rats via the following mechanisms.

a) Through potent hypoglycaemic and antioxidant agents present in A. *indica* leaves which may be mimicking or potentiating insulin release from the β-cells of the pancreas.

b) These antioxidants (SOD, CAT, GPx) most probably acted on the oxidative circle in the STZ treated pancreas and over time halted the process, hence re-establishing and regenerating islet cells by reduction of malondialdehyde released in tissues of the pancreas.

c) Reduction of oxidative stress in pancreas and hepatocytes through increased antioxidants such as superoxides dismutase, catalase and gluthathione peroxidases

d) Regeneration of hepatocytes.

ii) Melatonin showed it effects on STZ induced diabetes in Wistar rats through the following mechanisms;

a) Reduces blood glucose levels by regeneration of pancreatic islet cells thus increasing insulin secretion and restoration of glycogen stores in the liver cells.

b) Increased intracellular antioxidants (i.e. SOD, CAT, GPx) thereby speeding up the recovery, regeneration and restoration of pancreatic islets

c) Reduces liver enzymes in serum of treated animals

d) Hepato-protection by regenerating liver hepatocytes and sinusoids, and connective tissues

e) Mop up of free radicals and reactive oxygen species released by pancreatic beta cells selective destruction by STZ, by reducing Malondialdehyde (MDA) released from the pancreas.

iii) The extract and melatonin showed synergisticity in efficaciously ameliorating the hyperglycaemic changes better in all parameters examined in the study.

6.2 Conclusion

Ethanolic extract of *A. indica* leaves and melatonin posseses hypoglycaemic property, pancreatic regeneration and antioxidant effects in STZ induced diabetes in adult Wistar rats and that they are mediated by antioxidant release and decrease in lipid peroxidation. These findings supports the claim by traditional herbal healers that *Azadirachta indica* have hypoglycaemic properties, hence can be used for diabetic management with less side effects.

6.3 Recommendation

a) Further studies are needed to investigate the active principle and mechanisms involved in the combined therapy of A. *indica* and melatonin to quantify insulin release

b) Further studies are also needed to investigate the active principle involved in pancreatic regeneration and hepatoprotection

c) Further studies are required to understand the synergistic mechanisms involved in their combined hypoglycaemic effects.

REFERENCES

Ahmet. K., Turgut, T, Sukru. O and Dun-Xian R.J.R. (2008). Hyperglycemia Related Pathophysiologic mechanisms and potential beneficial actions of melatonin. *Mini-Reviews in Medicinal Chemistry,* 8: 1144-1153.

Ahsan, M.R., Islam, K.M., Bulbul, I.J., Musaddik, M.A and Haque, E (2009). Hepatoprotective activity of methanol extract of some medicinal plants against carbon tetrachloride-induced hepatotoxicity in rats. *European Journal of Science Research,* 37: 302-31.

Akenova, M.E. and Attackrah, A.N. (1986). Control of spear grass (imperata cylindrical {L} Bearuv in an alley cropping fallow. *Nitrogen fixing Tree research reports,* 4; 24-28.

Akinmoladun, A. C., Ibukun E.O., Afor E., Obuotor., E.M. and Farombi E.O. (2007). Phytochemical constituent and antioxidant activity of extract from the leaves of Ocimum gratissimum; Sciences Research Essay. 2: 163-166.

Akinpelu, D.A. and Onakoya, T.M. (2006). Antimicrobial activities of medicinal plant used in folk lore remedies in south-western. *African Journal of Biotechnology,* 5:1078-1081.

Aksoy, N., Vural, H., Sabuncu, T and Aksoy, S. (2003). Effects of melatonin on oxidative-antioxidative status of tissues in streptozotocin induceddiabetic rats: *Cell Biochemical Function*, 21: 121-125.

Aladakatt, A.R., Sukesh, B., Jadaramkunti, C.U and Hiremath, M.B. (2011). Aspects of the antiandrogenic/antifertility property of Azadirachtin-a from *Azadirachta indica* leaves in male albino rats: effect on the biochemical and cauda epididymal sperm parameters: *Recent Research in Science and Technology*; 3(2): 34-46.

