

Chapter One

Introduction

1.1 General Introduction

Diabetes mellitus is a heterogeneous group of metabolic disorders characterised by glucose intolerance and fasting hyperglycemia (Kantarova *et al*, 2006). Type 1 diabetes is immune-mediated and accounts for about 10% of all diabetes cases, affecting approximately 20 million people worldwide (Libman *et al*, 1993). Type 2 insulin-resistant diabetes accounts for about 90% of all diabetes. It afflicts an estimated 6% of the adult population, and its worldwide prevalence is expected to grow by 6% per annum, possibly reaching a total 200-300 million cases in 2010 (Amos *et al*, 1997).

Diabetes is associated with disabling and life-threatening complications such as retinopathy, nephropathy, neuropathy, hepatopathy and coronary artery disease, etc; and chronic hyperglycemia and oxidative stress are implicated in the pathogenesis of these complications (Baynes, 1991). Besides, impaired insulin levels/action in diabetes predisposes to dyslipidemia and increased risk of atherosclerosis (Rossetti and Goldberg, 2002).

Since the classic work of Banting and Best (1922) on insulin, myriad of findings had accumulated over the years. Currently, botanicals are being screened for their efficacy and safety in the management of diabetes and its complications. In this regard, there is laboratory-based evidence that the fruit juice of *Momordica charantia* reverses hyperglycemia in diabetic rats by decreasing gluconeogenesis and increasing insulin secretion (Shibib *et al*, 1993). Additional potential herbal sources of new chemical entities for the management of diabetes mellitus include *Coccinia indica* (Kimble *et al*, 1986), *Gymnema sylvestre* (Baskaran *et al*, 1990) and *Panax quinquefolius* (ginseng) (Sievenpiper *et al*, 2004). Others include *Annona muricata* (Adewole and Caxton-Martins, 2006), *Hypoxis hemerocallidea* (Ojewole, 2006), *Vernonia amygdalina* (Ebong *et al*, 2008), *Berberis lyceum* (Gulfraz *et al*, 2007) and *Aloe vera* (Noor *et al*, 2008).

Furthermore, multiherbal formulas have been reported to possess antidiabetic activity in animal studies and patients. The work of Yamabe and Yokozawa (2007) showed that the polyherbal drug *Hachimi-jio-gan* lowered blood glucose and protects against the development of pancreatic fibrosis and oxidative damage in Otsuka Long-Evans Tokushima fatty rats. Similarly, pancreatic tonic® (a mixture of ten antidiabetic herbs) shows antidiabetic activity in type 2 diabetic patients. Furthermore, the report of Kim *et al* (2007) showed that *Dangnyosoko*, a Chinese herbal medicine, has beneficial effects on glycemia and urine glucose, as well as on islet histology in diabetic rats. Moreover, Razdan *et al* (2008) studied the antidiabetic effect of *Diasansar*® (a polyherbal formulation containing *Gymnema sylvestre*, *Withania somnifera*, and *Azadirachta indica*, etc) on streptozotocin- and fructose-induced type 2 diabetes mellitus. Their findings showed that herbs are promising antidiabetic therapies.

Besides, the work of Murty *et al* (1978), Chattopadhyay (1996, 1999), Khosla *et al* (2000), Hsia *et al* (2004) and Chattopadhyay and Bandyopadhyay (2005) showed that the leaf extract of *Azadirachta indica* (*A. indica*) possesses hypoglycemic activity in experimental animals and human subjects. However, other than this effect on blood glucose, the effects of *A. indica* leaf extract on the microscopic anatomy of certain vital organs such as the kidney, pancreas and liver, which are frequently involved in diabetic complications, are yet to be reported.

This work was therefore designed to study the antidiabetic activity of the leaf extract of *A. indica* on a broader perspective: microanatomy of the pancreas, kidney, liver and jejunum; markers of oxidative stress in the pancreas and liver; markers of hepatic and renal function; and analysis of plasma lipids and insulin, etc.

1.2 Objectives of the Study

Azadirachta indica is currently being included as an active component of certain antidiabetic therapies. Such botanical formulations include Cogent db® (Shekhar *et al*, 2002) and

Pancreatic tonic[®] (Hsia *et al*, 2004), which were subjected to randomised clinical trials in Malaysia and USA, respectively. A couple of workers (Murty *et al*, 1978; Khosla *et al*, 2000; Chattopadhyay 1999; Rahman *et al*, 2005; Ebong *et al*, 2008) had reported the hypoglycemic effect of neem.

In diabetes, morphological changes are associated with organs such as the pancreas, liver, kidney and intestine. Such changes include loss of β cells and fibrosis of pancreatic islets (Ko *et al*, 2004), glomerulosclerosis and tubular lesions of the kidney (Heptinstall, 1991), accumulation of glycogen and fat in the liver (Torbensohn *et al*, 2006), and occasional lesions of the intestine (De Las Casas and Finley, 1999). However, no reports are available on the effects of the leaf extract of neem on the microscopic anatomy of these organs in diabetic rats, as well as on the oxidative status of the pancreas and liver. Furthermore, the effect of a chronic regimen of neem on diabetic dyslipidemia has not been reported. Thus, the objectives of this work are to:

- i. Study the effects of *A. indica* treatment on the microscopic anatomy of the pancreas, liver, kidney and jejunum of streptozotocin-induced diabetic rats;
- ii. Study oxidative stress biomarkers in the pancreas and liver of diabetic rats submitted to neem-leaf therapy. Plasma markers of hepatic and renal function will also be assessed;
- iii. Investigate the effect of neem treatment on plasma insulin levels; and
- iv. Study the effects of neem-leaf extract on diabetic dyslipidaemia.

The studies thus aimed at investigating the antidiabetic activity of the leaves of *A. indica* on a broader perspective. Such histopathological studies had been invaluable with respect to herbs such as *Aristolochia spp* and its reported nephrotoxicity (Ioset *et al*, 2003); *Mentha pulegium*

and its hepatotoxicity and nephrotoxicity (Anderson *et al*, 1996); and *Teuchrium chamaedrys* and its associated hepatic toxicity (Ben-Yahia *et al*, 1993).

1.3 Rationale for the Work

This study was predicated on the following:

- i. The traditional use (Abo *et al*, 2008) and reported hypoglycemic effect of *A. indica* (Chattopadhyay, 1999) require further studies;
- ii. No reports are currently available on the effect of neem-leaf extract on the micro-anatomy of certain vital organs that are frequently involved in diabetes complications (kidney, liver and pancreas); and on the epithelium of the jejunum following exposure of the intestinal mucosa of diabetic rats to neem leaf extract.
- iii. Insulin plays crucial roles in glucose homeostasis. Presently, no reports are available on the effect of *A. indica* leaf extract on plasma insulin levels of streptozotocin-induced diabetic rats.
- iv. Owing to the role of oxidative stress in the pathogenesis of diabetic complications (Baynes, 1991), the effects of *A. indica* treatment on diabetes-associated pancreatic and hepatic oxidative stress require investigation.
- v. Because dyslipidemia is associated with diabetes and its associated cardiovascular events (e.g. coronary artery disease), studies of the effects of acute and chronic *A. indica* treatment on diabetic dyslipidemia is pertinent.

1.4 Scope of the Work

This work was limited to (i) histological studies of the pancreas, kidney, liver, and intestine, using special histological stains; (ii) biochemical analysis of blood glucose, and plasma insulin, lipids, urea, creatinine and liver enzymes; and (iii) the use of oxidative stress

biomarkers to estimate the levels of reactive oxygen species and certain antioxidants in the pancreas and liver of diabetic rats treated with *A. indica*-leaf extract.

Chapter Two

Literature Review

2.1 Diabetes Mellitus

2.1.1 Definition and Description of Diabetes Mellitus

“Diabetes mellitus describes a metabolic disorder of multiple etiologies. It is characterised by chronic hyperglycemia, with perturbations of carbohydrate, lipid and protein metabolism that result from defects in insulin secretion, insulin action or both” (WHO, 1999). Chronic hyperglycemia leads to long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves and the cardiovascular system (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). At least three different factors are involved in the pathogenesis of diabetes mellitus (Lehmann and Spinas, 2000). These include:

1. Genetic predisposition, e.g. mutation of the glucokinase gene; mutation of different transcription factors; and mutation of the insulin receptor.
2. Insulin resistance (in muscle, liver, adipose tissue).
3. Defective insulin secretion by the pancreatic β cells; and autoimmune reactions.

Symptoms of diabetes mellitus include hyperglycemia, polyuria, polydipsia, weight loss, hyperphagia and sometimes blurred vision (WHO, 1999). Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia (WHO, 1999). Acute and life-threatening consequences of diabetes are hyperglycemia with ketoacidosis or the non-ketotic hyperosmolar syndrome. Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy, leading to renal failure; peripheral neuropathy with risk of foot ulcer, limb amputation and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms, and sexual dysfunction (Shamoon *et al*, 1993). Glycation of tissue proteins and other

macromolecules and excess production of polyol compounds from glucose are among the mechanisms that produce tissue damage from chronic hyperglycemia (Baynes, 1991). Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral vascular and cerebrovascular disease. Hypertension, abnormalities of lipoprotein metabolism and periodontal disease are often found in diabetic patients (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

2.1.2 Diagnosis of Diabetes Mellitus

There are different methods of diagnosing diabetes mellitus in humans (Mayfield, 1998):

1. Casual plasma glucose levels ≥ 200 mg/dl and clinical symptoms of diabetes mellitus
2. Fasting plasma glucose (FPG) (no caloric intake for at least 8 hours) ≥ 126 mg/dl
3. Plasma glucose two hours after an oral glucose load (75 g anhydrous glucose dissolved in water) ≥ 200 mg/dl

According to the American Diabetes Association (ADA) (1998), measurement of fasting plasma glucose (FPG) levels is sufficient for the diagnosis of diabetes mellitus (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). The World Health Organization (WHO) rather suggests performing an oral glucose tolerance test (OGTT), using 75 g glucose per person. Only if an OGTT cannot be performed, FPG levels should be used for diagnosis (Alberti and Zimmet, 1998). Epidemiological studies have shown that unlike FPG, impaired glucose tolerance (IGT) is a predictor of the risk of developing cardiovascular disease (Tominaga *et al*, 1999). The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997) recognizes an intermediate group of subjects whose glucose levels, although not meeting criteria for diabetes, are nevertheless too high to be considered normal. Diagnostic criteria for non-diabetic subjects, the intermediate group, and criteria for diagnosing diabetes mellitus are listed in table 1 (below).

Table 1: Criteria for diagnosing diabetes mellitus: fasting plasma glucose (FPG) values and two-hour post-load plasma glucose (PG) values after oral glucose challenge (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997)

Diagnosis	Criteria
Non-diabetic	FPG < 110 mg/dl 2-hour post-load PG values < 140 mg/dl
Impaired fasting glucose (IFG)	FPG $\geq$ 110 and < 126 mg/dl
Impaired glucose tolerance (IGT)	2-hour post-load PG values $\geq$ 140 mg/dl and < 200 mg/dl
Diabetes mellitus	FPG $\geq$ 126 mg/dl 2-hour post-load PG values $\geq$ 200 mg/dl

Since hyperglycemia can be transitory e.g., due to infections, cardiovascular episodes, trauma and other stress factors, the diagnosis should be confirmed on a different day, by one of the three methods (see above).

2.1.3 Classification of Diabetes Mellitus

Aetiology is the main criterion for the new classification of human diabetes mellitus, according to the ADA (1998) and WHO (1999). Therefore, terms such as insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) are being replaced. The same is true for the terms juvenile and adult diabetes, because 50% of type 1 diabetic patients are diagnosed when they are over 20 years of age and the incidence is equal for every decade after the 20th year of life. According to the ADA (1998) and WHO (1999), four main types of diabetes mellitus can be distinguished (table 2).

Table 2: Aetiologic Classification of Diabetes Mellitus according to the ADA (1998) and WHO (1999)

Type 1 Diabetes Mellitus

B-cell destruction, usually leading to absolute insulin deficiency

- It could be immune-mediated; or
- Idiopathic (rare, no indices for autoimmune disease)

Type 2 Diabetes Mellitus

Ranges from predominantly insulin resistance with relative insulin deficiency, to a predominantly insulin secretory defect with insulin resistance.

Gestational Diabetes Mellitus

Women who develop type 1 diabetes mellitus during pregnancy and women with undiagnosed asymptomatic type 2 diabetes mellitus that is discovered during pregnancy are classified under gestational diabetes mellitus. Most women with gestational diabetes mellitus develop relative insulin deficiency during the second half of pregnancy, leading to hyperglycemia. After delivery, hyperglycemia resolves in most women but places them with increased risk of developing type 2 diabetes mellitus later in life (Mayfield, 1998).

Other Specific Types of Diabetes

- Genetic defects of β -cell function; maturity onset diabetes of the young (MODY):

MODY 1: defective HNF-4 α (hepatocyte nuclear factor -4 α), chromosome 20

MODY 2: defective glucokinase, chromosome 7

MODY 3: defective HNF-1 α , chromosome 12

MODY 4: defect of the insulin promoter factor-1 (IPF-1)

MODY 5: defective HNF-1 β , mitochondrial diabetes and others

- Genetic defects in insulin action (type A insulin resistance, Rabson-Mendenhall syndrome: defective insulin receptor, lipotrophic diabetes and others)
- Diseases of the exocrine pancreas (pancreatitis, pancreatic trauma/pancreatectomy, cystic fibrosis, neoplasia, hemochromatosis, fibrocalculous pancreatopathy and others)
- Endocrine disorders (acromegaly, Cushing's syndrome, Conn's syndrome, pheochromocytoma, hyperthyroidism and others)
- Chemically-induced (steroids, pentamidine, diazoxide, thiazides, nicotinic acid and others)
- Infections (congenital rubella, measles, Coxsackie viruses, cytomegaly virus)
- Rare forms of immunogenic diabetes (Stiff-Man Syndrome, anti-insulin receptor antibodies and others)
- Other genetic syndromes associated with diabetes mellitus (Down syndrome, Klinefelter's syndrome, Turner's syndrome, Wolfram's syndrome, myotonic dystrophy and others)

2.1.4 Screening for Diabetes Mellitus

For screening and diagnosis of diabetes mellitus, FPG and 2-hour post-load PG values after oral glucose challenge are most suitable. Due to non-reproducible values in healthy subjects and a high range of variation, despite identical blood glucose values, glycated haemoglobin (HbA_{1C}) is unsuitable for the screening of diabetes mellitus (Simon *et al*, 1999). However, HbA_{1C} levels above 6.2% have been shown to correlate with the likelihood of having or developing macrovascular or microvascular disease. Measuring this parameter has therefore been recommended for monitoring the treatment of diabetes mellitus and glycemia (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

2.1.4.1 Screening for Type 1 Diabetes Mellitus

At the moment, a general screening for type 1 diabetes seems to be unsuitable (Ziegler and Scherbaum, 1998). Screening for auto-antibodies in healthy subjects, especially in relatives of

type 1 diabetics, is not recommended because to date, there are no effective intervention strategies for the prevention of type 1 diabetes (Mayfield, 1998).

2.1.4.2 Screening for Type 2 Diabetes Mellitus

Determination of FPG should be performed from 45 years of age onwards, and if normal, it is recommended to be repeated in three-year intervals (Mayfield, 1998). Screening for diabetes in younger subjects is advisable if one or more of the following criteria are true (Mayfield, 1998):

- Obesity (body mass index = body weight (kg) / height (m²) ≥ 27 kg/m²).
- First-degree relative with diabetes mellitus
- Member of high risk-ethnic group (Black, Asians, Hispanic, Native American and others)
- History of gestational diabetes mellitus or delivering a baby weighing more than 4 kg
- Hypertension (140/90 mm Hg)
- HDL-cholesterol < 0.9 mmol/l and/or triglyceride level ≥ 2.8 mmol/l
- History of IGT or IFG on prior testing (FPG ≥ 110 and < 200 mg/dl)

2.1.4.3 Screening for Diabetes-Associated Organ Lesions

Initial screening for diabetes-associated organ lesions in type 1 diabetic patients should be carried out about five years after diagnosis. In contrast to type 1 diabetes, onset of type 2 diabetes is not acute and cannot be clearly defined. Since 30-50% of type 2 diabetics, even in Western countries, are not diagnosed and taking into account that 50% of newly diagnosed diabetics already exhibit secondary organ lesions, a regular screening for type 2 diabetes is recommended. In case of diagnosis, cardiovascular risk factors and secondary organ lesions should be surveyed according to the criteria in table 3 below (Diabetes Control and Complications Trial Research Group, 1993).

Table 3: Screening for Organ Lesions in Types 1 and 2 Diabetes Mellitus (Diabetes Control and Complications Trial Research Group, 1993)

Complication	Screening
Retinopathy	Yearly ophthalmologic examination
Nephropathy	Determination of urinary albumin excretion [night urine < 20 µg/min; 24-hour urine < 30 mg/24h; albumin:creatinine ratio < 2.5 (male)/< 3.5 (female)]
Peripheral neuropathy	Yearly examination for the presence of peripheral sensorimotor neuropathy, testing of nerve conduction in at least two peripheral nerves and autonomic nerve testing
Cardiovascular risk factors	Measurement of blood pressure and analysis of plasma lipids. (In type 1 diabetics, disturbances in plasma lipid profile and hypertension only occur in coincidence with poor diabetes control and overt diabetic nephropathy, respectively)

2.1.5 Animal Models of Diabetes Mellitus

Animal models have been used intensively in order to clarify the pathogenesis of diabetes mellitus, as well as prevent its development, progression and complications.

2.1.5.1 Animal Models of Type 1 Diabetes Mellitus

Bio-Breeding (BB) Rats

The spontaneously diabetic rat syndrome was recognized in 1974 at the Bio-Breeding Laboratories, Canada. The inheritance pattern of susceptibility is that of an autosomal

recessive gene; however, only 50% of the susceptible animals develop diabetes (Janle-Swain, 1985). Diabetes onset is mainly between 60 and 120 days of age. An abrupt onset with glucosuria, hyperglycemia, ketonuria, hypoinsulinemia and death from ketoacidosis within one or two weeks is typically found in younger animals, whereas in older animals, diabetes development is slower and less dramatic (Smith and Hammond, 1991). Besides classic diabetic symptoms, rapid weight loss is a good predictor of diabetes development (Buschard, 1996). A variety of hormonal and metabolic abnormalities have also been found in this strain. Glucagon and somatostatin are elevated in diabetic animals; growth hormone secretion is initially normal but decreases with metabolic decompensation (Janle-Swain, 1985). Elevation of free fatty acids and branched chain amino acid levels are proportional to the severity of diabetes. There is an increased hepatic gluconeogenesis and decreased glucose utilization comparable to human type 1 diabetes (Bender and Mayes, 2006). The disease mechanisms have been intensively investigated. As in human type 1 diabetes, insulinitis is seen prior to clinical diabetes and in some BB rats, which do not develop diabetes mellitus. The infiltrate consists of CD4⁺ and CD8⁺ lymphocytes and NK cells. Macrophages are believed to be the first cells invading the islets. The BB rat shows lymphocytopenia, which can be recognized before clinical onset of diabetes. NK cells have been suggested to be responsible for the destruction of β cells, due to their ability to lyse islet cells *in vitro* (Janle-Swain, 1985). However, it now seems proven that the small number of CD4⁺ cells is ultimately responsible (Smith and Hammond, 1991). The BB rat displays islet cell antibodies, which can be detected up to two weeks before the onset of diabetes. Furthermore, antibodies against lymphocytes as well as against smooth muscles and gastric parietal cell antigens have been reported (Buschard, 1996).