Ali, B.H., Salih, A.M.M. (1982). Suspected *Azadirachta indica* toxicity in a sheep. *Veterinary Record*, 111: 494.

Ames, B.N and Shigenaga, M.K (1992). Oxidants are a major contributor to aging. *Annual New York Academy of Sciences Journal*, 663: 85–96.

American Diabetes Association (2008). Report of the Expert Committee on the diagnosis and Classification of diabetes mellitus. *Diabetes Care,* 31; 1:55-60.

Arendt, J. (1994**)**. Melatonin and the Mammalian Pineal gland: London; Chapman and Hall: 2: 189-192.

Arivazhagan, S., Balasenthil, S and Nagini, S. (2000a). Modulatory effects of garlic and neem leaf extracts on *N*-methyl-*N*_-nitro-*N*-nitrosoguanidine (MNNG)-induced oxidative stress in Wistar rats: *Cell Biochemistry and Function,* 18: 17–21.

Arivazhagan, S., Balasenthil, S and Nagini, S. (2000b). Garlic and neem leaf extracts enhance hepatic glutathione and glutathione dependent enzymes during *N*-methyl *N*_-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in rats: *Phytotherapy Research*, 14: 291–293.

Armagan, A., Uz, E., Yilmaz, H.R., Soyupek, S., Oksay, T and Ozcelik, N. (2006). Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic Rat testes: *Asian Journal of Andrology*: 8:595-600

Ar'Rajab, A and Ahrén, B. (1993). Long-term diabetogenic effect of streptozotocin in rats: *Journal of Surgical Research*, 35: 433-460.

Atangwho, I.J., Ebong, E.P., Godwin, E.E., Akpaso, M.I.,, and Asuquo, E.E. (2010). Histological effects of combined extracts of vernonia and Azadirachta indica on normal and diabetic rats: the pancreas and liver. *Research Journal of Agricultural and Biological Sciences*, 6(4):514-521.

Axelrod, J and Weissbach., H, (1960). Enzymatic O-methylation of N-acetylserotonin to melatonin. *Science Journal*, 131: 1312.

Baily, C.J and Day, C. (1989). Traditional plant medicines as treatments for diabetes. *Diabetes Care Journal*, 12: 553 –564.

Baydas, G., Canatan, H and Turkoglu, A (2003). Synthesis of melatonin. *Journal of Pineal Reearch, 32*: 225.

Baydas, G., Canatyan, H., and Toukoglu, A. (2002). Comparative analysis of the protective effects of Melatonin and Vitamin E on streptozotocin-induced diabetes mellitus: *Journal of Pineal research*, 32:225-230.

Beckman, J.A., Goodfine, A.B., Gordon, M.B., Garret. L.A., Keaney, F.J.Jr and Creager, M.A. (2001). Impaired Vasodilation: *American Journal of Physiology; 285:2392.*

Bell, R.H and Hye., R.J. (1983). Animal models of diabetes mellitus. *Physiology and Pathology Journal;* 35: 433-460.

Bhanwra, M. A. (2000). Evaluation of *Azadirachta indica* in Paracetamol Liver toxicity: *Indian Journal of Physiology and Pharmacology*, 44: 64-68.

Biswas, K, Chattepadhya, I, Banergee, R.K and Bandyopadhyayi, U. (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*): *Current Science Journal*, 82: 1336 –1345.

Bolzan, A.D and Bianchi, M.S., (2002). Effect of melatonin on hyperglycaemia and Dislipidaemia: *Mutation Research Journal*, *512*: 121-34.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature*, 414 ;6865: 813-820

Canadian Journal of Diabetes (2008). Classification of diabetes mellitus; An expert committee on the classification.

Centers for Disease Control and Prevention, (2011). National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease ControlandPrevention. Availableat:http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011

Chattopadhyay, R.R and Bandyopadhyay, M. (2005). Effect of Azadirachta indica leaf extract on serum lipid profile changes in normal and streptozotocin induced diabetic rats. *African Journal of Biomedical Research*, 8: 101-104.

Chattopadhyaya, R.R (1990). Possible mechanism of antihyperglycaemic effect of Azadirachta indica leaf extract: *Journal of Ethnopharmacology*; 67:373-376.