The Non-Obese Diabetic Mouse (NOD Mouse)

The NOD mouse strain was established in 1974 in Osaka, Japan (Buschard, 1996). In this strain, up to 80-90% of females develop diabetes between 80 and 200 days of age. The incidence in male mice is between 10-50%, but there is a substantial variation in different colonies (Buschard, 1996). Gonadal sex hormones are important modulators of diabetes development in NOD mice. Castrated males show higher incidence and oophorectomized females a lower incidence of diabetes (Buschard, 1996). The appearance of diabetes is followed by classic symptoms, such as hyperglycemia, hypoinsulinemia, glucosuria, ketonuria, polydipsia and polyuria (WHO, 1999). In contrast to BB rats, NOD mice can survive up to several months after the onset of diabetes. NOD mice display islet cell and islet cell surface antibodies with a peak after the age of six months and an incidence of up to 80%. Insulin autoantibodies are present in up to 80% of the mice after three months. Up to 95% of the mice show insulinitis that may be evident at 5-6 weeks of age (Anderson and Bluestone, 2005). The earliest changes are peri-insulinitis followed by invasion of the islet. In its final form, insulinitis in NOD mice is typically present in one part of the islet, which is heavily infiltrated, whereas the other part is completely normal. The mononuclear infiltrate looks more like a leukaemia infiltrate than an inflammatory process (Anderson and Bluestone, 2005). B and T-cells, as well as NK cells and activated lymphocytes are seen in insulinitis, the first infiltrating cells being monocytes. The odd-looking insulinitis could be a major disadvantage in this otherwise convenient model (Buschard, 1996).

Virus-Induced Diabetes

Environmental factors are hypothesised to play a pathogenetic role in the development of diabetes mellitus, and in this context, virus is a likely candidate (Forrest *et al*, 1969). For experimental models, encephalomyocarditis (EMC) virus is the most widely used. The result

of the virus infection is dependent on the virus strains (diabetogenic, non-diabetogenic) and is also highly dependent on the mouse strain used (Horwitz *et al*, 1998). EMC virus is a picornavirus possessing many of the biologic and pathogenetic properties of the group B Coxsackie viruses. Infection of genetically susceptible strains of mice leads to the development of lesions of the islets of Langerhans, accompanied by diabetes mellitus (Horwitz *et al*, 1998). The occurrence of diabetes in this model is affected by complex interrelated genetic, immunologic and environmental influences. After subcutaneous inoculation, EMC virus multiplies in the islets of Langerhans and causes lesions of the β cells. During early stages of infection, β cells degranulate and insulin content of the islet decreases markedly with hyperglycemia appearing concomitantly (Buschard, 1996). A mononuclear cell infiltrate appears transiently around the islet with the persistence of the infection. During convalescence (two to three weeks after inoculation), three patterns of metabolic disease can be observed. Some animals display profound hyperglycemia and ketoacidosis (Craighead, 1981). These mice lose weight and die several months after inoculation. Histologically, islets are shrunken, distorted and lack detectable amounts of insulin. In other animals, hyperglycemia is less marked for an extended period of time. After several months, animals can recover, apparently due to the regeneration of insular tissue. Finally, a number of animals exhibit normal fasting blood glucose concentrations and disturbed oral glucose tolerance (Craighead, 1981).

Four other human viruses are known to cause lesions of pancreatic islets in experimental animals. Venezuelan encephalitis virus damages islets of hamsters and nonhuman primates, resulting in carbohydrate intolerance. Furthermore, rubella virus infection, reovirus and cytomegalovirus can cause damage of pancreatic islets and β cells (Horwitz *et al*, 1998).

Alloxan and Streptozotocin-Induced Type 1 Diabetes

Since the initial findings in 1943 of alloxan (ALX)-induced beta cell necrosis in rabbits by Dunn *et al*, this compound has long been used for inducing experimental diabetes. Alloxan is a uric acid derivative and is highly unstable in water at neutral pH, but reasonably stable at pH 3 (Bailey, 2005). ALX acts by selectively destroying the pancreatic beta islets leading to insulin deficiency, hyperglycaemia and ketosis (Rerup, 1970). ALX causes diabetes in many rodent and non rodent animals and is most preferably used in case of rabbit because of the relative ineffectiveness of streptozotocin (STZ) in rabbits for induction of diabetes and development of well characterized diabetic complications (Rerup, 1970). However, guinea pigs and, recently, musk shrew have been reported to be resistant to the action of ALX due to certain unclear mechanisms (Ohno *et al*, 1998). Because of its low stability, relatively very shorter half-life (less than 1 min) and acidic nature of solution, intravenous route of administration of ALX is preferred. The hypoglycaemic phase may be quite severe and therefore ALX should not be given to fasted animals (Srinivasan and Ramarao, 2007). The ALX-treated animals exhibit severe hyperglycaemia, glucosuria, hyperlipidaemia, polyphagia, polydipsia and other symptoms of uncontrolled diabetes; and do also develop various complications such as neuropathy, cardiomyopathy, well marked retinopathy and others (Battell *et al*, 1999). ALX is disadvantageous as the percentage incidence of diabetes is quite variable and is not proportionately related to increasing doses of ALX (Battell *et al*, 1999). Further, the incidence of ketosis and resulting mortality is high. The reversal of hyperglycaemia due to pancreatic regeneration is early and common in case of ALX-treated animals. Because of these limitations, ALX is now almost replaced by STZ for induction of diabetes in laboratory animals.

Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and structurally is a glucosamine derivative of nitrosourea. Rakieten *et al* (1963) first demonstrated the

diabetogenic property of STZ in dogs and rats. Like ALX, it causes hyperglycaemia mainly by its direct cytotoxic action on the pancreatic beta cells (Ozturk *et al*, 1996).

There are wide variety of reports on doses and development of hyperglycaemia with STZ since the susceptibility of animals to STZ appear to depend on age, species and even strain (Verspohl, 2002). STZ is a preferred agent to induce experimental diabetes since it has some advantages over ALX such as relatively longer half-life (15 min.), sustained hyperglycaemia for longer duration and the development of well characterized diabetic complications with fewer incidences of ketosis and mortality (Ozturk *et al*, 1996). ALX and STZ diabetic animals are most widely used for screening compounds, including natural products, for their insulinomimetic, insulinotropic and other hypoglycaemic activities (Reed and Scribner, 1999).

2.1.5.2 Animal Models of Type 2 Diabetes Mellitus

***ob/ob* Mouse**

ob/ob mouse (obese mouse) is inherited as (monogenic) autosomal recessive mutation on chromosome 6 (obese) in C57BL/6J mouse strain (Shafrir, 2003). The mutation in *ob/ob* mice is now identified in leptin gene, which encodes for leptin; homozygous mutant mice exhibit rapid gain in body weight and may reach the three times of the normal weight of wild type control (Shafrir, 2003). Besides, there is marked hyperphagia and decreased energy expenditure resulting in increased carcass lipid with obvious obesity by approximately 4 weeks. In addition to obesity, *ob/ob* mouse exhibits hyperglycaemia, mild impaired glucose tolerance, severe hyperinsulinaemia, sub-fertility and impaired wound healing (Srinivasan and Ramarao, 2007). Hyperglycaemia is only transient on the C57BL/6J background. However, when *ob* gene is expressed on C57BL/KS background, mice become severely diabetic with regression of islets and early death (Bell and Hye, 1983). Since this model is severely obese, hyperinsulinaemic and insulin resistant throughout its life, the agents that

decrease the body weight and improves peripheral insulin sensitivity like insulin sensitizers, antiobesity and some other antihyperglycaemic agents have been largely tested and shown to produce antidiabetic activity in this model (Zhang *et al*, 1999).

***db/db* Mouse**

The *db/db* (diabetic) mouse is originally derived from an autosomal recessive mutation on chromosome 4 in mice of C57BL/KsJ strain originating from Bar Harbor, Maine (Shafir, 2003). The mutation in this diabetic animal was traced to *db* gene, which encodes for the leptin receptors. These mice are spontaneously hyperphagic insulin over-secretors, becoming obese, hyperglycaemic, hyperinsulinaemic and insulin resistant within the first month of age and develop hypoinsulinaemia, hyperglycaemia later, with a peak between 3-4 months of age (McNeil, 1999). Animals then exhibit ketosis, progressive body weight loss and do not survive longer than 8-10 months. Unlike *ob/ob* mice, exogenous administration of leptin fails to elicit effect on food intake and body weight as there is defect in leptin receptor9 (McNeil, 1999). *db/db* mice have been commonly and extensively used for the investigation of type 2 diabetes/diabetic dyslipidaemia and screening variety of agents such as insulin mimetic and insulin sensitizers (Zhang *et al*, 1999).

KK Mouse

KK (Kuo Kondo) mouse is a polygenic model of obesity and type 2 diabetes produced by selective inbreeding for the large body size (Velasquez *et al*, 1990). These animals are hyperphagic, hyperinsulinaemic, insulin resistant and show moderate obesity by 2 months of age (Velasquez *et al*, 1990). Insulin resistance precedes the onset of obesity. The increase in pancreatic insulin content is associated with increase in number and size of pancreatic islets, and histologically, hypertrophy of islets is found (Reddi and Camerini-Davalos, 1988). Besides, there is selective failure of insulin to suppress gluconeogenic pathway, while

exerting its inductive effect on glycolysis and lipogenesis as seen in hepatic insulin resistance of *db/db* mouse (Reddi and Camerini-Davalos, 1988).

KK/Ay Mouse

KK/Ay mouse (Yellow KK obese mouse) carries both lethal yellow obese (Ay) and diabetic gene, unlike KK mouse, where it carries only diabetic gene (Ramarao and Kaul, 1999). Mice homozygous for the yellow spontaneous mutation (Ay) die before implantation or shortly thereafter. KK/Ay mouse is heterozygous, and it shows severe obesity, hyperglycaemia, hyperinsulinaemia and glucose intolerance by 8 weeks of age. The strain KK/Ay mice serve as a good model for obesity and type 2 diabetes and for screening various classes of antidiabetic agents (Ramarao and Kaul, 1999)

New Zealand Obese (NZO) Mouse

The NZO strain is a polygenic model of obesity and diabetes obtained by selective inbreeding over several generation with the parents selected for their agouti coat colour (McNeil, 1999). It exhibits a polygenic syndrome of hyperphagia, obesity, mild hyperglycaemia, hyperinsulinaemia, impaired glucose tolerance and insulin resistance. Body weight rises rapidly during the first 2 months of life, due to hyperphagia (McNeil, 1999). Leptin resistance, which may account for hyperphagia, has been reported in NZO mice (McNeil, 1999). Hyperglycaemia and impaired glucose tolerance increase continuously with advancing age of animals. There is a marked hyperplasia and hypertrophy with islets composed of up to 90% of beta cells (Bell and Hye, 1983). Reduced glycogen synthase activity in liver has been considered as a primary early defect in the causation of diabetes (Thorburn *et al*, 1995). These mice have high prevalence of autoimmune disorder and become a useful model for studying relationship between autoimmunity, obesity and diabetes (Vogel and Vogel, 1997).

NONcNZO10 Mouse

This mouse is a recombinant congenic new mouse strain model of type 2 diabetes developed by introgressing 5 genomic intervals containing NZO/H1Lt (NZO) diabetogenic quantitative trait loci onto the non obese non diabetic (NON/Lt or NON) genetic background (Haskell *et al*, 2002). Whereas parental NZO males exhibit the unwanted phenotypes of hyperphagia, morbid obesity, poor fertility, and a variable frequency of hyperglycaemia, NONcNZO10 males are not hyperphagic, develop more moderate level of obesity, and reproduce normally (Haskell *et al*, 2002). They weigh more than NON males, but significantly less than the NZO males. Despite the reduced rate of weight gain compared to NZO, all NONcNZO10 males develop chronic hyperglycaemia by 12-20 weeks (Pan *et al*, 2005). Pancreatic islets show the similar atrophic changes as seen in diabetic NZO males. Increased serum triglycerides and liver steatosis are also observed. Unlike *ob/ob* and *db/db* mice, which are monogenic and exhibit morbid obesity, these mice are polygenic, not hyperphagic, have a normal leptin/leptin receptor axis, and do not exhibit hypercorticism (Haskell *et al*, 2002). Unlike parental NZO mouse, this model has recently gained attention for the investigation of diabetes as well as in drug testing for the treatment of type 2 diabetes (Pan *et al*, Reifsnnyder, 2005).

Tsumara Suzuki Obese Diabetes (TSOD) Mouse

By selective breeding of obese male mice of ddY strain, Suzuki and Tsumara described the two inbred strains, one with obesity and increased urinary glucose named TSOD (Tsumara Suzuki Obese Diabetes) and the other without these traits (TSNO, Tsumara Suzuki Non Obese) (Suzuki *et al*, 1999). TSOD mouse is of polygenic origin and characterized by polydipsia and polyuria at about 2 months of age (only in male mice), followed by hyperglycaemia and hyperinsulinaemia (Miura *et al*, 2001). Following these symptoms,

obesity gradually develops until about 12 months. Pancreatic islets of TSOD male mice are hypertrophic, but do not develop insulinitis or fibrosis (Suzuki *et al*, 1999). It has been shown that the reduced insulin sensitivity in diabetic TSOD mice is due, at least in part, to the impaired glucose transporter (GLUT4) translocation by insulin in both skeletal muscle and adipocytes (Miura *et al*, 2001).

M16 Mouse

M16 mouse is a new model for obesity and type 2 diabetes. These mice exhibit early onset of obesity and are larger at all ages (relative to institute of cancer research, ICR, mice), with increased body fat percentage, fat cell size, fat cell numbers, and organ weights (Allan *et al*, 2004). Besides, these mice exhibit hyperphagia, accompanied by moderate obesity; and are hyperinsulinaemic, hyperleptinaemic and hypercholesterolaemic. Both M16 males and females are hyperglycaemic, with 56% and 22% higher fasted blood glucose levels at 8 weeks of age (relative to ICR) (Srinivasan and Ramarao, 2007). M16 mice represent an outbred animal model to facilitate gene discovery and pathway regulation controlling early onset polygenic obesity and type 2 diabetic phenotypes. Phenotypes prevalent in the M16 model, with obesity and diabetes exhibited at a young age, closely mirror current trends in human populations (Allan *et al*, 2004).

Zucker Fatty Rat

The spontaneous mutation 'obese' (fatty) rat was found in the rat stock of Sherman and Merck, by Zucker and Zucker (1961). The Zucker (*fa/fa*) fatty or obese rat results from the simple autosomal recessive (*fa*) gene on chromosome 5. It is characterized by hyperphagia and early onset of obesity that appear at 4 weeks of age, along with increased growth of subcutaneous fat depot. It is also associated with mild hyperglycaemia, insulin resistance, mild glucose intolerance, hyperlipidaemia, hyperinsulinaemia and moderate hypertension

(Durham and Truett, 2006). The hyperphagia seen in this rat has been attributed to hypothalamic defect in leptin receptor signalling (McNeil, 1999). This model has been mostly used for screening the effects of different insulin sensitizing and antiobesity agents, and in few cases, for testing the potentiators of insulin secretion or incretin mimetic agents (Ramaraio and Kaul, 1999).

Zucker Diabetic Fatty Rat

It is a substrain of Zucker fatty rat (ZFR) selectively inbred for hyperglycaemia; and it is highly useful in the investigation of mechanism of type 2 diabetes (Shafrir, 1992). Unlike ZFR, male Zucker diabetic fatty (ZDF) rat progresses to frank diabetes due to failure to compensate adequately for insulin resistance. It is less obese but more insulin resistant than ZFR (Shafrir, 1992). Males are more prone to the development of diabetes usually at 7-10 weeks after birth. However, female littermates are obese, insulin resistant but do not develop diabetes. In contrast to *fa/fa* rats, the ability to over-secrete insulin to compensate peripheral insulin resistance is limited, and the β cells are brittle, and easily succumb to over secretion pressure (Shafrir, 1992). This ZDF rat has been most commonly used for investigating the mechanisms associated with insulin resistance and beta cell dysfunction in type 2 diabetes, as well as for testing insulin sensitizers, insulintropics and various other agents (Himms-Hagen and Danforth, 1996).

SHR/N- cp Rat

SHR/N-*cp* rat (spontaneously hypertensive rat/NIH-corpulent) is derived by inbreeding of SHR/N strains, a genetic model of obesity and type 2 diabetes with hypertension (Velasque *et al*, 2001). Male SHR/N-*cp* rats, if homozygous for corpulent gene (*cp*), exhibit hyperphagia and early onset of obesity, normal or slight hyperglycaemia, dyslipidaemia, profound hyperinsulinaemia, hyperleptinaemia, insulin resistance, impaired glucose tolerance and

essential hypertension (Velasque *et al*, 2001). This SHR/N-*cp* rats is highly useful for investigating obesity-associated type 2 diabetes and for studying the influence of dietary carbohydrate on the development of diabetes in certain genetically predisposed carbohydrate sensitive individuals (Velasque *et al*, 2001).

JCR/LA -cp Rat

Crossing of LA/N-*cp* male rats with hooded rat species produced an outbred substrain JCR (James C Russel):LA-*cp* with only 3% contribution of the *SHR* gene but the presence of *fa* allele (Clark and Pierce, 2000). The homozygous dominant (+/+) and heterozygous (+/*cp*) rats are metabolically unaffected. Animals that are recessive for the gene (*cp/cp*) exhibit extreme metabolic profile including insulin resistance, hyperinsulinaemia, pancreatic β -cell hyperplasia, obesity, glucose intolerance and severe hyperlipidaemia (Srinivasan and Ramarao, 2007). The major drawback of this rat as pure model of diabetes is that it is normoglycaemic when fasted. However, the principal attraction of JCR/LA-*cp* rat as research model is its development of atherosclerotic and myocardial lesions in association with the Syndrome-X metabolic profile (Clark and Pierce, 2000). The distinct feature of this animal is that vasculopathy progresses inherently without any dietary cholesterol and high fat diet interventions. This model is studied for the pharmacological and dietary intervention prior to the onset of cardiovascular lesions or hyperinsulinaemia to determine the efficacy for preventing or slowing the occurrence of cardiovascular lesions (Clark and Pierce, 2000)..

Otsuka Long Evans Tokushima Fatty (OLETF) Rat

OLETF (Otsuka Long Evans Tokushima Fatty) rat with mild obesity was obtained from the selective breeding of the spontaneous diabetic rats from the outbred colony of Long Evans rats (Kawano *et al*, 1992). This polygenic rat develops diabetes later in life at around 18-25 weeks of age. OLETF rats exhibit innate polyphagia, mild obesity, hyperinsulinaemia,

hypertriglyceridaemia, hypercholesterolaemia, chronic hyperglycaemia and impaired glucose tolerance at about 16 weeks of life (Kawano *et al*, 1992). In the recent years, OLETF rat model has been extremely used in pharmacological research while testing for a number of antidiabetic and antihypertensive drugs (Kosegawa *et al*, 1999).

Obese Rhesus Monkey (*Macaca mullata*)

Obese rhesus monkey, an excellent non-rodent model, develops obesity, hyperinsulinaemia and insulin resistance when maintained on *ad libitum* laboratory diet (Kim *et al*, 2005). This initial metabolic derangement gradually progresses to necrosis of β cells, severe fall in insulin levels and overt hyperglycaemia over a period of several years (Kim *et al*, 2005). Unlike conventional rodent models, the ultimate β -cell failure is associated with deposition of amylin/amyloid in islets and the development of diabetic complications similar to human type 2 diabetes (Bell and Hye, 1983). Pioglitazone was demonstrated to improve insulin resistance in obese rhesus monkeys (Kemnitz *et al*, 1994).

Cohen Diabetic Rat

The Cohen diabetic rat is an exceptional genetically-derived experimental model of diet-induced type 2 diabetes that reproduces many features of the disease in humans (Weksler-Zangen *et al*, 2001). It expresses genetic susceptibility (sensitivity and resistance) to a carbohydrate-rich diet, a central feature of type 2 diabetes (Weksler-Zangen *et al*, 2001). Recently, the Cohen rat strain was newly inbred and metabolic phenotypes of this rebred colony of CDs (Cohen diabetic sensitive) and CDr (Cohen diabetic resistant) rats and their genetic makeup render the Cohen diabetic rat a useful experimental model that is highly suitable for studying the interaction between nutritional, metabolic and environmental factors, and genetic susceptibility for the development of type 2 diabetes (Weksler-Zangen *et al*, 2001).

GK Rat

The GK (Goto-Kakizaki) rat, a polygenic model of type 2 diabetes, was established by Goto through repeated selective inbreeding of Wistar rats with abnormal glucose tolerance (Goto and Kakizaki, 1981). It is characterized by non obesity, moderate but stable fasting hyperglycaemia, hypoinsulinaemia, normolipidaemia, and impaired glucose tolerance, which appear at 2 weeks of age in all animals and an early onset of diabetic complications (Portha *et al*, 2001). In the adult GK rats, total pancreatic beta cell mass is decreased by 60%, along with decrease in pancreatic insulin stores. GK rat is one of the animal models used for studying the relation of changes in beta cell mass and occurrence of type 2 diabetes and diabetic complications (particularly diabetic nephropathy) (Dachicourt *et al*, 1998).