Champney, T.H., Steger, R.W., Christie, D.S and Reiter R.J (1985). Alterations in components of the pineal melatonin synthetic pathway by acute insulin stress in the rat and Syrian hamster: *Brain Research*, 338:25-32

Chouinard, S and Viau, C, (1992). Reversibility of renal tubular dysfunction in streptozotocin induced diabetic rats: *Canadian Journal of Physiolology and Pharmacology*, 70: 977-982.

Conti, A and Maestroni, G.J (1996). The Role of the pineal gland and melatonin in the development of autoimmune diabetes in non obese diabetic mice; *Journal of Pineal Research*, 20:164-172

Conti, A and Maestroni, G.J (1998). Melatonin rhythms in mice: role in autoimmune and lymphoproliferative diseases. *Annual New York Academy of Science*, 840:395-410.

Cormack, D. (2009). Histology of the pancreatic islets. Essential Histology 5th ED: 383-385.

Cvetko, M and Plosker, G.L (2007). Exanatide in type 2 diabetes mellitus (as an adjunct to metformin and/or a sulphonylurea): *Drugs Journal,* 67; 6: 935-54.

Deckert, T., Poulsen, J.E and Larsen, M. (1978). Prognosis of diabetics, with diabetes onset before the age of thirty one. *Diabetologia Journal,* 14: 363-377.

Dahiru T., Jibo A., Hassan A and Mande A. 2008. Prevalence of diabetes in a semi-urban community in Northern Nigeria, *Nigerian Journal of Medicine,* 17; 4: 14-16.

Das, A.R, Mostofo, M. E, Hoque, M. E, Das, S and Sarkar, A.K. (2010). Comparative efficacy of neem (*Azadirachta indica*) and Metformin chloride in streptozotocin induced diabetes mellitus in rats: *Bangledesh Journal of Veterinary Medicine*, 8; 1: 75-80.

Ding, H and Triggle, C.R, (2005). Antioxidant role of melatonin in vascular diseases, *Vascular Health Risk Management Journal*, 1; 55.

Dong, W.G., Mei, Q., Yu, J.P., Xu, J.M., Xiang, L and Xu, Y. (2003). Effects of melatonin on the expression of iNOS and COX-2 in rat models of colitis. *World Journal Gastroenterology*, 9; *6*: 1307-11.

Dubocovich, M.L and Markowska, M. (2005). Functional MT1 and MT2 melatonin receptors in mammals, *Endocrine*, 27:101–110

Ebenezar A., Nyenwe A., Osaretin J., Odia A., Anele E., Ihekwaba B., Ojule B and Babatunde B (2003). Type 2 diabetes in adult Nigerians: A study of its prevalence and risk factors in Port Harcourt Nigeria, *Diabetes Research and Clinical Practice*, , 62;23: 177-185.

Ebong Patrick E., (2006). Pancreatic beta cells regeneration: A probable parallel mechanism of hyperglycaemic actions of *Vernognia amygdalina Del* and *Azadirachta indica*. Paper presented at international neem conference in Kunming, China, pp. 234-238.

Ehud Ur., (2008). Definition, Classification and Diagnosis of Diabetes and Other Dysglycaemic Categories, *Canadian Diabetes Association Clinical Practice Guidelines Expert Committee: Canadian Journal of Diabetes Volume 32 Supplement 10-12.*

El-Hawary, Z.M and Kholeif., T.S. (1990). Biochemical studies of hypoglycaemic agents ; Effect of *Azadirachtan indica* leaf extract: *Archives of Phytotherapy Research Journal*, 13:108-112.

Ellis, H., (2006). Clinical Anatomy; A revision and applied Anatomy for clinical students: The Liver. 7th Edition, 93-98.

Espino, J., Pariente, A and Rodrigues, A.B. (2011). Role of melatonin on diabetes-related metabolic disorder. *World journal of diabetes*, 2;6:82-91

Evans, J.L., Goldfine, I.D., Maddux, B.A and Grodsky, G.M. (2003). Are oxidative stress mediated signaling Pathway mediators of insulin resistance and beta-cell disfunction? *Diabetes care Journal*, 52: 1-8.

Fimognari, A.,Pastorelli, R. and Incalzi, R.A. (2006). "Phenformin-Induced lactic acidosis in an older diabetic patient: a recurrent drama (phenformin and lacticacido)."*Diabetes care Journal,* 29; 40: 950-1.

Friedewald,W. T., Levy, R. and Fradrickson, D. S. (1972). Estimation of concentration of Low density lipoprotein cholesterol in plasma without the use of preparativeultracentrifugation. *Clinical chemistry journal, 19*; 449-452

Garg, G.P., Nigam, S.K., Ogle, C.W. (1993a). The gastric antiulcer effects of the leaves of the neem tree: *Planta Medica* 59: 215–217.

Garg, S., Taluja, V., Upadhyay, S.N., Talwar, G.P. (1993b). Studies on the contraceptive efficacy of Praneem polyherbal cream: Co*ntraception,* 48: 591–596.

Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A.L and Szabo, C (1997). *Life Science Journal*, *60*: 169.

Halliwel, B (1994). Free radicals, antioxidants and human diseases: causes or consequence? *Lancet,* 344:721-724.

International Diabetes Federation (2008). The IDF Consensus Worldwide Defination of Metabolic Syndrome: Bussels; IDF Communication; *http:/www.idf.org/webdata/docs/IDF_def_final.pdf.June, 15, 2012.*

Iwu, M.M, Obidoa, O. and Anazodo, M. (1986). The Anti-malarial activity of *Azadirachta indica:* Mode of action. *Pharmacy world, 34*: 16-30.

Jaiswal, A.K., Bhattacharya, S.K and Acharya, S.B., (1994). Anxiolytic activity of *Azadirachta indica* leaf extract in rats. *Indian Journal of Experimental Biology*, 32: 489–491

Javier, E, Pariente, J.A. and Rodríguez, A.B. (2011). Role of melatonin on diabetes-related metabolic disorders: *World Journal of Diabetes*, 15; 2; 6: 82-91.

Jelodar, G. A., Maleki, M., Motadayen., M. H and Sirus, S. (2005). Effect of Fenu greek onion and garlic on blood glucose and histopathology of pancreas oalloxan-induced diabetic rats. *Indian Journal of Medical Science*, 59(2): 64 –69.

Joshi, A.R., Ahamed, R.N., Pathan, K.M., Manivannan, B. (1996). Effect of *Azadirachta indica* leaves on testis and its recovery in albino rats. *Indian Journal of Experimental Biology* 34, 1091–1094.

Junod, A., Lambert A E., Orci L., Picket R., Gonet, A E and Renold A E (1967). Studies on nicotinamide-adenine dinucleotide. *Biochemical Journal*, 140; 487-491.

Kahler, W., Kuklinski, B., Rublman, C and Plotz, C. (1993). Diabetes mellitus a free radical associated disease: Results of adjuvant antioxidant supplementation. I: Z *GesamteInn Medicine*, 48(4): 223 –232.

Kaneto, H., Nakatani, Y., Kawamori, D., Miyatsuka, T., Matsuoka, T.A., Matsuhisa, M and Yamasaki, Y. (2005). *Inernational Journal of Biochemistry and Cell Biology, 37*: 1595.

Kasturi, M., Ahamed, R.N., Pathan, K.M., Parveen, D.S., Manivannan, B. (1997). Effects of *Azadirachta indica* leaves on the seminal vesicles and ventral prostate in albino rats. *Indian Journal of Physiology and Pharmacology*, 41: 234–260.

Karlsson, B, Knutsson, A and Lindahl B. (2001). Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occupational and Enviromental Medicine Journal*, 58: 747-752.

Kelly, D.E. (1995). Effects of weight loss on glucose homoestasis in NIDDM; *Diabetes Review*, 277; 21: 18919 –18927.

Khan, A., Safder, M., Alikhan, M.M., Khattah, K.N and Anderson, R.A (2003). '' Cinnamon improves Glucose and lipids of people with type 2 diabetes''. *Diabetes care Journal, 26: 12.*

Khosla, P., Sangeeta, B., Singh, J., Srivastava, R.K., (2000). Antinociceptive activity of *Azadirachta indica* (Neem) in rats; *Indian Journal of Pharmacology*, 32: 372–374.