Torri Rat

This is a new spontaneously diabetic non-obese rat from the Sprague-Dawley rat strain established at Torri Pharmaceutical Company, Japan (Shinohara *et al*, 2001). It is characterized by glucose intolerance, hyperglycaemia, hypoinsulinaemia and hypertriglyceridaemia. Histologically, haemosiderin deposition and marked fibrosis of pancreatic islets are observed in diabetic rats (Shinohara *et al*, 2001). Torri rats are able to survive for longer duration without insulin treatment; and are thus more useful for studies on diabetic complications. The distinct characteristics are ocular complications, cataract and retinopathy with fractional, retinal detachment, fibrosis and massive haemorrhage at 70-77 weeks of age (Shinohara *et al*, 2001).

Non Obese Mutant C57 BL/6 (Akita) Mouse

The non obese mutant mouse (Akita mouse) has been derived from the colony of C57 BL/6 (B6) mouse in Akita (Japan) (Yoshioka *et al*, 1997). The *Ins2* gene is the mouse homologue of human preproinsulin gene. Mice possess another active insulin gene, *Ins1*, which lacks an

intron present in the C-polypeptide-encoding region. The Akita (Ins2Akita) spontaneous mutation (referred as MODY) is an autosomal dominant mutation in the insulin II gene (*Ins2*) (Yoshioka *et al*, 1997). Ins2Akita mutation disrupts normal insulin processing and causes a failure in secretion of mature insulin, which results in early development of hyperglycaemia. It is characterized by hyperglycaemia, hypoinsulinaemia, polydipsia and polyuria, beginning around 3-4 weeks of age (Mathews *et al*, 2002). Obesity or insulinitis does not accompany diabetes. Histologically, at 4-35 weeks of age, there is a reduction in active pancreatic beta cell density without insulinitis; and the islets release very little mature insulin. The mice with reduced beta cell mass and absence of beta cell auto-immunity serve as an excellent substitute for mice made diabetic by treatment with chemical diabetogens for islet transplantation studies (Mathews *et al*, 2002).

ALS/Lt Mouse

Alloxan susceptible (ALS) new mouse model is produced by inbreeding outbred CD-1 mice with selection for susceptibility to alloxan (ALX), a generator of highly reactive oxygen free radicals and a potent beta cell toxin (Mathews *et al*, 2004). In ALS/Lt mice, hyperinsulinaemia and impaired glucose tolerance develop spontaneously between 6 and 8 weeks of age in alloxan-untreated males. This mouse model with reduced ability to diffuse free radical stress is of obvious interest because free radical-mediated damage is implicated in the pathogenesis and complications of both type 1 and type 2 diabetes (Mathews *et al*, 2004).

Sand Rat

Psammomys obesus (*P. obesus*; sand rat) develop obesity and diabetes mellitus in captivity when fed on standard laboratory chow (high-energy diet) instead of its usual low energy vegetable diet (Shafrir and Ziv, 1998). Initially, the sand rats develop hyperphagia, obesity, hyperinsulinaemia, and glucose intolerance. This is followed by β -cell degeneration and

necrosis, resulting in profound insulin deficiency and overt diabetes and ketosis, ultimately leading to death of the animal (Shafrir, 2001). In later stages of development, these animals show decreased adipose tissue, depletion of beta cell granules, apoptosis and ketoacidosis (Shafrir and Ziv, 1998). Thus, sand rats serve as an excellent polygenic model for the study of diabetes syndrome (Kaiser *et al*, 2005).

C57BL/6J Mouse

This type 2 diabetic model was initially developed in Japan by simply feeding high fat diet to non-obese, non diabetic C57BL/6J mouse strain. It is characterized by marked obesity, hyperinsulinaemia, insulin resistance and glucose intolerance (Surwit *et al*, 1988). In addition, they exhibit marked fasting as well as basal hyperglycaemia in contrast to normal basal glucose level seen in C57BL/6J (*ob/ob*) mice. These mice are demonstrated to develop peripheral leptin resistance (Surwit *et al*, 1988). They manifest most of the characteristic features of the patients with genetic predisposition to develop type 2 diabetes when they become obese. This animal model represents both genetic and environmental risk factors in contrast to C57BL/6J (*ob/ob*) mouse in which onset of symptoms is highly genetically determined. Thus, it is useful for testing and evaluation of antiobesity and antidiabetic drugs (Winzell and Ahren, 2004).

Goldthioglucose Obese Diabetic Mouse

Type 2 diabetes with obesity is induced in mice by goldthioglucose (GTG) (150-350 mg/kg, i.p.) injection. Mice gradually develop obesity, hyperinsulinaemia, hyperglycaemia, and insulin resistance over a period of 16- 20 weeks after GTG injection (Le Marchand *et al*, 1978). The GTG is transported in particular to the cells of ventromedial hypothalamus (VMH) and causes necrotic lesions, which subsequently is responsible for the development of hyperphagia and obesity. It also shows increased body lipid and hepatic lipogenesis and

triglyceride secretion; increased lipogenesis in adipose tissue and decreased glucose metabolism in muscle. In addition, it exhibits many molecular defects in relation to insulin signaling pathways (Le Marchand, 1999). However, it is disadvantageous as it takes very long time to develop obesity/diabetes and the number of mortality following GTG injection is extremely high, which limits its use in diabetes research (Srinivasan and Ramarao, 2007).

Streptozotocin-Induced Type 2 Diabetes in Adult Rats

Recently, a new animal model of type 2 diabetes was produced by the combination of STZ and NAD administration in adult rats (Masiello *et al*, 1998). The rats administered NAD (230 mg/kg, intraperitoneal) 15 min before STZ (65 mg/kg, intravenous) has been shown to develop moderate and stable non-fasting hyperglycaemia without any significant change in plasma insulin level (Masiello *et al*, 1998). NAD is an antioxidant that exerts protective effect on the cytotoxic action of STZ by scavenging free radicals; thus, STZ causes only minor damage to pancreatic beta cell mass, thereby producing type 2 diabetes. This model is a tool for the investigation of insulinotropic agents in the treatment of type 2 diabetes ((Masiello *et al*, 1998). Recently, moderate insulin deficiency and type 2 diabetes was developed in a non-rodent model of Gottingen pig by combination of STZ and NAD, which provides good opportunity to investigate diabetes in much closely similar pathophysiologic situation as in human (Larsen *et al*, 2002).

Neonatal Streptozotocin Diabetic Rats

Injection of STZ to rats at birth or immediately after birth produces type 2 diabetes in the adult age. Single injection of STZ at 80-100 mg /kg of STZ (intravenous or intraperitoneal or subcutaneous) to one- or two- or five-day old Wistar or Sprague-Dawley neonatal rats has been reported to produce type 2 diabetic conditions (Bonner-Weir *et al*, 1981). The neonatal STZ-treated rats are considered better tools for the elucidation of the mechanisms associated

with regeneration of the β cells, the functional exhaustion of the β cells and the emergence of defects in insulin action (Portha *et al*, 1994). Some investigators have also developed neonatal type 2 diabetic models by injecting alloxan (ALX) (200 mg/kg, i.p) to male neonatal rats at age of 2, 4 or 6 days after birth (Kodama *et al*, 1993). These STZ/ALX models are useful for the investigation of long-term complication of type 2 diabetes as well as regular drug-screening experiments (Kodama *et al*, 1993).

Non Obese Partial Pancreatectomized Diabetic Animals

Diabetes caused by 70-90% (usually 90%) partial pancreatectomy has been reported in various animal species, mostly in dogs, pigs, rabbits and rats (Sasaki *et al*, 2000). It is characterized by moderate hyperglycaemia, without reduction in body weight. However, better degree of glycaemia or stable form of diabetes for long duration can be achieved by the combination of partial pancreatectomy with certain diabetogens (ALX and STZ) in animals such as dogs, pigs, and monkeys (Guerrero and Hernandez, 2005). Recently, yet another model of stable form of type 2 diabetes was produced by the combination of 50% partial pancreatectomy with NAD (350 mg/kg) and STZ (200 mg/kg) treatment in BALB/c mice (Kurup and Bhonde, 2000). These partially pancreatectomized models alone or in combination are useful in the investigation of transplantation of pancreatic grafts or islets. Further, they help in the study of factors involved in islet regeneration (Risbud and Bhonde, 2002).

Transgenic and Knockout Type 2 Diabetic Models

In general, transgenic animals, particularly mice, are usually created by transferring and altering the site or level of expression of functional gene (transgene) or by deleting specific endogenous genes (knockout) or placing them under the control of alternate promoter regions (Livingston, 1999). The transgenic and knockout models are developed for studying the role

of genes and their effects on peripheral insulin action and signalling cascade (Livingston, 1999). These genetically modified animals exhibit various phenotypic features of type 2 diabetes varying from mild to severe hyperglycaemia, insulin resistance, hyperinsulinaemia, and impaired glucose tolerance (Plum *et al*, 2005). Currently, transgenic/knockout animals are used mostly for the mechanistic study in diabetes research and are not usually recommended for screening programme as they are more complicated and costly (Plum *et al*, 2005).

2.2 Pancreatic Islet of Langerhans

The pancreas comprises both exocrine and endocrine portions. The latter is made up of the islets of Langerhans, which are multihormonal endocrine micro-organs. Islets of Langerhans are rounded clusters of cells embedded within the exocrine pancreas (Fig. 1 A). Most islets measure 100–200 μm across. The human pancreas contains more than a million islets and these are most numerous in the tail of the organ (Junqueira and Carneiro, 2005). Trichrome stain allows for the recognition of acidophils (α) and basophils (β) within the islets (Junqueira and Carneiro, 2005) (Fig. 1B). With immunocytochemical staining, four cell types – α , β , δ and PP cells – can be recognised (Yukawa *et al*, 1999; El-Naggar, 2000). Beta cells constitute about 70% of all islet cells, and they are mostly located in the central part of the islet (Fig. 1B). These cells produce and secrete insulin (Yukawa *et al*, 1999).

Furthermore, about 20% of islet cells are α cells (Fig. 1B); and these cells produce glucagon. Delta and PP cells constitute a minor population of islet cells and they produce somatostatin and pancreatic polypeptide, respectively (Yukawa *et al*, 1999). Somatostatin receptors have been identified on α and β cells of the islet, and this hormone acts in a paracrine manner to inhibit the activity of these islet cells (Hauge-Evans *et al*, 2009). The function of pancreatic polypeptide is to self-regulate the secretory activities of the pancreas (endocrine and

exocrine); it also has effects on hepatic glycogen levels and gastrointestinal secretions. Its secretion in humans is increased after a protein meal, fasting, exercise, and acute hypoglycaemia; and is decreased by somatostatin and intravenous glucose (Yukawa *et al*, 1999).

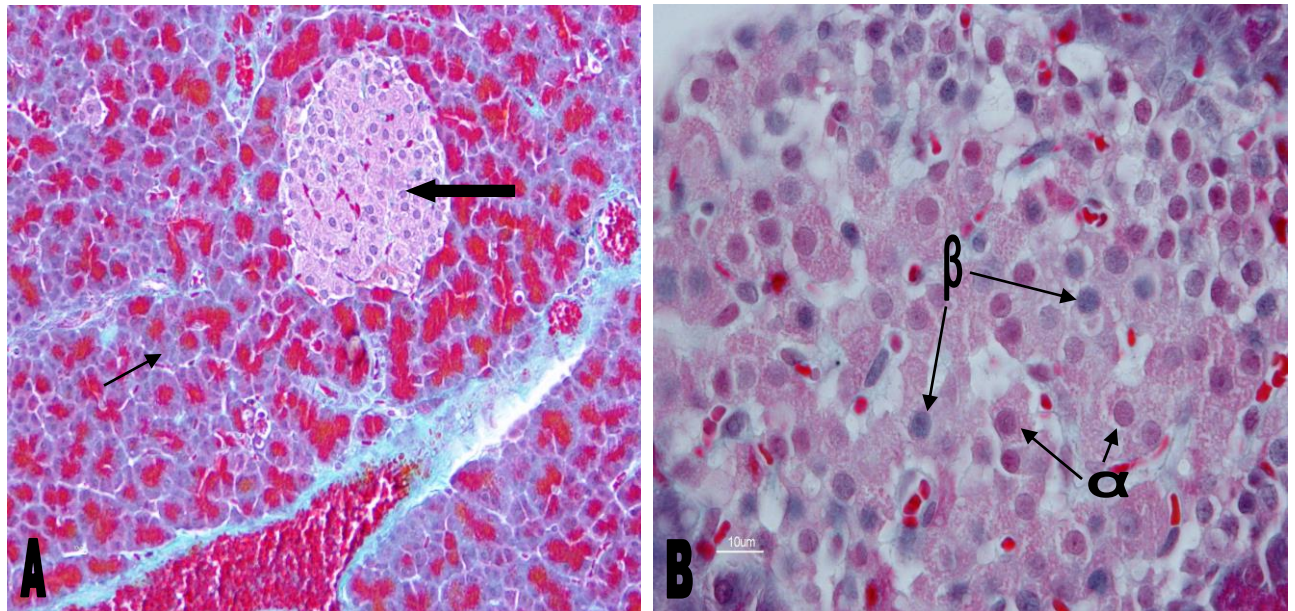


Figure 1. Sections of rat pancreas; **(A)** islet of Langerhans (thick arrow); exocrine pancreas (thin arrow); x200; Masson's trichrome stain. **(B)** Islet of Langerhans showing alpha (α) and beta (β) cells; x600. Masson's trichrome stain. (Akinola *et al*, 2009).

2.3 Insulin

Insulin is a small protein with a molecular weight of 5808 in humans. It contains 51 amino acids arranged in two chains (A and B) linked by disulphide bridges (Nolte and Karam, 2007) (Fig. 2). Pro-insulin, a long chain protein molecule, is processed within the Golgi apparatus and packaged into granules, where it is hydrolysed into insulin and a residual connecting segment called C-peptide, by the removal of four amino acids (Nolte and Karam, 2007).

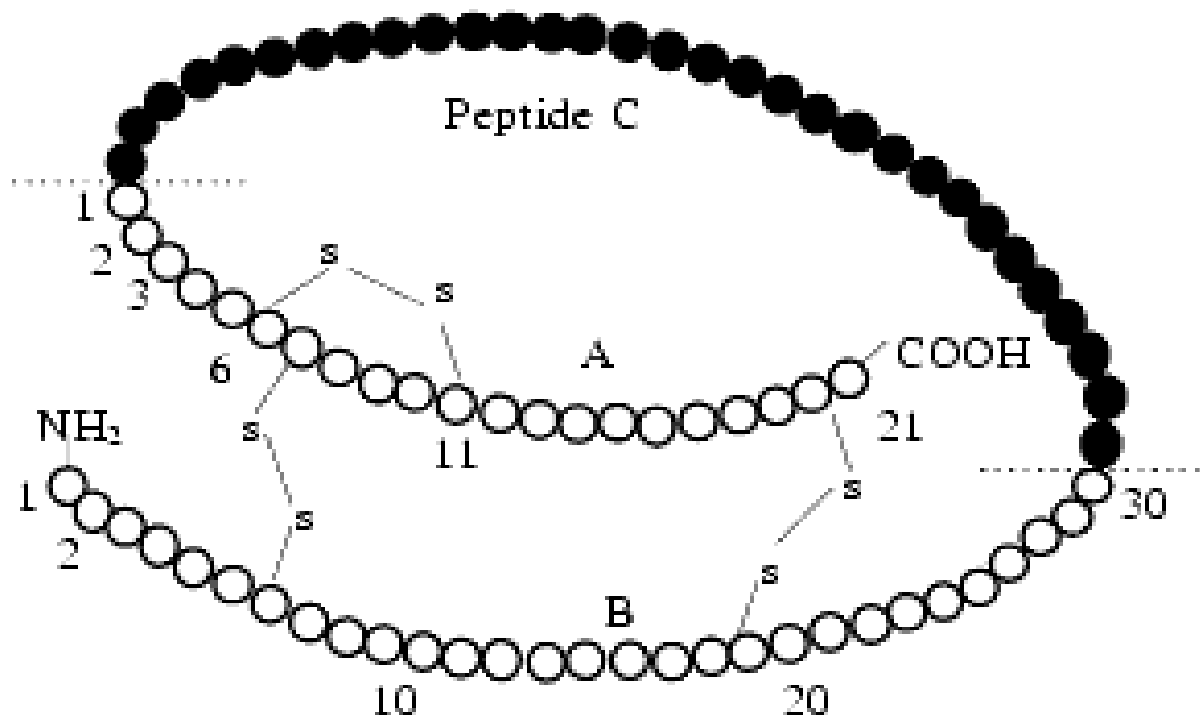


Figure 2: Structure of insulin (Nolte and Karam, 2007)

Insulin and C-peptide are secreted in equimolar quantities by pancreatic β cells in response to elevated levels of nutrients in the blood (Henquin *et al*, 2002) (Fig. 3). Increased plasma levels of insulin secretagogues, such as glucose, results in the uptake of this nutrient by the beta cells, via GLUT-2 transporter (Fig. 3). In the beta cells, glucose is metabolised to yield ATP. The latter mediates the closure of ATP-sensitive potassium channels in the membrane of beta cell, resulting in depolarisation of the cell membrane. This effect results in the opening of the voltage-gated calcium channels in beta cell membrane, followed by influx of Ca^{2+} into the cells. This action results in exocytosis of insulin from the secretory granules (Henquin *et al*, 2002) (Fig. 3).

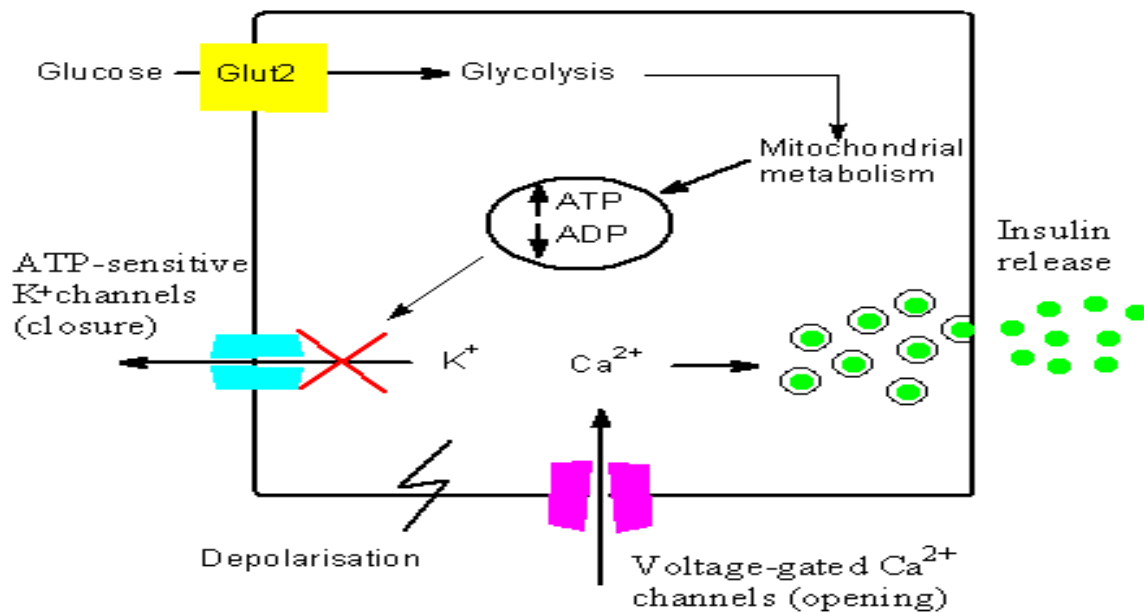


Figure 3: Metabolic regulation of insulin secretion (Nolte and Karam, 2007).

Insulin has a half-life of 3-5 minutes, and it is metabolized by insulinase in the liver, kidney, and placenta. About 50% of insulin secreted by the pancreas is removed by first-pass extraction in the liver (Bevan, 2001). Insulin promotes glycogenesis in the liver and inhibits glycogenolysis. It promotes protein, cholesterol, and triglyceride synthesis and stimulates formation of very-low-density lipoprotein cholesterol (VLDL-c). It also inhibits hepatic gluconeogenesis, stimulates glycolysis, and inhibits ketogenesis (Goalstone and Draznin, 1997). Insulin triggers the uptake of glucose, fatty acids and amino acids into adipose tissue, muscle and the liver and promotes the storage of these nutrients in the form of glycogen, lipids and protein, respectively (Goalstone and Draznin, 1997). Failure to uptake and store nutrients is characteristic of diabetes mellitus. Type 1 diabetes is characterised by the inability to synthesise insulin, whereas in type 2 diabetes, the body becomes resistant to the effects of insulin presumably because of defects in the insulin signalling pathway (Libman *et al*, 1993; Raskin *et al*, 2005).