Khosla, P., Bhanwra, S., Singh, J., Seth, S., Srivastava, R.K., (2000). A study of hypoglycamic effects of *Azadirachta indica* (NEEM) in normal and alloxan diabetic rabbits; *Indian Journal of Physiology and Pharmacology*, 44: 69–74.

Kiersztan, A., Winiarska, K., Drozak, J., Przedlacka, M., Wegrzynowic Z , Landmesser, U., Spiekermann, S., Dikalov, S., Tatge, H., Wilke,R., Kohler, C., Harrison, D.G., Hornig, B and Drexler, H. (2002). Antioxidants and diabetes. *Journal of Circulation, 106*: 3073.

King, H, Aubert, R.E and Herman, W.H. (1995). Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care,* 1998, 21: 1414-1431.

Knutson, K.L., Ryden, A.M., Mander. B. A and Van Cauter E. (2006). Role of sleep duration and quality in the risk and severity of type 2 diabetes mellitus. *Archieve Internal Medicine*, 166: 1768-1774.

Korkamaz, A and Reiter, R.J. (2008). Melatonin Regulation of antioxidant enzymes. Journal of Pineal Reaearch, 44: 41.

Krause, D.N., Geary G.G., Doolen S and Duckles S.P (1999). Melatonin and cardiovascular functions. *Advanced Experimental Medical Biology*, 460: 299-310.

Kroes, B.H., Van den Berg, A.J.J., Labadie, R.P., Abeysekera, A.M., De Silva, K.T.D. (1993). Impact of the preparation process on immunomodulatory activities of the ayurvedic drug Nimba arishta: *Phytotherapy Research*, 7: 35–40

Kvetnoy, I., Sandvik A.K and Waldum H.L. (1997). The diffuse neuro-endocrine system and extrapineal Melatonin. *Journal of Molecular Endocrinoloy*: 18: 1-3.

Kvetnoy, I.M. (1999). Extrapineal melatonin: location and role within diffuse neuroendocrine system. *Histochemical Journal,* 31: 1-12.

Lapshina, E.A. Sudnikovich, E.J., Maksimchik, J.Z. Zabrodskaya, S.V and Zavodnik, L.B. (2006). Melatonin; *Life Science, 79*: 1804.

Leuenberger, P., Cameron, D., Stauffacher, W., Renold, A.E and Babel, J (1971). Ocular lesions in rats rendered chronically diabetic with streptozotocin. *Ophthalmol*ogy *Research Journal, 47: 144-148.*

Lupi, R., Del Guerra, S., Mancarella, R., Novelle M., Valgimigli, L., Pedulli, G.F., Paolini M., Soleti, A., Filliponi F., Mosca, F., Boggi, U., Del Prato S., Masiello, P and Machetti P. (2007).Melatonin and diabetes metabolism: *Diabetes Metabolism Journal*, 33: 340

Maisaa, M.A. (2007). Inhibitory effect of melatonin on histological changes induced in rat liver by thiocetamide intoxication: *Journal of Biological Sciences*, 7 (1): 10-18.

Medhi, A. A., Chandra, A., Singh, R. K., Shukla, S., Mishra, L. C and Ahmad, S. (2003) Effect of herbal hypoglycemic agents on oxidative stress and antioxidant status in diabetic rats. *Indian Journal of Clinical Biochemistry,* 18(2): 8-15.

Meigs, J.B.. Hu, F.B., Rifai, N and Manson, J.E. (2007). Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *Journal of American Medical Association,* 291; (16):1978-86.

Meshchyshen, I, Kushner, O and Yaremii, I (2010): Hypoglycaemic and antioxidant action of melatonin in alloxan-induced diabetic rats. *Annales Universitatis Marie Curie-Sklodowska Lublin-Polonia,* 23; 31: 227-230.

Mohamed, A.K., Bierhaus, A., Schiekofer, S., Tritschler, H., Ziegler, H and Nawroth P.P. (1999). The role of oxidative stress and NF (B) activation in late diabetic complications. *Biofactors*, 10:179-175.

Mokini, Z and Chiarelli, F (2006). Pancreatc β-cells and Hyperglycaemia: *Pediatrics. Endocrinology Revision, 4*: 138.

Montilla, P.L., Vargas, J.F., Túnez, I.F., Muñoz de Agued,a M.C,, Valdelvira., M.E and Cabrera, E.S. (1998). Oxidative stress in diabetic rats induced by streptozotocin: protective effects of melatonin. *Journal Pineal Research*, 25:94-100.

Moore, K.L and Dalley A.F, (2005). Anatomy of the liver. Clinical oriented anatomy, 5TH ed; 289-298.

Mossa, J.S., (1985). A study on the crude antidiabetic drugs used in Arabian folk medicine. *International Journal of Crude Drug Research* , 23: 137– 145.

Mühlbauer, E, Wolgast, S., Finckh, U., Peschke., D and Peschke, E (2004). Indication of circadian oscillations in the rat pancreas. *FEBS Letters*. 2004: 564:91-96.

Mühlbauer, E and Peschke, E. (2009) Evidence for the expression of both the MT1- and in addition, the MT2-melatonin receptor, in the rat pancreas, islet and beta-cell. *Journal of Pineal Research,* 42:105-10.

Mulder H, Nagorny CLF, Lyssenko V, Groop L (2009). Melatonin receptors in pancreatic Islets Good morning to a novel Type 2 Diabetes gene. *Diabetologia*, 52: 1240-124

Njoku, O., Nwanguwa, A and Akunyili, D. (2004) Effect of aqueous leaf extract of *Vernonia amygdalina* on blood glucose and triglyceride levels of alloan-induced diabetic rats (*Rattus rattus*). *Animal Research International*, 1(2): 90-94.

Obaseki, O and Jegede-Fadunsin, H.A (1986). The antimalarial activity of *Azadirachta indica*. *Fitoterapia Journal*, 57: 247–251.

Okwu., D.E and Josiah, C. (2006). Evaluation of the chemical composition of two Nigerian Medicinal plants *African Journal of Biotechnology,* 5(4): 357-361.

Ozaki, Y and Lynch H.J. (1976). Presence of melatonin in plasma and urine of Pinealectomized rats. *Endocrinology Journal,* 99: 641-644.

.

Parveen, D.S., Manivannan, B., Pathan, K.M., Kasturi, M., Ahamed, R.N. (1993). Antispermatic activity of *Azadirachta indica* leaves in albino rats. *Current Science*, 64 : 688–689

Peschke, E. (2008). Melatonin endocrine Pancreas and Diabetes. *Journal of Pineal Research,* 44: 26-40.

Pescke, E., Stumpf, I., Bazwinsky, I., Litvak, L., Dralle, H, and Muhlbauer, E. (2007). Melatonin and β-cell receptors: *Journal of Pineal Research, 42: 350.*

Picinato, M.C, Haber EP., Cipolla-Neto J., Curi R., de OliveiraCarvalho C.R., and Carpinelli, A.R . (2002). Melatonin inhibits insulin secretion and decreases PKA levels without interfering with glucose metabolism in rat pancreatic islets. *Journal of Pineal Research*, 33: 156-160.

Pietrosemoli, S., Olavez, R., Montilla, T. (1999). Empleo de hojas de neem (*Azadirachta indica* A. Juss) en control de nematodos gastrointestinales de bovinos a pastoreo. Revista de la Facultad de Agronomia, Universidad Del Zulia 16, 220–225 (abstract).

Rakieten, N., Rakieten, M.L and Nadkarm, M.V. (1963).Studies on the diabetogenic actions of streptozotocin. *Cancer Chemotherapy Research Journal,* 29: 91-98.

Ramanadham. S., Mongold, J.J., Brownsey, R.W., Cros, G.H and McNeill, J.H. (1989). Oral vanadyl sulfate treatment of diabetes mellitus in rats. *American Journal Physiology,* 257: 904-910.

Rasmussen, D.D., Boldt, B.M., Wilkinson, C.W., Yellon, S.M and Matsumoto, A.M. (1999). Daily melatonin administration at middle age suppresses male rat visceral fat, plasma leptin, and plasma insulin to youthful levels. *Endocrinology,* 140:1009-1012.