2.3.1 Insulin Signaling and Glucose Uptake

The insulin receptor is composed of two extracellular α subunits and two transmembrane β subunits linked together by disulphide bonds. Binding of insulin to α subunit induces a conformational change resulting in autophosphorylation of a number of tyrosine residues present in the β subunit (Van Obberghen *et al*, 2001). These residues are recognised by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS) (Goalstone and Draznin, 1997). Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognised by the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI-3-kinase), a lipid kinase (Fig. 4). The catalytic subunit of PI3-kinase, p110, then phosphorylates phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P₂) leading to the formation of phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P₃). A key downstream effector of PtdIns(3,4,5)P₃ is AKT (also known as PKB), which is recruited to the plasma membrane. Activation of AKT also requires the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1), which in combination with a yet unidentified kinase leads to the phosphorylation of AKT (Goalstone and Draznin, 1997). Once active, AKT enters the cytoplasm where it leads to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3). A major substrate of GSK3 is glycogen synthase, an enzyme that catalyses the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore, the inactivation of GSK3 by AKT promotes glucose storage as glycogen (Lizcano and Alessi, 2002).

In addition to promoting glucose storage, insulin inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis (Lizcano and Alessi, 2002). Insulin directly controls the activities of a set of metabolic enzymes by phosphorylation and dephosphorylation events and regulates the expression of genes

encoding hepatic enzymes involved in gluconeogenesis. Recent evidence suggests that forkhead transcription factors, which are excluded from the nucleus following phosphorylation by AKT, play a role in hepatic enzyme regulation by insulin (Barthel *et al.*, 2001).

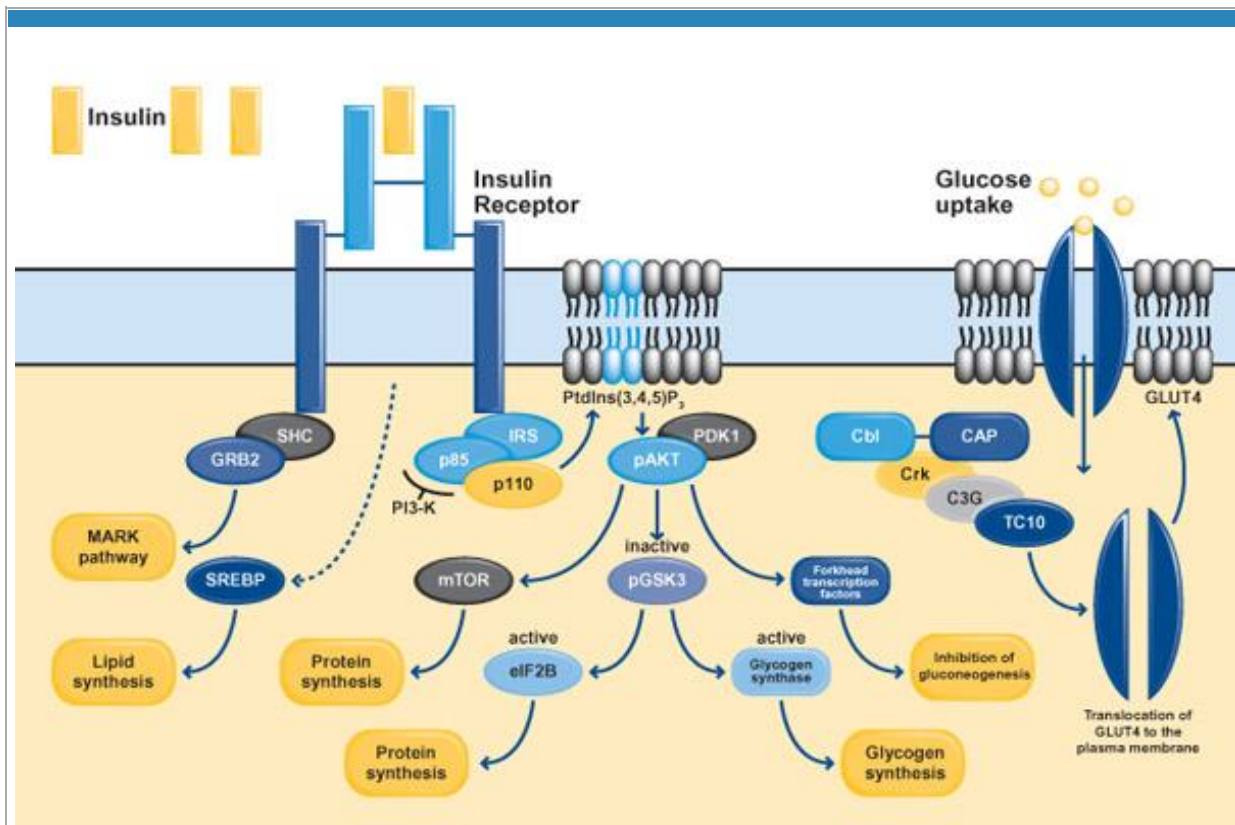


Figure 4: Insulin signalling pathways. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism (Van Obberghen *et al.*, 2001).

Abbreviations:

IRS: Insulin receptor substrate

SHC: Src-homology-2-containing protein

GRB2: Growth factor receptor-bound protein 2

MAPK: Mitogen-activated protein kinase

SREBP: Steroid regulatory element-binding protein

PtdIns(3,4,5)P₃: Phosphatidyl inositol 3,4,5, triphosphate

PI3-K: Phosphatidylinositol 3-kinase

mTOR: Mammalian target of rapamycin
PDK1: 3-Phosphoinositide-dependent protein kinase-1

pGSK3: Glycogen synthase kinase 3

CAP: Cbl-associated protein

GLUT-4: Glucose transporter-4

PKC: Protein kinase C

P110: Phosphoinositide-3-kinase, catalytic, beta polypeptide

P85: Phosphoinositide-3-kinase, regulatory subunit 2 (beta)

eIF2B: Eukaryotic initiation factor 2B

C3G: Guanine nucleotide-releasing factor 2

Cbl: Casitas B-lineage lymphoma proto-oncogene

A key action of insulin is to stimulate glucose uptake into cells by inducing translocation of the glucose transporter 4 (GLUT4) from intracellular storage to the plasma membrane. PI 3-kinase and AKT are known to play a role in GLUT4 translocation (Lizcano and Alessi, 2002). In addition, a PI-3-kinase independent pathway provides a second cue for GLUT4 recruitment to the plasma membrane (Goalstone and Draznin, 1997). In this pathway, insulin receptor activation leads to the phosphorylation of Cbl, which is associated with the adaptor protein CAP. Following phosphorylation, the Cbl-CAP complex translocates to lipid rafts in the plasma membrane. Cbl then interacts with the adaptor protein Crk, which is constitutively associated with the Rho-family guanine nucleotide exchange factor, C3G. C3G in turn activates members of the GTP-binding protein family, TC10, which promote GLUT4 translocation to the plasma membrane through the activation of unknown adaptor molecules (Lizcano and Alessi, 2002).

2.3.2 Insulin and Protein Synthesis

Insulin stimulates amino acid uptake into cells, inhibits protein degradation and promotes protein synthesis (Goalstone and Draznin, 1997). Under basal conditions, the constitutive activity of GSK3 leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation (Lizcano and Alessi, 2002). Therefore, upon receipt of an insulin signal, inactivation of GSK3 by AKT leads to the dephosphorylation of eIF2B, thereby promoting protein synthesis and the storage of amino acids (Lizcano and Alessi, 2002). AKT also activates mammalian target of

rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) (Asnaghi *et al*, 2004).

2.3.3 Insulin and Lipid Synthesis

Insulin promotes the uptake of fatty acids and the synthesis of lipids, while inhibiting lipolysis. Recent studies indicate that lipid synthesis requires an increase in the transcription factor steroid regulatory element-binding protein (SREBP)-1c (Shimomura *et al*, 1999). However, the pathway leading to changes in SREBP expression are unknown. Insulin inhibits lipid metabolism by decreasing cellular concentrations of cAMP through the activation of a cAMP-specific phosphodiesterase in adipocytes (Kitamura *et al*, 1999).

2.3.4 Mitogenic Responses to Insulin

Other signal transduction proteins interact with IRS, including GRB2, an adaptor protein that contains SH3 domains, which in turn associates with the guanine nucleotide exchange factor son-of sevenless and elicits activation of the MAPK cascade, leading to mitogenic responses to insulin (Ogawa *et al*, 1998). SHC is another substrate for the insulin receptor. Upon phosphorylation, SHC associates with GRB2 and can therefore activate the MAPK pathway independently of IRS (Ogawa *et al*, 1998).

2.3.5 The Pancreas in Diabetes Mellitus

In diabetes mellitus, impairment/loss of β cells occurs; and islet degeneration is a feature of types 1 and 2 diabetes (Mathis *et al*, 2001). In type 1 diabetes, immune-mediated loss of β cells does occur, such that more than 90% of these cells are already destroyed at diagnosis (Ryu *et al*, 2001). Beta cells are susceptible to auto-immune destruction owing to the presence of autoantigens such as insulin and glutamic acid decarboxylase (GAD) (Dotta *et al*, 2005). It is now widely agreed that destruction of β cells is both autoantibody- and T-cell-

mediated (Dotta *et al*, 2005). CD4⁺ (helper) and CD8⁺ (cytotoxic) T cells are involved in the development of type 1 diabetes. B lymphocytes, macrophages and dendritic cells are also mobilised (Haskins *et al*, 2003). In NOD mice and BB rats, macrophages and/or dendritic cells are the first to infiltrate the islets. The presentation of B-cell autoantigens by macrophages and/or dendritic cells to CD4⁺ T-helper cells, in association with MHC class II molecules, is considered the initial step in the development of type 1 diabetes (Dotta *et al*, 2005). Activated macrophages and CD4⁺ lymphocytes release cytokines (IL1- β , TNF- α , and INF- γ) and induce the migration of CD8⁺ peripheral T cells to the inflamed islets (insulinitis). The activated cytotoxic CD8⁺ T cells then damage β cells by releasing perforin and granzyme; and by Fas-mediated apoptosis (Kay *et al*, 2000).

Furthermore, oxidative stress has a role in the pathogenesis of type 1 diabetes. Studies showed that incubation of human islets with a combination of IL1- β + TNF- α + INF- γ resulted in upregulation of inducible nitric oxide synthase (iNOS) in β cells and macrophages (Corbett *et al*, 1993), and subsequent synthesis of NO. Other radicals such as O₂⁻ and ONOO⁻ are also produced, and together with NO, contribute to β -cell damage. NO impairs β -cell function by blocking aconitase and by inducing DNA breaks (Corbett *et al*, 1993). NO could also induce Fas expression on β cells, thereby mediating β -cell destruction (Dotta *et al*, 2005). In long-standing type 1 diabetes, β -cell population in islets is less than 1% of the normal mass (Meier *et al*, 2005).

In type 2 diabetes, amyloidosis and fibrosis are the major structural modifications of the islets (Hayden, 2007). This is in addition to β -cell failure associated with this disease. The work of Hull *et al* (2005) showed that amylin and transthyretin are deposited in the islets of type 2 diabetics. Islet amyloid, β -cell deficit and increased β -cell apoptosis had also been reported by Butler *et al* (2003).

Islet amyloid deposits are found in >90% of type 2 diabetics (Khan *et al*, 1999). Studies on diabetic primates showed that formation of islet amyloid precedes the development of hyperglycaemia (de Koning *et al*, 1993); and this presupposes that islet amyloid may have an important role in the pathogenesis of type 2 diabetes. Formation of islet amyloid is associated with a decrease in β -cell mass in human (Clark *et al*, 1988) and non humans (de Koning *et al*, 1993). Islet amyloid is mainly composed of the β -cell derived peptide – islet amyloid polypeptide (IAPP) or amylin. It is produced and co-secreted with insulin in response to β -cell stimulation by glucose and non-glucose secretagogues (Khan *et al*, 1990). High concentrations of amylin have been shown to inhibit insulin secretion (Degano *et al*, 1993) and action (Leighton and Cooper, 1988).

Besides amyloidosis, fibrosis is also a common feature of type 2 diabetes, as was demonstrated in Long-Evans Tokushima fatty rats by Ko *et al* (2004). Chronic hyperglycemia had been implicated in the aetiology of islet fibrosis (Hong *et al*, 2007); and therapies that suppress islet fibrosis and improve islet morphology are beneficial in type 2 diabetes (Ko *et al*, 2004). Thus, conventional and botanical therapies that improve islet morphology could be beneficial in the amelioration of islet lesions in diabetes.

2.4 The Liver in Glucose Homeostasis

An appreciation of the role of the liver in the regulation of carbohydrate homeostasis is essential to understanding the many physical and biochemical alterations that occur in the liver in the presence of diabetes and to understanding how liver disease may affect glucose metabolism (Levintall and Tavill, 1999). The liver uses glucose as a fuel and stores it as glycogen; it also synthesises it from noncarbohydrate precursors such as glycerol and lactate (during gluconeogenesis) (Levintall and Tavill, 1999). Mann and Magath (1922) demonstrated that total hepatectomy in a dog results in death within a few hours (from

hypoglycemic shock); and this underscores the important role the liver plays in maintaining normoglycemia. Glucose absorbed from the intestinal tract is transported via the portal vein to the liver. Some suggest that much of the absorbed glucose is retained by the liver so that the rise in peripheral glucose concentration reflects only a minor component of postprandial absorbed glucose (Bjornstorp and Sjostrom, 1978). Therefore, it is possible that the liver plays a more significant role than does peripheral tissue in the regulation of systemic blood glucose levels following a meal (Bjornstorp and Sjostrom, 1978). Katz *et al* (1983) however suggest that the bulk of the absorbed glucose is not taken up by the liver but is rather metabolized via glycolysis in the peripheral tissues.

Many cells in the body, including fat, liver, and muscle cells, have specific cell membrane insulin receptors, and insulin facilitates the uptake and utilization of glucose by these cells. Glucose rapidly equilibrates between liver cytosol and the extracellular fluid. Transport into certain cells, such as resting muscle, is tightly regulated by insulin, whereas uptake into the nervous system is not insulin-dependent (Guyton and Hall, 2006). Glucose can be used as a fuel or stored in a macromolecular form as polymers (glycogen in animals). Glycogen storage in cells is promoted by insulin (Guyton and Hall, 2006).

The glucose that is taken up by a cell may be oxidized via glycolysis. It is oxidized to pyruvate in the cytosol; and electrons generated from this process are transferred to the mitochondria (Levintall and Tavill, 1999). Pyruvate generated by the Emden-Meyerhof pathway is oxidized to acetyl CoA in the mitochondria, which in turn undergoes further oxidation by the Krebs tricarboxylic acid cycle (Bender and Mayes, 2006). Nearly 36 moles of high energy phosphates are generated from each molecule of glucose by aerobic glycolysis (Bender and Mayes, 2006). In the absence of oxygen, pyruvate is converted to lactate by the action of lactate dehydrogenase. Lactate is a potential fuel, or it may be converted to glucose

in the liver. The formation of glucose from lactate, glycerol, alanine, etc, is known as gluconeogenesis; and this occurs mainly in the liver and kidneys (Bender and Mayes, 2006).

2.4.1 Diabetes Mellitus and Liver Disease

2.4.1.1 Glycogen Deposition in Hepatocytes

Excess glycogen accumulation in the liver (hepatic glycogenosis; glycogenic hepatopathy) is seen in 80% of diabetic patients (Stone and VanThiel, 1985). Glycogen synthesis in the liver is impaired in diabetes due to defective activation of glycogen synthase. In patients with chronic diabetes, glycogen accumulation is seen, and it is postulated that long-standing insulin deficiency may actually facilitate glycogen synthase activity (Ferrannini *et al*, 1990). This may account for the net accumulation of glycogen in diabetes. The exact mechanism of cytoplasmic glycogen deposition is uncertain but may be related to the large variations in glucose concentration and frequent insulin dosing (Munns *et al*, 2000).

In diabetes, glycogen deposition is also seen in nuclei of hepatocytes. The mechanism of nuclear glycogen deposition is unclear, with the stored glycogen resembling muscle glycogen more than hepatocyte cytoplasmic glycogen (Manderson *et al*, 1968). Nuclear glycogen deposition was first described by Ehrlich in 1883. It is postulated that glycogen is actually synthesized in the nucleus and has been found in 60–75% of diabetic patients (Kautzsch, 1963). Nuclear glycogen deposition is also seen in sepsis, tuberculosis, some patients with hepatitis (particularly autoimmune chronic hepatitis), Wilson's disease, and cirrhosis (Kautzsch, 1963).

The finding of nuclear glycogen deposition in a patient with fatty liver is useful confirmatory evidence that the fatty liver is secondary to diabetes even if the glucose tolerance test is normal (Levintall and Tavill, 1999). However, Creutzfeldt *et al* (1970) have also reported this

combination in obese patients. Patients showing solely excessive glycogen deposition may exhibit hepatomegaly and liver enzyme abnormalities and may have abdominal pain, nausea, vomiting, and rarely, ascites. These abnormalities may improve with sustained glucose control (Duvnjak *et al*, 2004).

2.4.1.2 Fatty Liver (Steatosis and Steatohepatitis)

Hepatic fat accumulation is a well-recognized complication of diabetes with a reported frequency of 40-70% (Levinthal and Tavill, 1999). Unfortunately, associated obesity is a frequently occurring confounding variable. Type 1 diabetes is not associated with fat accumulation if glycemia is well controlled, but type 2 diabetes may have a 70% correlation regardless of blood glucose control (Levinthal and Tavill, 1999).

Fat is stored in the form of triglyceride and may be a manifestation of increased delivery of exogenous fatty acids to hepatocytes; increased endogenous hepatic production of fatty acids; decreased hepatic disposal of fatty acids; or impaired export of VLDL from the liver (Fong and Lindor, 2000). The steatosis may be microvesicular or macrovesicular and may progress to fibrosis, cirrhosis and hepatocellular carcinoma (Matteoni *et al*, 1999). The most common clinical presentation is hepatomegaly, and most patients have normal or only mildly abnormal transaminases and normal bilirubin (Levinthal and Tavill, 1999). A negative ultrasound does not exclude the presence of microscopic fatty infiltration (O'Connor *et al*, 1997). A liver biopsy is obviously the best method for detecting hepatic fat accumulation (Schaffner and Thaler, 1986).

The morphological pattern of diabetic steatohepatitis resembles that seen in alcoholic hepatitis. However, the histopathological changes in diabetes tend to be periportal (situated in zone I), while those in alcoholic steatohepatitis are predominantly pericentral (in zone III)

(Levinthal and Tavill, 1999). In an animal model of type 1 diabetes, there is a high incidence of perisinusoidal hepatic fibrosis; while in humans, perisinusoidal fibrosis often parallels diabetic microangiopathy (Bernuau *et al*, 1982).

2.5 Diabetes Mellitus and Kidney Disease (Diabetic Nephropathy)

Diabetic kidney disease is known for a long time, but it was only after insulin therapy that renal complications became an important problem in diabetic patients. Longer life expectancy is probably the reason since the increased life span would give a longer time for the various structural changes to develop (Heptinstall, 1991). Kimmelstiel and Wilson (1936) first described the glomerular lesions characteristic of diabetes. The knowledge of the structure of diabetic kidney lesions has extended ever since. Today, diabetic kidney disease is the single commonest cause of end-stage renal failure worldwide (Thomas and Viberti, 2000).

2.5.1 Diabetic Glomerulosclerosis

The first morphological change of the human diabetic kidney after onset of diabetes is an increase in glomerular size and therefore, a rise in filtration area, resulting in an increase in glomerular filtration rate (Romen, 1980). Approximately two years after onset of diabetes, glomerular basement membranes begin to thicken and the expansion of capillary walls and the mesangium leads to the development of diffuse form of glomerulosclerosis. Nodular glomerulosclerosis may develop in more advanced kidney damage. This lesion is the type of change originally described by Kimmelstiel and Wilson in 1936. It typically consists of a rounded, homogeneous, eosinophilic, PAS-positive area situated in the central part of a glomerular lobule, usually toward the periphery. Several lobules within a given glomerulus can be affected, and the nodules are usually of varying sizes. Nodules are often acellular, but elongated cells may appear concentrically arranged near the periphery (Kelly *et al*, 1998). Around the periphery of the nodule, the capillaries are usually patent with the capillary walls

appearing thickened. Glomerular capillaries may be dilated and lipophages are sometimes seen, apparently in capillary lumens. The glomerular tuft often retains size, which is in contrast to the shrinkage that accompanies the purely ischemic glomerulus. In late stages, exudative lesions appear more frequently and glomeruli are reduced in size (Heptinstall, 1991). It has been postulated that nodules result from repair processes of microaneurysmal glomerular capillaries, breakage and lysis of the mesangium, as well as organization of mesangial destruction (Ritz and Usadel, 1999).

The diffuse lesion consists of an increase in eosinophilic, PAS-positive material in the mesangium (Dijk and Berl, 2004). The process also includes thickening of the capillary walls, which is often widespread within a glomerulus and may affect all glomeruli. In later stages, as thickening progresses, capillary lumens become reduced. The capsule of Bowman becomes thickened in more advanced lesions and both afferent and efferent arterioles are hyalinized. Unlike the nodular lesion, the diffuse lesion is not specific for diabetes and can occur in other conditions such as glomerulonephritis (Kelly *et al*, 1998).

Another type of lesion seen in diabetic patients is the exudative lesion, which includes fibrinoid caps and capsular drops, as well as arteriolar hyalin changes (Dijk and Berl, 2004). The fibrinoid cap appears as an eosinophilic, smooth, homogenous crescentic structure lying in the concavity of a capillary, usually at the periphery of a lobule. It has the general appearance of fibrin; is sometimes foamy, and contains lipid. The lesion is mostly common in diabetic kidneys with severe vascular disease but not specific for diabetic glomerulosclerosis (Heptinstall, 1991). Capsular drops appear as small, rounded, eosinophilic masses on the inside of Bowman's capsule between the basement membrane and the parietal epithelial cells (Kelly *et al*, 1998), 1991). This lesion was first described in Kimmelstiel and Wilson's (1936) original paper, but only later was it regarded as diagnostic aid for diabetic glomerulosclerosis. The arterial hyaline change is a lesion of both afferent and efferent arterioles, the walls of

which are thick, homogenous or slightly fibrillar and vacuolated; and strongly eosinophilic and PAS-positive (Heptinstall, 1991). The normal arterial morphology disappears and no muscle coat can be seen in an arteriole with well-developed hyaline change. The PAS+ arteriolar lesion is continuous with the diffuse lesion in the lobules through a PAS+ thickening in the capillary stalk (Olsen, 1969). Arteriolar hyaline change of the afferent arterioles may also be seen in patients with essential hypertension, whereas the hyaline change of the efferent arterioles is considered diabetes specific (Ritz and Usadel, 1999).

2.5.2 Diabetes Mellitus and Changes in Renal Tubules

In diabetes mellitus, the proximal convoluted segment of the tubules often appears finely vacuolated. Ebstein (1882) demonstrated vacuolization of kidney tubules in patients who died from diabetic coma. In more advanced lesions, there is tubular loss, with atrophic tubules showing thickened basement membranes (Levinthal and Tavill, 1999). Vacuoles of Armanni-Ebstein cells had been reported to contain glycogen (Kang *et al*, 2005).

2.5.3 Renal Interstitial Changes in Diabetes Mellitus

In diabetes mellitus, renal interstitial tissue is frequently fibrotic and may contain inflammatory cells. Lymphocytes and plasma cells may be so numerous that the reaction is indistinguishable from that seen in chronic infections (Wang *et al*, 2000). Interstitial infiltrates become more numerous with increasing duration of diabetes and with the degree of arteriosclerosis (Kelly *et al*, 1998). Such morphological changes in the human diabetic kidney are parts of the underlying cause of functional abnormalities, such as proteinuria, with urinary albumin excretion being the most powerful predictor of progression of renal injury (Wang *et al*, 2000).

2.6 Diabetes Mellitus and the Small Intestine

The small bowel may be greatly involved in diabetes mellitus, resulting in diarrhoea and malabsorption (Taub *et al*, 1979). In diabetic patients, a group of chronic disorders of the intestine consists of the following (Bjelakovic *et al*, 2005):

1. Chronic diabetic diarrhoea without steatorrhoea, which is considered to be genuine diabetic diarrhoea.
2. Diabetic diarrhoea with steatorrhoea.
3. Diarrhoea and steatorrhoea in diabetics owing to chronic insufficiency of exocrine function of the pancreas.
4. Primary malabsorption syndrome and diabetes.

2.6.1 Chronic Diabetic Diarrhoea without Steatorrhoea

The first case of diabetic diarrhoea as a rare complication of diabetes was described by Joslin in 1912. The exact pathogenesis of diarrhoea-steatorrhoea is still undetermined (Bjelakovic *et al*, 2005); and many factors may be involved (Berge *et al*, 1956):

- Autonomous neuropathy
- Lack of vitamins
- Insufficiency of exocrine function of the pancreas
- Bacterial contamination of the superior parts of the digestive tract; and
- Damage to intestinal mucosa.

There is a high incidence of neuropathy in these patients, including all of its forms (peripheral, autonomous and visceral); and many are of the opinion that neuropathy is the cause of diabetic diarrhoea-steatorrhoea (De Las Casas and Finley, 1999). Other possible causes may include lack of vitamins, disorder of exocrine pancreas function, bacterial contamination of the superior parts of digestive tract, and damage to intestinal mucosa (Berge

et al, 1956). This type of diarrhoea is typical of younger people with long-standing anamnesis and inappropriately controlled diabetes that is characterized by frequent hyperglycaemia, hypoglycaemia and ketoacidosis. The symptoms usually occur 5-10 years after the diagnosis of diabetes; and it is more frequent in men than in women (De Las Casas and Finley, 1999). Regularly, in anamnesis of the patient, there is diarrhoea that lasts a few days, weeks or months, but rarely years. The most typical symptom is intermittence of diarrhoea attacks with exacerbations and remissions of different duration which cannot be foreseen. Diarrhoea is usually abundant, watery, urgent and frequent (up to 20 times per day). It can be induced by diet and emotional factors; although in most cases it is not easy to identify the provoking factors (Berge *et al*, 1956). Night incontinency of stool is frequent. Stools are abundant, watery, rare, brownish in colour, and homogeneous, without the presence of blood. The time of barium transit through the small intestine is usually prolonged; intestinal lumen is dilated and the barium meal can be segmented with mucous villous atrophy which may show roughness, irregularity and even obliteration. Barium can produce pools in the small intestine (Yang *et al*, 1984).

2.6.2 Diabetic Diarrhoea with Steatorrhoea

Diabetic diarrhoea and steatorrhoea manifestations are of the same basic process. In some patients, steatorrhoea occurs only when diarrhoea worsens. Some report that this complication is not the consequence of diabetes but of reduced exocrine function of the pancreas (Frier *et al*, 1976). Clinically, diabetic steatorrhoea shows intermittent flow. It can last several hours, weeks, as well as several months and years. Symptoms are, more frequently, postprandial and they appear at night when incontinency of the stool is possible. Stools are not typically fatty, but they are rare, watery and abundant (Bjelakovic *et al*, 2005).

2.6.3 Histological Changes in the Small Intestine in Diabetes

In diabetes, histologically established changes in intestinal mucosa include atrophic, thicker, and reduced villi; and stromal infiltration in a certain number of patients (De Las Casas and Finley, 1999). In many patients, the medical report is normal, so that it is difficult to state whether or not histological changes do exist in diabetes (Bjelakovic *et al*, 2005).

2.7 Diabetic Dyslipidaemia

Ninety-seven per cent (97%) of adults with diabetes have one or more lipid abnormalities (Fagot-Campagna *et al*, 2000). The central characteristic of dyslipidemia in patients with diabetes is an elevated triglyceride level, particularly triglyceride-rich VLDL levels; and decreased HDL cholesterol levels. In diabetic patients, the concentration of LDL cholesterol is usually not significantly different from that seen in non-diabetic individuals (Lamarche *et al*, 1997). However, diabetic patients typically have a preponderance of smaller, denser, oxidized LDL particles, which may increase atherogenicity, even if the absolute concentration of LDL cholesterol is not elevated (Lamarche *et al*, 1997). Besides, LDL may as well be glycated in chronic hyperglycemia (Kesaniemi *et al*, 1983). This lipid triad, referred to as atherogenic dyslipidemia, is usually present in patients with premature coronary artery disease. When this characteristic lipid profile is seen in diabetes, it is referred to as diabetic dyslipidemia and confers a risk of cardiovascular disease (or cerebrovascular/peripheral vascular disease) that equals or exceeds that of a high-risk LDL cholesterol concentration of 150–220 mg/dl (Grundy, 1997). According to the American Diabetes Association (ADA, 1998), the presence of increased triglyceride and decreased HDL levels is the best predictor of vascular disease in patients with diabetes. Other predictive factors include a history of cigarette smoking and hypertension. The evidence regarding the predictive value of elevated LDL cholesterol is still accumulating. Most recently, results of

the Strong Heart Study indicate that LDL cholesterol is an independent predictor of vascular disease in patients with diabetes, along with age, albuminuria, HDL cholesterol (inverse predictor), and percent body fat (inverse predictor) (Howard *et al*, 2000). Starting with LDL levels as low as 70 mg/dl, every 10 mg/dl increase in LDL cholesterol was reported to be associated with a 12% increase in risk of CVD (Howard *et al*, 2000). This finding is supported by results of prospective, long-term clinical trials in which reduction of LDL levels was associated with a significantly reduced risk of cardiovascular events in both diabetic and non-diabetic participants (Haffner *et al*, 1999).

2.7.1 Pathogenesis of Diabetes-Associated Vascular Disease

The pathogenesis of vascular disease (VD) associated with diabetes is not yet fully understood. However, because atherosclerotic macrovascular complications occur at an earlier age and with greater severity in people with diabetes, it is likely that its pathogenesis is directly influenced by the diabetic state. Long-term exposure to elevated glucose levels alone can contribute to the endothelial cell dysfunction observed in diabetes (Cohen, 1993). Increasing evidence suggests that endothelial dysfunction may play a central role in the development of atherosclerosis (Laight *et al*, 1999). Endothelial dysfunction is characterized by inhibited vasodilation, increased vascular smooth-muscle proliferation, increased thrombogenesis, and proatherogenic cellular processes (Glasser *et al*, 1996). Abnormal endothelium-dependent vasodilation also occurs in the microcirculation of diabetic patients, where it may contribute to ischemia and its sequelae (Luscher *et al*, 1996). In addition to accelerated atherosclerosis, endothelial dysfunction has been linked with increased thrombosis, hypertension, and dyslipidemia, all of which contribute to the pathogenesis of vascular disease in diabetes (Cohen, 1993). Hyperglycemia might contribute to atherosclerosis in diabetes in a number of other ways. For example, hyperglycemia causes

glycosylation of proteins in a process that induces cross-linking of collagen and other extracellular matrix proteins in the arterial wall (Brownlee, 1992). The end products of glycation modify LDL cholesterol, prolonging its half-life and producing changes in the artery, rendering it more susceptible to atherosclerosis (Bucala *et al*, 1993). Among other proposed biochemical pathways in the pathogenesis of diabetic macrovascular disease are glucose-induced activation of protein kinase C isoforms and increased intracellular oxidative stress (Nishikawa *et al*, 2000a).

2.8 Oxidative Stress and Diabetes Mellitus

2.8.1 Diabetes: Multiple Sources of Oxidative Stress

Fasting hyperglycemia, defining established diabetes, can induce oxidative stress by various mechanisms. Excessive levels of glucose reaching the mitochondria lead to an overdrive of the electron transport chain, resulting in overproduction of superoxide anions normally scavenged by mitochondrial superoxide dismutase (SOD). When the latter fails, oxidative stress develops and it was recently proposed that this mechanism is responsible for the activation of all major pathways underlying the different components of vascular diabetic complications (glycation, PKC activation, sorbitol pathway) (Nishikawa *et al*, 2000a). The *in vitro* supplementation of SOD-like drugs corrected most of these defects, supporting the importance of these mechanisms (Rechioni *et al*, 2002). Another mechanism whereby high glucose can stimulate oxidative stress is the autooxidation of glucose in the presence of transition metals, as well as the generation of reactive oxygen species (ROS) during the process of glycation (Wolff *et al*, 1989). Indeed, the development from Schiff base to Amadori to advanced glycation end-products (AGEs) is accompanied by ROS-generating reactions at various steps (Yim *et al*, 2001). Once AGEs are formed, they bind to various receptors termed RAGE and this step also generates ROS (Chappey *et al*, 1997). It was also

proposed that carbonyl stress, rather than OST, involving both sugars and lipids, would be the relevant source of oxidative stress in diabetes (Baynes and Thorpes, 1999).

2.8.2 Hyperglycemia Reduces Antioxidant Potential

Oxidative stress acts on signal transduction, and via NFkB, affects gene expression, thereby the expression of antioxidant enzymes can be reduced (Kaneto *et al*, 1994). Moreover, hyperglycemia can simply inactivate existing antioxidant enzymes by glycation of these proteins; glycation of SOD, for example, also leads to DNA cleavage (Kaneto *et al*, 1994). This finding suggests that oxidative stress may develop by insufficient antioxidant activity, even if reactive oxygen species (ROS) production is within a physiological range (Wiernsperger, 2003).

2.8.3 Oxidative Stress in Pre-diabetes

Many studies report the presence of oxidative stress in prediabetic stages. For example, children have increased oxidative stress and SOD levels at the onset of type 1 diabetes (Guzik *et al*, 2002). In subjects with impaired glucose tolerance (IGT), the increased level of inflammatory cytokines is linked to oxidative processes (Exposito *et al*, 2002). Increased levels of vitamin A, increased TBARS, increased isoprostanes, and reduced antioxidants have all been reported in IGT (Vijayalingam *et al*, 1996). Data on antioxidants are variable, since, according to the origin of the insult and tissue/cell type, antioxidants may even be increased (Paget *et al*, 1998). In view of the data on insulin and some lipid fractions, it is likely that normoglycemic insulin-resistant subjects may already exhibit oxidative stress. Indeed, obesity, characterized by hyperinsulinemia and dyslipidemia, is accompanied by elevated oxidative stress (Ciccone *et al*, 1999). Even in a healthy population, variations in insulin sensitivity are related to lipid hydroperoxide levels and reduced catalase and vitamin E levels (Facchini *et al*, 2000). Low vitamin E levels had also been reported to be better predictors of

diabetes than age, BMI or smoking (Salonen *et al*, 1995). Thus, oxidative stress may be a very early event in the long history of diabetes, similar to what is reported for functional microvascular defects (Wiernsperger, 2000). That oxidative stress can precipitate diabetes development is suggested by an experiment showing the appearance of fasting hyperglycemia within days after administration of a pro-oxidant to insulin resistant, obese Zucker rats (Laight *et al*, 2000). It is therefore conceivable that chronic exposure of insulin resistant tissues to oxidative stress, generated, for example, by the daily iterative postprandial periods might constitute an important factor in the aetiology of diabetes.

2.8.4 Diabetes and Organ-Wide Oxidative Stress

Evidence that oxidative stress is present in diabetes originates from the frequent observation that both ROS and antioxidants are increased. The latter is logically seen in early stages of diabetes and is interpreted as a tentative compensation of cells against increasing oxidative stress (Zobali *et al*, 2002). According to tissue and cell type, the nature of antioxidant elevation may vary, indicating specificities that may be important for therapeutic interventions (Wiernsperger, 2003). Countless publications exist showing the existence of various indicators of oxidative stress *in vitro*, and this has finally led to an ongoing debate about the pertinence and relevance of parameters such as thiobarbituric acid reactive substances (TBARS), malondialdehyde, isoprostanes and nitrotyrosines as typical examples (De Zwart *et al*, 1999). Oral intake of high glucose in animals increases TBARS and reduces the activity of hepatic enzymes susceptible to thiol group oxidation (Folmer *et al*, 2002). In humans, oxidative stress is also seen in postprandial periods in normal individuals but diabetic patients are unable to compensate for the increased ROS (Ceriello *et al*, 1998). This increase may be attributed to acute effects of high glucose and/or lipids. Increased oxidative stress is also found in the basal state in both types of diabetes, and certain studies suggest that

oxidative stress is much more pronounced in type 2 than in type 1 diabetes (Seghrouchni *et al*, 2002).

2.9 Streptozotocin

Streptozotocin (STZ) is an antimicrobial agent produced by *Streptomyces achromogenes*; and is used as a chemotherapeutic alkylating agent (Schein *et al*, 1974). In 1963, Rakieten *et al* reported that STZ is diabetogenic. This insulinopenia syndrome, called streptozotocin diabetes, is caused by the specific necrosis of the pancreatic β cells; and streptozotocin has been the agent of choice for the induction of diabetes mellitus in animals ever since (Arison *et al*, 1967).

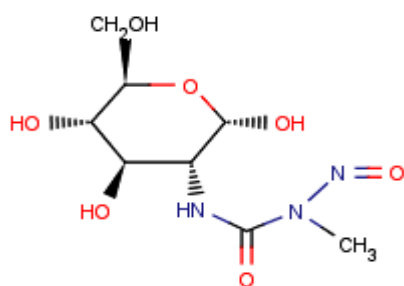


Figure 5: Molecular structure of streptozotocin (Lenzen, 2008)

2.9.1 Mechanism of Action of Streptozotocin

Streptozotocin inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus. Both effects can be attributed to its specific chemical properties, namely its alkylating potency (Fig. 6). As with alloxan, its beta cell specificity is mainly the result of selective cellular uptake and accumulation (Lenzen, 2008).

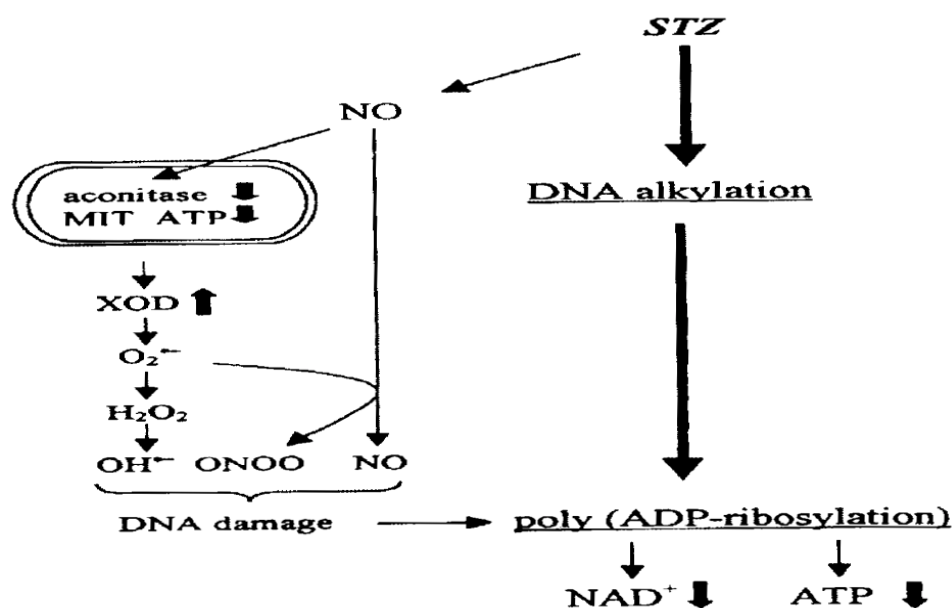


Figure 6: The mechanism of streptozotocin-induced toxic events in β cells of rat pancreas. MIT: Mitochondria; XOD: xanthine oxidase; NO: nitric oxide. (Szkudelski, 2001).