Rasmussen, D.D, Mitton, D.R, Larsen, S.A and Yellon, S.M, (2001). Aging-dependent changes in the effect of daily melatonin supplementation on rat metabolic and behavioral responses. *Journal of Pineal .Research,* 31:89-94.

Reiter, R.J. (2003). Melatonin a multifunctional hormone. *Journal of Pineal Research,* 34: 75.

Reiter, R.J., Calvo J.R., Karbownik., M, Qi., W and Tan, D.X. (2000). Melatonin and it's relation to the immune system and inflammation. *Annual New York Academic Science Journal,* 917: 376-386.

Reiter, R.J., Tan D.X., Sainz R.M., Mayo, J.C and Lopez-Burillo, S. (2007). Melatonin: A multifunctional molecule,*Journal of Pharmacology, 54*:1299.

Reiter, R.J., Tan, D.X and Burkhardt, S. (2004). Melatonin a versatile antioxidant: *Mechanics of Ageing Development, 123:* 1007.

Reiter, R.J., Tan, D.X and Allegra, M. (2002). Antioxidant functions of melatonin. *Neuro-Endocrinolology Letters, 23: 1-* 3.

Reiter, R.J., Tan, D.X., Manchester, L.C., Lopez-Burillo, S., Sainz, R.M and Mayo, J. C., (2003). Melatonin; A versatile antioxidant: *The Advanced Expermental Medical Biology*, 87: *527-* 539.

Reiter, R.J., Tan, D.X., Manchester, L.C., Pilar Terron, M and Flores, L.J (2007). The Hormone Melatonin; *Advanced Medical Science journal*: *52*, 11.

Reppert, S.M, Weaver D.R and Ebisawa T. (1994). Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron*, 13; 1177-1185.

Reyes-Toso, C.F., Linares, L.M., Ricci, C.R., Obaya-Naredo, D., Pinto, J.E., Rodriguez, R.R and Cardinali., D.P. (2004). Melatonin and the circadian rhythm; *Journal of Pineal Research, 39*: 386.

Richard, A. H. and Pamela,C.C. (2009). Lippincott's illustrated Reviews: Pharmacology (4thed.) Lippincott Williams and Wilkin. A Wolters Kluwer Company, Baltimore pp. 249-295.

Robertson, R., Zhou, H., Zhang, T and Harmon, J.S. (2007). Pathogenesis of diabetes mellitus.s: *Cell Biochemistry and Biophysiology Journal*, *48*: 139.

Rodrigues, B., Cam, M.C., Kong, J., Goyal, R.K., and Mc Neil, J.H. (1997). Strain differences in and susceptibility to streptozotocin-induced diabetes Effect on hypertriglyceridemia in cardiomyopathy*: Journal of Cardiovascular Research*, 34: 99-205.

Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolin, I., Herrera, F, Martin, V and Reiter, R.J. (2005). Antioxidant action of melatonin: *Journal of Pineal Research,* 36: 1.

Sadir, S., Deveci, S., Korkmaz, A and Oter, S. (2007). Pathophysiology of diabetes: *Cell Biochemical Function, 25*: 521.

Sasaki, M., Jordan, P., Joh, T., Itoh, M., Jenkins, M., Pavlick, K., Minagar, A and Alexander, S.J (2002). Hyperglycaemia and diabetes, *Gastroenterology Journal, 2*:9

Sartori, M. (2009). Melatonin improves glucose homeostasis and endothelial vascular function in high fat diet fed insulin resistant mice, *Endocrinology*, 10:25.

Scheer, F.A, Hilton, M.F., Mantzoros, C.S and Shea. S.A (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Process of National Academy of Sciences USA*, 106: 4453-4458

Shailey, S and Basir, S.F. (2011). Evaluation of *Azadirachta indica* seed extract for *invivo* antioxidant potential and protective effects against diabetes induced oxidative damage. *International Journal of Chemical and Analytical Science*, 2:9: 1202-1207.

Shukla, R., Sharma, S.B., Prabhu K.M., and Murthy P.S (2000). Medicinal plants for the treatment of Diabetes Mellitus: *Indian Journal of Clinical Biochemistry*, 15: 169-177.