2.9.2 Beta-Cell Selectivity of Streptozotocin

Streptozotocin is a nitrosourea analogue in which the *N*-methyl-*N*-nitrosourea (MNU) moiety is linked to the carbon-2 of a hexose (Lenzen, 2008). The toxic action of STZ and chemically related alkylating compounds requires their uptake into the cells. Nitrosoureas are usually lipophilic and tissue uptake through the plasma membrane is rapid. However, as a result of the hexose substitution, STZ is less lipophilic (Lenzen, 2008). Streptozotocin is selectively accumulated in pancreatic beta cells via the low-affinity GLUT-2 glucose transporter in the plasma membrane (Tjälve *et al*, 1976). Thus, cells that do not express this glucose transporter are resistant to STZ (Elsner *et al*, 2000). This observation also explains the greater toxicity of STZ compared with *N*-methyl-*N*-nitrosourea in cells that express GLUT 2, even though both substances alkylate DNA to a similar extent (Ledoux *et al*, 1986). The importance of the GLUT 2 glucose transporter in this process is also shown by the observation that STZ damages other organs expressing this transporter, particularly kidney and liver (Rerup, 1970).

2.9.3 Beta-Cell Toxicity of Streptozotocin

It is generally assumed that the toxicity of streptozotocin is dependent upon the DNA alkylating activity of its methylnitrosourea moiety (Bennett and Pegg, 1981), especially at the O⁶ position of guanine (Lenzen, 2008). The transfer of the methyl group from streptozotocin to the DNA molecule causes damage, which, along a defined chain of events (Pieper *et al*, 1999), results in the fragmentation of the DNA (Yamamoto *et al*, 1981). Protein glycosylation may be an additional damaging factor (Konrad & Kudlow, 2002). In the attempt to repair DNA, poly(ADP-ribose) polymerase (PARP) is overstimulated. This diminishes cellular NAD⁺, and subsequently ATP stores (Yamamoto *et al*, 1981). The depletion of the cellular energy stores ultimately results in β -cell necrosis. Although STZ also methylates proteins (Bennett and Pegg, 1981), DNA methylation is ultimately responsible for β -cell death; but it is likely that protein methylation contributes to the functional defects of the β cell after exposure to STZ (Lenzen, 2008). Inhibitors of poly ADP-ribosylation suppress the process of DNA methylation. Thus, injection of nicotinamide and other PARP inhibitors in parallel with, or prior to the administration of STZ is well known to protect β cells against the toxic action of STZ, and to prevent the development of a diabetic state (Schein *et al*, 1967). Also, mice deficient in PARP are resistant to β -cell death mediated by STZ, in spite of DNA fragmentation. The absence of PARP prevents the depletion of the cofactor NAD⁺ and the subsequent loss of ATP (Pieper *et al*, 1999), and thus cell death. The role of alkylation in β -cell damage has also been examined by the use of ethylating agents, which are less toxic than their methylating counterparts, on account of O⁶- ethylguanine being less toxic than O⁶-methylguanine (Delaney *et al*, 1995). The fact that *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate are significantly less toxic to insulin-producing cells than MNU and methyl methanesulphonate (Delaney *et al*, 1995) has been taken as support for the notion that in insulin-producing cells, as in other cell types, the mechanism of toxic action is due to

alkylation, with methylation of DNA bases being more toxic than ethylation (Bennett and Pegg, 1981). An alternative hypothesis proposes that part of the diabetogenic effect of streptozotocin may relate not to its alkylating ability but to its potential to act as an intracellular nitric oxide (NO) donor (Turk *et al*, 1993). Both streptozotocin and MNU contain a nitroso group (Fig. 5) and can liberate NO. In fact, STZ has been shown to increase the activity of guanylyl cyclase and the formation of cGMP, which are characteristic effects of NO. However, the alkylating agent methyl methanesulphonate is the most toxic compound, though unlike MNU, it is not a NO donor (Delaney *et al*, 1995), indicating that NO is not an indispensable prerequisite for the toxic action of the family of alkylating agents to which STZ belongs. Finally, some minor generation of ROS, including superoxide and hydroxyl radicals originating from hydrogen peroxide dismutation during hypoxanthine metabolism (Nukatsuka *et al*, 1990), may accompany the effect of STZ and accelerate the process of β -cell destruction.

2.9.4 Inhibition of Insulin Secretion by Streptozotocin

The effects of STZ on glucose and insulin homeostasis reflect the toxin-induced abnormalities in β -cell function. Initially, insulin biosynthesis, glucose-induced insulin secretion and glucose metabolism (both glucose oxidation and oxygen consumption) are all affected (Nukatsuka *et al*, 1990). On the other hand, STZ has no immediate, direct inhibitory effect on glucose transport (Elsner *et al*, 2000) or on glucose phosphorylation by glucokinase (Lenzen *et al*, 1987). However, at later stages of functional β -cell impairment, deficiencies in terms of gene expression and protein production lead to the deterioration of both glucose transport and metabolism (Wang and Gleichmann, 1998). Even before the negative effects of mitochondrial DNA and protein alkylation and glycosylation become evident, streptozotocin-induced depletion of NAD⁺ may result in the inhibition of insulin biosynthesis and secretion

(Yamamoto *et al*, 1981). Later, inhibition of glucose-induced and amino acid-induced insulin secretion as a result of mitochondrial enzyme dysfunction (Rasschaert *et al*, 1992) and damage to the mitochondrial genome (Eizirik *et al*, 1991) become apparent. This impairment is more marked for nutrient- than for non-nutrient-induced insulin secretion. This interpretation has been confirmed through studies which have shown that pre-treatment of isolated pancreatic islets with the PARP inhibitor, nicotinamide, prevents early inhibition of β -cell function during the first day after STZ exposure; while long-term inhibition of insulin secretion six days after STZ exposure was not counteracted by nicotinamide (Strandell *et al*, 1989).

2.10 *Azadirachta indica* A. Juss (Neem)

Azadirachta indica (A. Juss) is an evergreen tree of the Mahogany family. It is native to the Indian subcontinent and South-east Asia (Pennington, 1981). The other specie of the Meliaceae family, which is closely related to *A. indica*, is *M. azadirach*. *Azadirachta indica* (A. Juss) is also known as Indian neem, margosa tree, Indian lilac, *dogoyaro* (Hausa), *igi Oba* (Yoruba), etc. Taxonomic details of neem are as follows:

Order	Rutales
Suborder	Rutinae
Family	Meliaceae (Mahogany family)
Subfamily	Melioideae
Tribe	Meliaeae
Genus	<i>Azadirachta</i>
Specie	<i>indica</i>



Figure 7: *Azadirachta indica* (A. Juss).

Though of Indo-Malayan origin (Pennington, 1981), neem is generally found in the Tropics, including Nigeria, etc. Almost all parts of the tree – stem, bark, roots, leaves, seed, fruits, flowers, etc, are used as traditional medicines (Schmutterer, 1995). In India, neem is referred to as the ‘village dispensary’ owing to its multiple medicinal uses (Brahmachari, 2005). Detailed phytochemical analysis of the stem, roots, seeds, seed coats and leaves of neem had been done by Farhana (2002) and Siddiqui *et al* (2004) (tables 4 and 5). Besides, recent review by Brahmachari (2005) outlined the major pharmacologically-active principles of neem to include isoprenoids (diterpenoids and triterpenoids) and non-isoprenoids (including polyphenolics such as flavonoids and their glycosides). The most abundant bioflavonoids in neem have been reported to include quercetin, rutin, myricetin, kaempferol, and their glycosides (Chattopadhyay, 1999).

Table 4. Terpenoidal and steroidal constituents of *Azadirachta indica* (Farhana, 2002; Rasheed, 2002)

SN	Name of Constituent	Molecular formula	Molecular weight	Plant material
1	Nimbiol	C ₁₈ H ₂₄ O ₂	272.386	Trunk bark
2	Nimbidiol	C ₁₇ H ₂₂ O ₃	274.395	Root bark
3	Nimbione	C ₁₈ H ₂₂ O ₃	286.370	Stem bark
4	methylnimbiol	C ₁₉ H ₂₆ O ₂	286.413	Stem bark
5	Nimbisonol	C ₁₈ H ₂₄ O ₃	288.0	Stem bark
6	Margosolone	C ₁₇ H ₂₂ O ₄	288.386	Stem bark
7	Demethylnimbionol	C ₁₉ H ₂₄ O ₃	290.0	Stem bark
8	Margolone	C ₁₉ H ₂₄ O ₃	300.397	Stem bark
9	Nimbosodione	C ₂₀ H ₂₈ O ₂	300.397	Stem bark
10	Sugiol (7 oxoferruginol)	C ₂₀ H ₂₈ O ₂	300.0	Trunk bark
11	Nimbosone	C ₂₀ H ₂₈ O ₂	300.440	Stem bark
12	Nimbonone	C ₂₀ H ₂₈ O ₂	300.440	Stem bark
13	nimbonolone	C ₂₀ H ₂₈ O ₂	300.440	Stem bark
14	Margosin	C ₂₀ H ₂₈ O ₂	300.440	Root bark
15	Azadirin A	C ₂₀ H ₂₈ O ₂	300.0	Root bark
16	Azadirin B	C ₂₀ H ₂₈ O ₂	300.0	Root bark
17	Nimbinone	C ₁₈ H ₂₂ O ₄	302.0	Stem bark
18	Nimbionone	C ₁₈ H ₂₂ O	302.0	Trunk bark
19	Nimbocidin	C ₂₀ H ₃₀ O ₂	302.0	Root bark
20	Nimbionol	C ₁₈ H ₂₄ O ₄	304.0	Stem bark
21	Nimbilicin	C ₂₀ H ₂₄ O ₃	312.408	Stem bark
22	Margolonone	C ₁₉ H ₂₂ O ₄	314.380	Stem bark

23	isomargolonone	C ₁₉ H ₂₂ O ₄	314.380	Stem bark
24	Nimosone	C ₂₀ H ₂₆ O ₃	314.0	Stem bark
25	Azadirilin	C ₂₀ H ₂₆ O ₃	314.0	Root bark
26	Methylnimbionone	C ₁₉ H ₂₄ O ₄	316.0	Stem bark
27	Margosone	C ₂₀ H ₂₈ O ₃	316.439	Stem bark
28	Nimolinin	C ₂₀ H ₂₈ O ₃	316.0	Root bark
29	Margocilin	C ₂₀ H ₂₈ O ₃	316.0	Root bark
30	Nimbocinin	C ₁₉ H ₂₆ O ₄	318.0	Root bark
31	Margocinin	C ₂₀ H ₂₆ O ₄	330.0	Root bark
32	Desacetylnimbinolide	C ₂₈ H ₃₄ O ₁₀	350.0	Twigs
33	Desfuranoazadiradione	C ₂₄ H ₃₂ O ₄	384.514	Fresh ripe fruits
34	7 α -Acetoxy-4,4,8-trimethyl-5 α -(13 α Me)-17-oxo-androsta-1,14-diene-3,16-dione	C ₂₃ H ₃₀ O ₅	386.487	Fresh ripe fruits
35	7 α -Acetoxy-4,4,8-trimethyl-5 α -17-oxa-androsta-1,14-dien-3,16-dione	C ₂₃ H ₃₀ O ₅	386.211	Fresh ripe fruits
36	Nimbocinol	C ₂₆ H ₃₂ O ₄	408.536	Fruits
37	17-epinimbocinol	C ₂₆ H ₃₂ O ₄	408.536	Oil
38	Meldenindiol	C ₂₆ H ₃₄ O ₄	410.0	Fresh leaves
39	4 α -methyl-5 α -ergosta-8,24,(28)-dien-3 β -ol	C ₂₉ H ₄₈ O	412.0	Heart wood
40	β -Sitosterol	C ₂₉ H ₅₀ O	414.0	Blossoms, cotyledon, wood oil
41	7-Deacetyl-17 β -hydroxyazadiradione	C ₁₆ H ₃₂ O ₅	424.536	Seeds

42	Limocinone	C ₃₀ H ₄₈ O	424.370	Fruits
43	Limoconol	C ₃₀ H ₅₀ O	426.386	Fruits
44	Cycloeucalenol	C ₃₀ H ₅₀ O	426.0	Wood oil
45	4,14 α -Dimethyl-5 α -ergosta-8,24(28)-dien-3 β -ol	C ₃₀ H ₅₀ O	426.0	Heart wood
46	Margocidin	C ₂₉ H ₄₈ O ₂	428.446	Root bark
47	Vilasinin	C ₂₆ H ₃₆ O ₅	428.567	Leaves
48	Azadirone	C ₂₈ H ₃₆ O ₄	436.590	Oil
49	Neeflone	C ₂₈ H ₃₆ O ₄	438.606	Flowers
50	7-Deacetyl-7 α -hydroxygedunin	C ₂₆ H ₃₂ O ₆	440.0	Oil
51	6-Deacetylnimbiene	C ₂₆ H ₃₂ O ₆	440.536	Bark, leaves, oil
52	24,Methylene-cycloartenol	C ₃₁ H ₅₂ O	440.0	Wood oil
53	Nimbidinin	C ₂₆ H ₃₄ O ₆	442.551	Oil
54	Nimolicinoic acid	C ₂₆ H ₃₄ O ₆	442.551	Fruits
55	Epoxyazadiradione	C ₂₈ H ₃₄ O ₆	446.573	Oil
56	17-hydroxyazadiradione	C ₂₈ H ₃₄ O ₆	446.573	Fruits
57	Nimbolide	C ₂₇ H ₃₀ O ₇	446.530	Leaves
58	azadiradione	C ₂₈ H ₃₄ O ₅	450.574	Seed oil
59	Nimolicin	C ₂₈ H ₃₄ O ₅	450.574	Fruit
60	17-Epiaazadiradione	C ₂₈ H ₃₄ O ₅	450.574	Fruit
61	28-Deoxonimbolide	C ₂₇ H ₃₃ O ₆	452.546	Leaves
62	Vepinin	C ₂₈ H ₃₆ O ₅	452.589	Oil
63	Trichilenone	C ₂₈ H ₃₆ O ₅	452.586	Seeds

64	Nimocinol	C ₂₈ H ₃₆ O ₃	452.589	Leaves
65	Nimolinone	C ₃₀ H ₄₄ O ₅	452.676	Fruits
66	Meldenin	C ₂₈ H ₃₈ O ₅	454.605	Leaves
67	Isomeldenin	C ₂₈ H ₃₈ O ₅	454.605	Leaves
68	Azadironic acid	C ₂₈ H ₃₈ O ₅	454.0	Fruit coat
69	Nimbandiol	C ₂₆ H ₃₂ O ₇	456.535	Leaves, oil
70	Nimbidic acid (salannic acid)	C ₂₆ H ₃₄ O ₇	458.550	Oil
71	Azadiradionolide	C ₂₈ H ₃₄ O ₆	460.0	Fruit coat
72	Epoxyazadiradione	C ₂₈ H ₃₄ O ₆	460.0	Seeds
73	6-Deacetylnimbinal	C ₂₇ H ₃₂ O ₇	468.456	Leaves
74	4 α ,6 α - Dihydroxyhomoazadirone	C ₂₈ H ₃₆ O ₆	468.0	Leaves
75	Azadironolide	C ₂₈ H ₃₆ O ₆	468.589	Fruit coat
76	isoazadironolide	C ₂₈ H ₃₆ O ₆	468.589	Fruit coat
77	Limocin A	C ₂₉ H ₄₂ O ₅	470.648	Fruits
78	Limocin B	C ₂₉ H ₄₂ O ₅	470.648	Fruits
79	Nimbocinone	C ₃₀ H ₄₈ O ₄	472.0	Leaves
80	Odoratone	C ₃₀ H ₄₈ O ₄	472.0	Seeds
81	Nimolicinol	C ₂₈ H ₃₄ O ₇	482.572	Fruits
82	Gedunin	C ₂₈ H ₃₄ O ₇	482.572	Oil
83	1,2,14,15-Diepoxy- azadiradione	C ₂₈ H ₃₄ O ₇	482.572	Seeds
84	Nimbione	C ₂₈ H ₃₄ O ₇	482.572	Bark, leaves, oil
85	Margosinolide	C ₂₇ H ₃₂ O ₈	484.545	Twigs
86	Isomargosinolide	C ₂₇ H ₃₂ O ₈	484.545	Twigs

87	Nimocinolide	C ₂₈ H ₃₆ O ₇	484.588	Leaves
88	isonimocinolide	C ₂₈ H ₃₆ O ₇	484.588	Leaves
89	Azadiradionol	C ₃₀ H ₄₄ O ₅	484.0	Fresh fruit coat
90	Limocin C	C ₃₀ H ₄₄ O ₅	484.3158	Fruit coat
91	Limocin D	C ₃₀ H ₄₄ O ₅	484.3158	Fruit coats
92	Meliantriol	C ₃₀ H ₅₀ O ₅	490.0	Seed oil
93	Desacetylnimbin	C ₂₈ H ₃₄ O ₈	498.0	Bark, seeds
94	6-0-acetylnimbandiol	C ₂₈ H ₃₄ O ₈	498.572	Seed oil, leaves, bark
95	1,2-Dihydro-1 α -methoxy-epoxyazadiradione	C ₂₉ H ₃₈ O ₇	498.615	Seeds
96	Nimolide	C ₂₉ H ₃₈ O ₇	498.615	Twigs
97	Isonimolide	C ₂₉ H ₃₈ O ₇	498.615	Twigs
98	Nimocin	C ₃₃ H ₃₈ O ₄	498.661	Fruits
99	Azadironic acid	C ₃₀ H ₄₂ O ₄	498.0	Fresh fruit coats
100	Nimbanal	C ₂₉ H ₃₄ O ₈	510.583	Seeds
101	Nimbinol	C ₂₉ H ₃₆ O ₈	512.599	Seed kernels
102	1,3-Diacetylvilasinin	C ₃₀ H ₄₀ O ₇	512.642	Seed oil
103	7-Deacetyl-7 α -benzoylazadiradione	C ₃₃ H ₃₆ O ₅	512.0	Seeds
104	Salimuzzalin	C ₃₀ H ₄₀ O ₇	512.0	Fresh fruit coats
105	Meliacinin	C ₃₁ H ₄₄ O ₆	512.3145	Fresh fruit coat
106	Azadironol	C ₃₂ H ₄₂ O ₆	522.0	Fresh fruit coat
107	Melialactol	C ₃₇ H ₅₀ O ₉	522.2972	Fresh fruit

				coat
108	Azadirachtol	C ₃₂ H ₁₆ O ₆	526.712	Fruits
109	Mahmoodin	C ₃₀ H ₃₈ O ₈	526.0	Fresh fruit coat
110	Naheedin	C ₃₂ H ₄₈ O ₆	528.0	Fresh fruit coat
111	Desacetylnimbinolide	C ₂₈ H ₃₄ O ₁₀	530.571	Twigs
112	Desacetyl isonimbinolide	C ₂₈ H ₃₄ O ₁₀	530.571	Twigs
113	Nimbin	C ₃₉ H ₃₆ O ₉	540.509	Oil, root bark, cotyledon, trunk
114	Isonimolicinolide	C ₃₀ H ₃₆ O ₉	540.509	fruits
115	4-epinimbin	C ₃₀ H ₃₆ O ₉	540.509	--
116	Isolimbolide	C ₃₀ H ₃₈ O ₉	542.525	Twigs
117	7-deacetyl-7 α -benzoylgedunin	C ₃₃ H ₃₆ O ₇	544.0	Seeds
118	Azadirol	C ₃₂ H ₄₈ O ₇	544.727	Fresh fruit coat
119	Limocinin	C ₃₄ H ₄₄ O ₆	548.0	Fresh fruit coat
120	3-deacetylsalannol	C ₃₂ H ₄₂ O ₈	554.679	Seed oil
121	2',3'-dehydrosalannol	C ₃₂ H ₄₂ O ₈	554.0	Leaves
122	Nimbinlactone	C ₃₀ H ₃₆ O ₁₀	556.0	Oil
123	Salannol	C ₃₂ H ₄₄ O ₈	556.695	Seed oil
124	Deoxyazadiratolide	C ₃₃ H ₄₈ O ₇	556.738	Leaves
125	Azadirachtolide	C ₃₃ H ₄₆ O ₈	570.722	Leaves
126	3-acetoxy-7-tigloylvilasininlactone	C ₃₃ H ₄₆ O ₈	570.0	Seed

127	isonimbinolide	C ₃₀ H ₃₆ O ₁₁	572.608	Stem bark
128	B-sitosterol-β-D-glucoside	C ₃₅ H ₆₀ O ₆	576.0	Blossoms, heart wood, leaves
129	Nimbocinolide	C ₃₂ H ₄₂ O ₁₀	586.678	Leaves
130	isonimbocinolide	C ₃₂ H ₄₂ O ₁₀	586.678	Leaves
131	Isoazadirolide	C ₃₂ H ₄₂ O ₁₀	586.0	
132	3-acetylsalannol	C ₃₄ H ₃₆ O ₉	588.0	Leaves
133	Salannin	C ₃₄ H ₄₄ O ₉	596.716	Seeds
134	Azadirachtanin	C ₃₂ H ₄₀ O ₁₁	600.0	Seed oil
135	Salannolactam-(21)	C ₃₄ H ₄₅ NO ₉	611.731	Leaves
136	Salannolactam-(23)	C ₃₄ H ₄₅ NO ₉	611.731	Seed kernel
137	salanninlactone	C ₃₄ H ₄₄ O ₁₀	612.0	Oil
138	Azadirachtin 1	C ₃₂ H ₄₂ O ₁₂	618.677	Seed kernel
139	1,3,-Diacetyl11,19-deoxa-11-oxo-meliacarpin	C ₃₁ H ₄₀ O ₁₃	620.649	Seeds
140	3α-Acetoxy-1-α-hydroxyazadirachtol	C ₃₀ H ₃₈ O ₁₄	622.622	Seeds
141	Salannolide	C ₃₄ H ₄₄ O ₁₁	628.0	Trunk wood
142	Limbocinin	C ₃₃ H ₄₀ O ₁₂	628.0	Fruit coat
143	Nimoblin A	C ₃₉ H ₄₆ O ₈	642.788	Trunk wood
144	Limbocidin	C ₃₃ H ₄₀ O ₁₄	660.0	Fruit coat
145	Nimbocin	C ₄₀ H ₅₄ O ₈	662.0	Seeds
146	Azadirachtin H	C ₃₃ H ₄₂ O ₁₄	662.686	Seed kernels
147	11α-Hydroxy-12-norazadirachtin	C ₃₃ H ₄₂ O ₁₄	662.686	Seeds
148	Azadirachtin K	C ₃₄ H ₄₀ O ₁₅	668.681	Seed kernels

149	Nimbolin B	C ₃₉ H ₄₆ O ₁₀	674.786	Trunk wood
150	Azadirinin	C ₃₉ H ₄₆ O ₁₀	674.786	Root bark
151	1-Tiglogyl-3-acetyl 11 α -hydroxy-4- β -methyl-meliacarpin	C ₃₄ H ₄₄ O ₁₄	676.713	Seeds
152	Limbonin	C ₃₅ H ₄₈ O ₁₃	676.756	Fruits
153	11-epiazadirachtin D	C ₃₄ H ₄₄ O ₁₄	676.713	Seeds
154	Deacetylazadirachtinol	C ₃₃ H ₄₄ O ₁₅	680.0	Fresh fruit oil
155	Nimbolicin	C ₄₁ H ₄₈ O ₁₀	700.0	Root bark
156	13,14-desepoxy-azadirachtin A	C ₃₅ H ₄₄ O ₁₅	704.724	Seed kernel
157	Nimbilin	C ₄₂ H ₅₀ O ₁₀	714.0	Root bark
158	Azadirachtin A	C ₃₅ H ₄₄ O ₁₆	720.723	Neem kernels
159	Melianol	C ₃₅ H ₄₈ O ₉	612.327	Leaves
160	Desfurano-6 α -hydroxy-azadiradione	C ₂₄ H ₃₂ O ₅	400.230	Leaves
161	Zeeshanol	C ₂₉ H ₄₀ O ₆	484.273	Leaves
162	7 α -senecieryl(7-deacetyl)-23-O-methylnimocinolide	C ₃₂ H ₄₂ O ₇	538.0	Leaves
163	22,23-Dihydronimocinol	C ₂₈ H ₃₈ O ₅	454.264	Leaves
164	Zafaral	C ₂₉ H ₄₀ O ₆	484.279	Leaves
165	23-O-Methylnimocinolide	C ₂₉ H ₃₈ O ₇	498.259	Leaves
166	Meliacinol	C ₃₂ H ₄₂ O ₈	554.291	Leaves
167	Desfurano-desacetylnimbin-17-one	C ₂₄ H ₃₀ O ₈	446.190	Leaves

168	Meliatetraone	C ₂₉ H ₃₆ O ₇	496.244	Leaves
169	Meliatetraolenone	C ₃₂ H ₄₂ O ₁₀	586.0	Leaves
170	Meliacinanhydride	C ₃₁ H ₃₈ O ₁₀	570.0	Leaves

Table 5. Non-terpenoidal/steroidal constituents of *Azadirachta indica* (Farhana, 2002; Rasheed, 2002)

SN	Name of constituent	Molecular formula	Molecular weight	Plant material
1	5-hydroxymethyl-furfural	C ₆ H ₆ O ₃	126.0	Fruits
2	Methylgrevillate	C ₁₀ H ₁₀ O ₄	194.0	Stem bark
3	6-hydroxymellcin	C ₁₀ H ₁₀ O ₄	194.187	Twigs
4	Margoccin	C ₁₂ H ₁₄ O ₄	222.24	Twigs
5	Tridecylbenzene	C ₁₉ H ₃₂	260.462	Leaves
6	Kaempferol	C ₁₅ H ₁₀ O ₆	286.0	Blossoms
7	Quercetin	C ₁₅ H ₁₀ O ₇	302.0	Blossoms, leaves
8	Myricetin	C ₁₅ H ₁₀ O ₈	318.0	Blossoms
9	Nimbothalin	C ₂₃ H ₂₆ O ₄	366.0	--
10	Nimbocetin	C ₂₄ H ₂₈ O ₄	390.0	Fruits
11	Nimbaflavone	C ₂₆ H ₂₀ O ₅	422.52	Leaves
12	Nimbochalicin	C ₂₂ H ₂₆ O ₉	434.0	Fruits
13	2,3-Dihydroxy-3'-(3-hydroxy-3-methyl-butyl)-4'-methoxy-8-prenyl flavone	C ₂₆ H ₃₂ O ₆	440.535	Leaves
14	Quercitrin	C ₂₁ H ₂₀ O ₁₁	448.685	Leaves
15	Kaempferol-3-	C ₂₁ H ₂₀ O ₁₁	448.685	Blossoms

	glucoside(Astragolin)			
16	Glucoside of kaempferol	C ₂₁ H ₂₀ O ₁₁	448.685	Leaves
17	Margosinolone	C ₂₈ H ₅₀ O ₄	448.685	Stem bark
18	Margosinone	C ₂₈ H ₄₈ O ₄	448.685	Stem bark
19	Melicitrin	C ₂₀ H ₁₈ O ₁₂	450.0	Blossoms
20	Nimilicidin	C ₂₉ H ₆₀ O ₃	456.0	Root bark
21	Glucoside f quercetin	C ₂₁ H ₂₀ O ₁₂	464.0	Leaves
22	Quercetin-3-galactoside (hyperin)	C ₂₁ H ₂₀ O ₁₂	464.0	Blossom, leaves
23	rutin	C ₂₇ H ₃₀ O ₁₆	610.0	Leaves

Various parts of *A. indica* had been reported to possess hypoglycaemic effects (Khosla *et al*, 2000), antiulcer effects, antimalarial effect, anticarcinogenic and antioxidant activity (Arivazhagan *et al*, 2000). Other reported medicinal uses of neem include hepatoprotective activity against paracetamol-induced liver damage (Chattopadhyay, 2003); anxiolytic activity (Jaiswal *et al*, 1994); diuretic function (Abraham *et al*, 1986); and contraceptive importance (Aladakatti *et al*, 2001). *Azadirachta indica* has always been and will continue to be part of herb-based therapies for human use.

With respect to diabetes mellitus, a number of animal and human studies have reported the antidiabetic potential of herbal therapies, as reviewed recently by Dham *et al* (2006) and Abo *et al* (2008). Botanicals such as *Anarcadium occidentale* have been reported to possess hypoglycemic activity in fructose-induced diabetic rats (Olatunji *et al*, 2005). Recently, Adewole and Caxton-Martins (2006) reported hypoglycemic activity of the aqueous extract of the leaves of *Annona muricata* in STZ diabetic rats. Besides, the work of Ojewole and Drewes (2007) also showed that the hydro-ethanolic extract of *Eriosema kraussianum*

rootstock possessed hypoglycemic activity in STZ diabetic rats. Furthermore, STZ-induced mice pretreated with *Cortex cinnamomi* extract maintained normoglycemia; and this showed the antidiabetic effect of the extract (Kwon *et al*, 2006). Similarly, the hypoglycaemic activity of *Bauhinia forficata* was reported by da Cunha *et al* (2010).

Similarly, *Azadirachta indica* (A. Juss) had also been reported to possess hypoglycemic function in dogs (Murty *et al*, 1978), rabbits (Khosla *et al*, 2000), rats (Chattopadhyay, 1999; Ebong *et al*, 2008) and even humans (Hsia *et al*, 2004). Its hypoglycemic activity in rats had been reported to be comparable to glyburide (Rahman *et al*, 2005).

Chapter Three

Materials and Methods

3.1 Animals

One hundred male Wistar rats (Harlan, Milan, Italy) were used. Animals were 10 weeks old and weighed 175.5 g. Animals were kept in cages (5 animals per cage) and housed in the animal room of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Italy. Animals were exposed to 12 hour light, 12 hour dark cycle at a room temperature of 21-23 °C. They were maintained on Harlan Global Diet 2018 (Harlan, Milan, Italy). The composition of the feed are shown in table 6. Water was given freely, and feed intake was quantified. The study was approved by the Animal Use Committee of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Lecce, Italy.

Table 6. Composition of Harlan Global Diet 2018

Feed Composition	Quantity (%)
Protein	18.5
Fat	5.5
Carbohydrate	57.3
Ash	6.0
Fibre	4.5

3.2 *Azadirachta indica* Leaves

3.2.1 Collection of *Azadirachta indica* Leaves

Mature fresh leaves of *Azadirachta indica* (A. Juss) were collected from neem trees in the premises of the University of Ilorin Mini Campus, Nigeria, between June and August 2007. A

sample of the collection was compared to the Voucher specimen at the herbarium of the Botany Department of the same University (Voucher Number 542).

3.2.2 Colourimetric Estimation of Total Flavonoid Content of *Azadirachta indica* Leaves

Total flavonoid content of the leaf powder of *A. indica* was determined by spectrophotometry. The reagents were prepared as described in section 8.1. HEPES buffer (40 mM, pH 7.4) was used as homogenizing buffer. Dry leaf powder of *A. indica* (3 g) was homogenized in 15 ml of HEPES-NaOH buffer using a potter homogenizer (GPE, Bedfordshire, England). An aliquot of the homogenate (0.3 g) was transferred into three Eppendorf tubes (Greiner Bio-One, Frickenhausen, Germany), and 1.5 ml of absolute methanol was added to each. The solutions were mixed overnight at 300 rpm on a rotary mixer (Chemic, ALS, Milan, Italy) at 4 °C. Thereafter, they were centrifuged at 10,000 rpm for 10 min in a Sigma benchtop centrifuge (Celbio, Milan, Italy) at 4 °C. Each supernatant was transferred into separate tubes and evaporated to dryness in the water bath at 40 °C. The residue from each was suspended in 200 µl of absolute methanol and an aliquot of 50 µl was placed in an Eppendorf tube. The volume was made up to 500 µl with bidistilled water. This was followed by the addition of 30 µl of 5% NaNO₃, and the solution was allowed to stand for 5 min. Thereafter, 60 µl of 10% AlCl₃ was added and the solution was again allowed to stand (for 6 min). Then, 200 µl of 1 M NaOH and 210 µl of bidistilled water were added. The solution was vortex-mixed, and absorbance was read at 361 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Absolute methanol constituted the blank, while rutin (Sigma, MO, USA) served as standard.

3.2.3 Extraction of *Azadirachta indica* Leaves

Fresh leaves of *A. indica* were air-dried (under shade) and extracted by percolation as previously described (Chattopadhyay and Bandyopadhyay, 2005). A total of 2.4 kg of the dry leaf powder was extracted at room temperature with 70% ethanol. The initial ethanolic solution of the extract was concentrated under vacuum in a Buchi rotavapor R-114 (Buchi, Switzerland) at 50 °C (bath temperature). This concentrated extract was dissolved in bidistilled water and filtered with Whatman No.1 paper. The filtrate was then concentrated in the rotavapor at 50 °C (bath temperature). The final residue (a total of 120 g) was a dark-brown mass. This was stored at 4 °C.

3.3 Induction of Diabetes Mellitus

Hyperglycemia was induced in 64 overnight-fasted, randomly selected animals by a single intraperitoneal injection of streptozotocin (Sigma, MO, USA) at 70 mg/kg bw (Lal *et al*, 2000). Streptozotocin (STZ) was dissolved in citrate buffer (0.1 M, pH 4.5) just prior to injection. Animals were allowed free access to feed and water after STZ injection. Hyperglycemia was allowed to develop for at least 72 hours (Lenzen, 2008). Animals with fasting blood glucose ≥ 250 mg/dl were considered hyperglycemic (Gupta *et al*, 2004) and were thus included in the study. Control animals (n=18) received a single intraperitoneal injection of 0.1 M citrate buffer (1 ml/kg bw; pH 4.5). The reagents used are shown in section 8.2.

3.4 *Azadirachta indica* Treatment

The dose of ethanolic leaf extract of *Azadirachta indica* used in this study was based on the pilot study on this work, and is supported by the report of Chattopadhyay (1999) and Rahman *et al* (2005). This dose was much less than the oral LD50 of 4.57 g/kg bw reported for ethanolic leaf extract of neem in acute toxicity study in rats (Chattopadhyay, 1998). Ethanolic

extract of *A. indica* was dissolved in physiological saline (to make a stock) and was administered by gavage to a group of hyperglycemic rats (n=18) at 500 mg/kg bw/d (at 9.00-10.00 hour each day) for a maximum of 50 d. Fresh stock solution was prepared each week and stored at 4 °C. A biguanide, metformin, (Pharmacia and Upjohn, Milan, Italy) was given at 350 mg/kg bw/d (Kakemi *et al*, 1983; Penicaud *et al*, 1989) to another group of hyperglycemic Wistar rats (n=18) for a maximum of 50 d. This group served as a positive control and its blood glucose, histological findings, oxidative stress markers and plasma parameters were compared with those of neem-treated hyperglycemic animals.

Furthermore, a separate group of normoglycemic rats (n=18) were administered 500 mg/kg bw/d of *A. indica* only. Control rats comprised a group of normoglycemic rats (n=18) that received neither STZ nor neem. These rats received an intraperitoneal dose of 0.1 M citrated buffer (pH 4.5). Eighteen (18) hyperglycemic rats, which received neither *A. indica* nor metformin, constituted the diabetic group.

In addition, two groups of diabetic rats (n=5 each) were treated with 250 mg/kg bw/d and 750 mg/kg bw/d of neem leaf extract, respectively. Blood glucose responses to neem treatment at these doses were compared with the hypoglycemic effect of the extract at 500 mg/kg bw/d for a period of 21 d.

3.5 Measurement of Blood Glucose

Blood glucose was estimated in overnight-fasted rats at 9.00-10.00 hour. Blood glucose was measured with One Touch Ultra 2 Glucometer (Lifescan, CA, USA). Blood was obtained from the dorsal vein of the tail. At day 0 of *A. indica* and metformin treatment, blood glucose was monitored at alternate hours (0, 1, 3, 5, 7, 9 and 11 hour); then each day for the first three days; and thereafter, at 72-hour interval, for seven weeks.

3.6 Body Weight

Body weights of the rats were taken prior to the induction of hyperglycemia, and on a daily basis during treatment, for 7 weeks. Weight was taken with an electronic balance (EU-C500, Gibertini Elettronica, Milan, Italy).

3.7 Feed and Water Intake

The quantity of feed and water consumed on a daily basis were documented each morning (9:00 hour) prior to neem and biguanide (metformin) treatment.

3.8 Termination of Treatment

For the purpose of assessing the morphological and biochemical changes occurring in the untreated diabetic and neem-treated animals with the progression of diabetes, 6 rats were sacrificed in each group (control, diabetic, diabetic + neem, diabetic + metformin, and neem only) at 7 days (7 d), 21 days (21 d), and 50 days (50 d) of treatment. Animals were anaesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg bw) (Pharmacia and Upjohn, Milan, Italy). Laparotomy was performed and blood was drawn from the inferior vena cava with a 10-ml syringe (Novico, Barcelona, Italy) into heparinised BD vacutainersTM (BD Vacutainer Systems, Plymouth, UK). The blood was centrifuged at 860 x g (2000 rpm) in an Eppendorf 5810R refrigerated centrifuge (GMI, Minnesota, USA) at 4 °C for 10 minutes (Yousef *et al*, 2006). Plasma was collected into plain tubes and stored at –20 °C for analysis.

The pancreas, liver, kidney and intestine were also harvested, trimmed free of adipose tissue, rinsed in phosphate buffered solution (PBS) and fixed in pre-cooled Bouin's fluid and 4 % paraformaldehyde for histological processing. Portions of the liver and pancreas of the same animals were stored at -80 °C for the estimation of oxidative stress markers.

3.9 Plasma Analysis

Having collected the plasma as described in section 3.8, the following parameters were analysed: total cholesterol, HDL-c, LDL-c, VLDL-c, total triglyceride, insulin and biomarkers of hepatic and renal function.

3.9.1 Total Cholesterol

Plasma total cholesterol (free cholesterol + cholesteryl ester) was assayed in samples from all treatment and control groups at 7 d, 21 d, and 50 d of treatment using Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, CA, USA) according to the method of Allain *et al* (1974). In this assay, cholesteryl esterase hydrolyses cholesteryl ester into cholesterol. Cholesterol is oxidised to H_2O_2 by cholesterol oxidase. Hydrogen peroxide then reacts with a cholesterol probe to produce resorufin. The latter is detectable by spectrophotometry at O.D. 570 nm. The contents of the kit include: cholesterol reaction buffer, cholesterol probe, dimethylsulphoxide (DMSO), enzyme mix, cholesterol esterase, and cholesterol standard. Reagents were prepared as shown in section 8.3.

Standard Curve for Total Cholesterol

For cholesterol standard curve, cholesterol standard solution (5 $\mu\text{g}/\mu\text{l}$) was diluted to 0.5 $\mu\text{g}/\text{dl}$ by adding 20 μl cholesterol standard solution to 180 μl of cholesterol reaction buffer, followed by vortex-mixing. Thereafter, 0, 4, 8, 12, 16, and 20 μl of the cholesterol standard (0.5 $\mu\text{g}/\mu\text{l}$) was placed in separate wells (in duplicate), and the volume of each was made up to 50 $\mu\text{l}/\text{well}$ with cholesterol reaction buffer (to generate 0, 2, 4, 8, and 10 μg of cholesterol/well). To each of these wells was added 50 μl of the reaction mix.

Samples

For the samples (plasma), the assay was also done in duplicate. Into each well was placed 48 μl of cholesterol reaction buffer, followed by 2 μl of plasma (sample), and 50 μl of reaction

mix. The reaction was incubated at 37 °C for 60 min in a SANYO CO₂ incubator (SANYO, Milan, Italy). Absorbance was read at O.D. 570 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy). Total cholesterol levels of the samples were extrapolated from the cholesterol standard curve.

3.9.2 Plasma High Density Lipoprotein Cholesterol (HDL-C) and Low Density Lipoprotein/Very Low Density Lipoprotein Cholesterol (LDL-C/VLDL-C)

Estimation of plasma HDL-c and LDL/VLDL-c in the samples was done using the HDL and LDL/VLDL Cholesterol Quantitation Kit (BioVision, CA, USA) according to the method of Albers *et al* (1978). Contents of the kit are same as for cholesterol/cholesteryl ester quantitation kit except for an extra reagent – 2x LDL/VLDL precipitation buffer. Preparation of reagents and reaction mix was as described for total cholesterol assay in section 8.3. Cholesterol standard curve also followed similar procedures.

Samples

For the test samples (plasma), HDL-c and LDL-c/VLDL-c were first precipitated from the plasma using 2x LDL/VLDL precipitation buffer. In this procedure, 100 µl of 2x precipitation buffer was added to 100 µl of each plasma sample in a microcentrifuge tube. The mixture was incubated for 10 min at room temperature and centrifuged for 10 min at 2000 x g (5000 rpm) in a Sigma benchtop microcentrifuge (Celbio, Milan, Italy). The supernatant was transferred to plain tubes and these constituted the HDL-c fraction. The pellets were re-centrifuged as above and the trace supernatants were discarded. The pellets were dissolved in 200 µl of PBS, and these constituted the LDL-c/VLDL-c fraction. The fractions (HDL-c and LDL-c/VLDL-c) served as samples for the assay. Subsequent procedures were as described for total cholesterol assay (section 3.9.1).