Sies H. (1991). Oxidative stress: Oxidants and antioxidants: *New York Academic press*, 2: 8.

Standrig, S. (2006). Histology of the Pancreatic islets; Gray's Anatomy; The antomical Basis of clinical practice. Elsevier churchhill 39th ed; 857-559.

Stockklauser-Farber, K., Ballhausen, T., Laufer, A and Rosen, P. (2000). Potential beneficial effects of melatonin: *Biochimisty and Biophysiological Journal, 1535*: 10-11.

Szkudelski, T (2001). The mechanism of Alloxan and streptozotocin action in beta-cells of the rat pancreas. *Physiology Research Journal*, 50: 536 –546.

Tan, D.X., Manchester L.C., Hardeland, R., Lopez-Burillo S., Mayo, J.C and Sainz, R.M. (2003). N-nitrosomethylurea on the pancreatic B cells with special regard to the role of Streptozotocin and alloxan-induced damage in rat β cells: *Diabetológia Journal*, 27: 587-591.

Tanveer, A., Mustafa, R., Anwar, Z., (1998). Control of hydatidosis in rabbits through feeding local plants. Punjab *University Journal of Zoology* 13, 99–113 (abstract).

Tesfamariam, B and DeFelice, A. F.(2007). Melatonin effect on the vasculature: *Vascular Pharmacology Journal, 46*: 229.

Topal, T., Oztas, Y., Korkmaz, A., Sadir, S., Oter, S., Coskun, O and Bilgic, H (2005). Oxidixing properties of melatonin: *Journal of Pineal Research*, 38: 272.

Ucar, M., Korkmaz, A., Reiter, R.J., Yaren, H., Oter, S.; Kurt, B and Topal, T. (2007). *Toxicology Letter, 173*: 124.

Wang, H., Wei, W., Shen, Y.X., Dong, C., Zhang, L.L., Wang, N.P., Yue, L and Xu, S.Y. (2004). Effects of diabetes. *World Journal of Gastroenterology, 10*: 2690.

Wardle, E.N. (1999). Diabetes and its complications. *Nephrology Diaysis Transplantant Journal, 14*: 1336.

Weidig, P., McMaster, D and Bayraktutan, U. (2004): High glucose mediates prooxidant and antioxidant enzyme activities in coronary endothelial cells. *Diabetes of Obesity Metabolism*, 6: 432-441.

Weiss, R., Dziura, J., Burgurt, T.S., Tamborlane, W.V., Taksali, S.E., Yeckel. C.W., Allen, K., Lopes, M., Savoye, M., Morrison, J., Sherwin, R.S and Caprios, S. (2004): Obesity and the metabolic syndrome in children and adolescents. *New England Journal of Medicine*, 350: 2362-2374.

Wild, S., Roglic, G., Green, A., Sicree, R and King, H. (2004). Global prevalence of diabetes, Estimates for the year 2000 and projections for 2030. *Diabetes Care*, 27: 1047-1053.

Wilson, G.L., Patton, N.J., McCord, J.M., Mullins, D.W and Mossman, B.T. (1984). Mechanisms of streptozotocin: *Cancer Chemotherapy Research Journal*, 29: 91-98.

Winiarska, K., Fraczyk, T., Malinska, D., Drozak, J and Bryla, J. (2006). Melatonin. *Journal of Pineal Reseach, 40*: 168.

Wolfgang, K., (2003). Histology of the pancreas: Color Atlas of Cytology, Histology, and Microscopic Anatomy: 4TH ed, 268-269.

Vijayanand, S and Wesley, E.G. (2011).Evaluation of antidiabetic activity of *Melia Azadirachta indica* on alloxan induced diabetic rats. *International Journal of Current Pharmaceutical Research*, 3; 4: 37-4.

Vivian E.M (2006). Type 2 diabetes in children and adolescents, the next epidemic. *Current Medical Research Journal*, 22; 2: 297-306.

Yerneni K.K., Bai, W., Khan, B.V., Medford, R.M., Natarajan, R (1999). Vascular endothelium and hyperglycaemia related disorders. *Journal of Diabetes*, 48: 855.