Assay was done in duplicate. Cholesterol reaction buffer (48 µl) was placed in each well and to this was added 2 µl of the test sample (HDL-c fraction or LDL-c/VLDL-c fraction), followed by 50 µl of reaction mix. After vortex-mixing, the mixture was incubated at 37 °C for 60 min in an incubator (SANYO, Milan, Italy). Absorbance was read at 570 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy).

3.9.3 Plasma Total Triglyceride Assay

Plasma total triglyceride levels were determined using a triglyceride determination kit (Sigma, MO, USA), according to the method of McGowan *et al* (1983). The kit contains the following reagents:

- Triglyceride reagent (5x), 10 ml
 - 250,000 units/L lipase
 - 0.05 % sodium azide
- Free glycerol reagent (10x), 40 ml
 - 0.75 mM ATP
 - 3.75 mM magnesium salt
 - 0.188 mM 4-aminoantipyrine
 - 2.11 mM N-Ethyl-N-(3-sulphopropyl) m-anisidine, sodium salt
 - 1,250 units/L glycerol kinase
 - 2,500 units/L glycerol phosphate oxidase
 - 2,500 units/L peroxidase (horseradish)
 - Buffer, pH 7.0±0.1
 - 0.05 % sodium azide, added as preservative

Reagents were prepared as shown in section 8.4.

Assay Procedures for Total Plasma Triglyceride

- Labelled Eppendorf tubes (Greiner Bio-One, Frickenhausen, Germany) were set up in duplicate for the blank, standard and each of the samples;
- Into each tube was placed 1 ml of triglyceride working reagent, followed by the addition of 10 µl of deionised water, glycerol standard solution (2.5 mg/dl) and samples, into the tubes labelled blank, standard and samples respectively;
- Mixing was done by inversion, followed by incubation for 5 min at 37 °C. Absorbance was read at 540 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Deionised water served as blank.

Total plasma triglyceride concentration was estimated according to the following expression:

$$\text{Total triglyceride} = [(A_{\text{sample}} - A_{\text{blank}}) \times (\text{Concentration of standard})] / (A_{\text{standard}} - A_{\text{blank}})$$

Concentration of standard = 2.5 mg/dl; A=Absorbance.

Estimation of VLDL-c

$$\text{VLDL-c} = \text{Triglyceride}/5 \text{ (Friedwald } et al, 1972)$$

Estimation of LDL-c

$$\text{LDL-c} = (\text{LDL-c} + \text{VLDL-c}) - (\text{triglyceride}/5) = (\text{LDL-c} + \text{VLDL-c}) - \text{VLDL-c}$$

3.9.4 Plasma Insulin

Plasma insulin concentrations of each treatment group were estimated using Mercodia Ultrasensitive Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden) (Jaekel *et al*, 2004). The kit contains the following reagents:

- | | |
|--|---|
| • Coated 96-well plate (mouse monoclonal anti-insulin) | • Calibrator 1 (0.02 µg/l), 1 vial, 1000 µl |
| • Calibrator 0 (0.00 µg/l), 1 vial, 600 µl | • Calibrator 2 (0.05 µg/l), 1 vial, 1000 µl |

- Calibrator 3 (0.15 µg/l), 1 vial, 1000 µl
- Calibrator 4 (0.35 µg/l), 1 vial, 1000 µl
- Calibrator 5 (0.75 µg/l), 1 vial, 1000 µl
- Calibrator 6 (2.00 µg/l), 1 vial, 1000 µl
- Calibrator 7 (5.50 µg/l), 1 vial, 1000 µl
- Enzyme conjugate 11x, 600 µl
- Enzyme conjugate buffer, 6 ml
- Wash buffer 21x, 40 ml
- Substrate TMB, 22 ml
- Stop solution, 0.5 M H₂SO₄, 7 ml

Reagents were prepared as shown in section 8.5.

Assay Procedure for Plasma Insulin

All reagents were brought to room temperature and the assay was performed using the mouse monoclonal anti-insulin-coated 96-well plate. Assay was performed as summarised below.

- Wells were designated (in duplicate) as calibrators (standard curve) and samples (plasma)
- 25 µl of calibrator 0 was added to each well (calibrator and sample wells)
- 5 µl of each of calibrator 0 and calibrators 1–7 was added to the respective calibrator wells
- 5 µl of each of the samples (plasma) was added to each of the sample wells
- Enzyme conjugate solution (50 µl) was then added to each of the wells (calibrator and sample wells)
- Incubation was done (with gentle mixing on a shaker) at room temperature (22 °C) for 2 hours, followed by repeated washing of the wells six times with 350 µl of the wash buffer. For each wash, the content of the wells was completely aspirated. After the 6th wash, the multiwell plate was inverted and tapped firmly on an absorbent paper
- After washing, 200 µl of substrate TMB was added to each well, followed by incubation for 30 min at room temperature

- The reaction was stopped by the addition of 50 µl of 0.5 M H₂SO₄ (stop solution) to each well. The reaction volume was mixed on a shaker for 5 seconds
- Absorbance of the coloured product was read at 450 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy)
- A calibration curve was constructed (absorbance vs. insulin concentration of calibrators 1–7), and the concentrations of insulin in each sample volume was estimated. Results were expressed as µg insulin/L of plasma.

3.9.5 Plasma Markers of Hepatic and Renal Function

Markers of hepatic and renal function were assayed in the plasma. These include alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, urea and creatinine.

3.9.5.1 Alkaline Phosphatase Assay

Plasma levels of alkaline phosphatase (ALP) were estimated using QuantiChrom alkaline phosphatase kit (BioAssay Systems, Hayward, USA), according to the method of Morgenstern *et al* (1965). This assay utilizes p-nitrophenyl phosphate (p-NPP) that is hydrolysed by ALP into a yellow product that is detectable by spectrophotometry at 405 nm.

The kit contains the following: Assay buffer (pH 10.5); p-NPP (1 M); Mg acetate (0.2 M); tartrazine (calibrator).

The reagents were prepared as described in section 8.6.

Assay Procedure

The assay, performed in triplicate, was done in a 96-well plate. Distilled water (200 µl) and calibrator (200 µl) were transferred into separate wells. For the samples (plasma), 20 µl of each sample was transferred (in triplicate) into separate wells, followed by the addition of 180 µl of working solution to each sample well. Mixing was gentle and brief. Absorbance (at 405 nm) was read promptly, first at time 0 minute, and then 4 minute later, in a microplate

reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy). ALP levels of the samples were calculated as follows:

$$\text{ALP (IU/L)} = \frac{[(A_{\text{sample } t} - A_{\text{sample } o}) \times \text{reaction volume}]}{[\text{sample volume} \times t] \times 35.3}$$

$t = 4$ (time after the initial absorbance at time o); A = absorbance

3.9.5.2 Plasma Aspartate Aminotransferase

Plasma aspartate aminotransferase (AST) was estimated with an IDTox aspartate transaminase enzyme assay kit (ID Labs Inc., Ontario, Canada) according to the method of Karmen (1955). The kit contains the following: A microtiter plate (96-well plate), reagent mix, AST enzyme control, and AST dilution buffer. The reagents were prepared as described in section 8.7.

Assay Procedure

Each plasma sample (10 μl) was transferred in triplicate to separate wells of a 96-well plate. To each was added 240 μl of reagent mix. Absorbance was read twice, first at time 0 and then 5 minutes later, in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy).

Calculation of AST levels

AST levels were calculated as decrease in absorbance of each sample multiplied by 1072, and the results were expressed as unit/L (U/L).

3.9.5.3 Plasma Alanine Aminotransferase (ALT)

ALT assay was performed in plasma samples using alanine transaminase activity assay kit (Cayman Chemical Company, Ann Arbor, USA), according to the method of Ishiguro *et al* (1991). The assay is based on the oxidation of NADH to NAD^+ in a coupled reaction system

that employs lactate dehydrogenase (LDH). The decrease in absorbance at 340 nm is directly proportional to ALT activity in the sample.

The kit contains the following: ALT assay buffer, ALT substrate (crystalline L-alanine), ALT cofactor (powder of NADH and LDH), ALT initiator (α -ketoglutarate, 150 mM), ALT positive control (porcine heart ALT, 1 U/ml), and a 96-well plate.

The reagents were prepared as described in section 8.8.

Assay Procedure

Three wells were designated as positive control wells, and to each was added 150 μ l of substrate, 20 μ l of cofactor and 20 μ l of positive control. For each sample (plasma), the assay was done in triplicate; and to separate wells were added 150 μ l of substrate, 20 μ l of cofactor and 20 μ l of each of the samples, followed by incubation at 37 °C for 15 minutes. Thereafter, 20 μ l of ALT initiator was added to each well and absorbance was read at 340 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy) for a period of 5 min (at one minute interval). ALT activity was calculated as follows:

ALT activity (U/ml) = $[(\Delta A_{340}/\text{min} \times 0.21 \text{ ml}) / 4.11 \text{ mM}^{-1} \times 0.02 \text{ ml}] \times \text{sample dilution}$

$$\Delta A_{340}/\text{min} = [A_{340} (\text{Time } 2) - A_{340} (\text{Time } 1)] / [\text{Time } 2 (\text{min.}) - \text{Time } 1 (\text{min.})]$$

$$4.11 \text{ mM}^{-1} = \text{NADH extinction coefficient; } 0.21 = \text{assay volume in each well}$$

$$0.02 \text{ ml} = \text{sample volume.}$$

3.9.5.4 Plasma Urea Assay

Urea concentration of the plasma was estimated using QuantiChrom urea assay kit (BioAssays Systems, Hayward, USA), according to the method of Jung *et al* (1975). The

assay utilizes a chromogenic reagent that forms a coloured complex with urea. This coloured product can be measured at 520 nm.

The kit contains the following: Reagent A, Reagent B, and urea standard (50 mg/dl). Reagents were prepared as described in section 8.9

Assay procedure

The assay used a 96-well plate. Into separate wells was transferred (in duplicate) μl of distilled water (blank), 5 μl of urea standard, and 5 μl of each of the samples. This was followed by the addition of 200 μl of the working reagent to each well. Mixing was by light tapping, followed by reading of absorbance at 520 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy). Urea levels of each sample was calculated as follows:

$$\text{Urea (mg/dl)} = [(A_{\text{sample}} - A_{\text{blank}}) / (A_{\text{standard}} - A_{\text{blank}})] \times [\text{standard}]$$

$$[\text{standard}] = 50$$

3.9.5.5 Plasma Creatinine Assay

Plasma creatinine levels were assayed using QuantiChrom creatinine assay kit from BioAssays Systems (Hayward, USA), according to the method of Rajs and Mayer (1992). The assay utilizes picrate, which forms a red coloured complex with creatinine. This product is measured at 510 nm by spectrophotometry.

The kit contains the following: Reagent A, reagent B, and creatinine standard (50 mg/dl). The reagents were prepared as described in section 8.10.

Assay Procedure

To separate wells were transferred 30 µl of standard and plasma samples (in duplicate), followed by the addition of 200 µl of working reagent (and gentle mixing). Incubation followed at room temperature, and absorbance was read (at 510 nm), first at one minute and then 5 minutes, in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy). Creatinine levels of plasma samples were calculated as follows:

$$\text{Creatinine (mg/dl)} = [(A_{\text{sample5}} - A_{\text{sample1}}) / (A_{\text{standard5}} - A_{\text{standard1}})] \times 2$$

3.10 Oxidative Stress Markers

Levels of lipid peroxides, aqueous hydroperoxide, reduced glutathione (GSH), and superoxide dismutase (SOD) were estimated in homogenates of the pancreas and liver as markers of oxidative stress in these organs. GSH was not estimated in the pancreas.

3.10.1 Preparation of Liver and Pancreas Homogenates

For each sample of the pancreas and liver, 10% (w/v) homogenate was prepared with cold 0.15 M KCl-Tris buffer, pH 7.4 (Bouchet *et al*, 1998) in a potter homogeniser (GPE, Bedfordshire, England). The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4 °C (Scott *et al*, 2000) in an Eppendorf 5810R centrifuge (GMI, Minnesota, USA). The supernatant was used for the estimation of oxidative stress markers (except GSH) and protein assay. This was stored at -80 °C before analysis.

Preparation of 0.15 M KCl-Tris buffer is described in section 8.11.

3.10.2 Lipid Hydroperoxide Levels of the Pancreas and Liver

Lipid hydroperoxide contents of the pancreas and liver were estimated with a PeroxiDetect™ kit (Sigma, MO, USA) according to the method of Jiang *et al* (2006). The kit contains the following reagents:

- Organic peroxide colour reagent containing 480 μM butylated hydroxytoluene (BHT) and 15 μM xylenol orange
- Ferrous ammonium sulphate reagent containing 25 mM ferrous ammonium sulphate in 2.5 M H_2SO_4
- *tert*-Butyl hydroperoxide (t-BuOOH), 70 % aqueous solution

The assay was based on the oxidation of Fe^{2+} to Fe^{3+} by lipid peroxides in the samples. Fe^{3+} then forms a coloured adduct with xylenol orange (3,3'-bis[N,N-bis[carboxymethyl]aminomethyl]-o-cresolsulphonaphthalein, sodium salt) (Jiang *et al*, 2006). This adduct is detectable by spectrophotometry at O.D. 560 nm.

The reagents were prepared as described in section 8.12.

Assay Procedure

Standard Curve

For t-BuOOH standard curve, 0, 5, 10, 20, 40, 60 and 80 μl of 200 μM t-BuOOH standard solution were placed in separate Eppendorf tubes (Greiner Bio-One, Frickenhausen, Germany), and the volume of each was made up to 100 μl with 100 % methanol (J .T Baker, Deventer, Holland). This was incubated with 1 ml of working colour reagent at room temperature for 30 min. Absorbance of the coloured product was read at 560 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Absolute methanol served as blank.

Samples

For each sample (homogenates of liver and pancreas), the assay, in duplicate, was as described for the standard curve. t-BuOOH standard solution (200 μM) was replaced by 20 μl of the homogenate (sample).

Calculation

Lipid hydroperoxide levels of each sample were estimated as follows:

$$\text{nmole peroxide/ml} = \frac{[A_{560}(\text{sample}) - A_{560}(\text{blank})] \times \text{dilution factor}}{[A_{560}(\text{1 mole peroxide})] \times \text{sample volume in ml}}$$

Dilution factor= 0.2; sample volume= 0.02 ml; A_{560} = Absorbance at O.D. 560 nm

3.10.3 Aqueous Hydroperoxide Levels of the Pancreas and Liver

Aqueous hydroperoxide levels of the pancreas and liver were estimated using PeroxiDetect™ kit (Sigma, MO, USA) according to the method of Jiang *et al* (2006). The kit contains:

- Aqueous peroxide colour reagent containing 100 mM sorbitol and 125 µl xylenol orange
- Ferrous ammonium sulphate reagent containing 25 mM ferrous ammonium sulphate in 2.5 M sulphuric acid
- Hydrogen peroxide colour reagent, 30% aqueous solution

The assay principle was as described for lipid hydroperoxides. However, colour formation was enhanced by the addition of sorbitol to the reaction volume. Sorbitol is converted by H_2O_2 in the samples to a peroxy radical. The latter oxidises Fe^{2+} to Fe^{3+} , which forms a coloured adduct with xylenol orange. Deionised water served as blank, and absorbance was read at 560 nm.

The reagents were prepared as described in section 8.13.

Assay Procedure

Standard Curve

For the standard curve, 0, 10, 20, 40, 60, and 80 µl of 100 µM H_2O_2 standard solution was placed in labelled Eppendorf tubes, and the volume of each was made up to 100 µl with

deionised water. 1 ml of working colour reagent was added to each. The solution was vortex-mixed and incubated at room temperature for 30 min.

Samples

For the test samples, 20 µl of each homogenate of the liver and pancreas was placed (in duplicate) in Eppendorf tubes containing 80 µl deionised water. This was incubated with 1 ml of the working colour reagent for 30 min at room temperature. Absorbance of the colour product was read at 560 nm in a UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Deionised water served as blank. Aqueous hydroperoxide levels of the samples were calculated using the equation in section 3.10.2.

3.10.4 Reduced Glutathione (GSH)

GSH levels of liver homogenates were estimated with a glutathione assay kit (Sigma, MO, USA) according to the method of Akerboom and Sies (1981). The kit contains:

- Glutathione assay buffer (5x). This contains potassium phosphate, 500 mM, pH 7.0, with 5 mM EDTA
- Glutathione reductase, 400 U/ml, in 3.6 M ammonium sulphate (pH 7.0) containing 0.1 mM dithiothreitol
- Glutathione reduced, standard
- 5,5'-Dithiobis (2-nitrobenzoic acid)[DTNB]
- 5-Sulphosalicylic acid
- NADPH
- Dimethyl Sulphoxide (DMSO)

This assay depends on the continuous reduction of DTNB to TNB by GSH. The GSSG formed is also recycled by glutathione reductase and NADPH. TNB, the yellow product, is

detectable by spectrophotometry at 412 nm. Its rate of production is proportional to the concentration of GSH in the samples. The reagents were prepared as described in section 8.14.

Assay Procedures

Samples were first deproteinised using 5% SSA. Each sample (0.1 g) was homogenised with 1 ml of 5% SSA using a 7-ml Dounce tissue grinder equipped with a PTFE resin pestle (Wheaton, USA). The homogenate was allowed to stand for 10 min at 4 °C, and then centrifuged at 10,000 x g for 10 min. The supernatant was diluted 5 folds for use in this assay.

For the standard curve, serial dilution of glutathione standard solution (50 µM) was done as shown in table 7.

Table 7. Preparation of glutathione standard solutions by serial dilution of the 50 µM glutathione solution with 5% SSA

Well No.	1	2	3	4	5
5% SSA (µl)	0	25	25	25	25
GSH standard solution (µl)	50	25(taken from well 1)	25(taken from well 2)	25(taken from well 3)	25(taken from well 4)

The assay was done in duplicate. An aliquot (10 µl) of each sample and standard solutions was placed in a 96-well plate. To each was added 150 µl of the working mixture, and this was mixed by pipetting, followed by 5 minutes of incubation at room temperature (23 °C). Thereafter, 50 µl of the diluted NADPH solution was added to each. Mixing was done by pipetting, and absorbance read at O.D 412 nm in a 4000 UV/visible spectrophotometer

(Pharmacia Biotech, Uppsala, Sweden). 5% SSA (10 µl) constituted the blank reagent. The concentrations of GSH in the samples were estimated as follows:

$$\text{nmoles GSH/ml of sample} = \frac{[A_{412/\text{min}} (\text{sample}) \times \text{dilution factor}]}{[A_{412/\text{min}} (1 \text{ nmole}) \times \text{sample volume}]}$$

Dilution factor = 0.2; sample volume = 20 µl (0.02 ml)

3.10.5 Superoxide Dismutase (SOD)

SOD assay was done using 19160 SOD determination kit (Fluka, Buchs, Switzerland) according to the method of Paoletti *et al* (1986). The kit contains the following reagents:

- WST solution
- Enzyme solution
- Buffer solution
- Dilution buffer

The reagents were prepared as described in section 8.15. Before use, enzyme solution was centrifuged for 5 seconds and mixed by pipetting.

SOD assay utilises a highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium). This salt produces a water-soluble formazan dye upon reduction with superoxide anion. The coloured formazan dye can be detected by spectrophotometry at 450 nm.

Assay Procedure

Homogenates of the liver and pancreas were prepared as described in section 3.10.1. The assay was performed as shown in table 8. A 96-well plate was used.

Table 8. Quantity of reagents for sample, blank 1, blank 2, and blank 3 for SOD assay

Reagent/Well	Sample	Blank 1	Blank 2	Blank 3
Sample solution	20 µl		20 µl	
Bidistilled water		20 µl		20 µl
WST working solution	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20 µl		
Dilution buffer			20 µl	20 µl

Wells were designated (in duplicates) as sample wells (for each sample), blank 1, blank 2, or blank 3 wells. The following procedures were observed:

- To begin, 20 µl of each sample was placed in blank 2 and sample wells; while 20 µl of bidistilled water was placed in each blank 1 and blank 3 well
- 200 µl of WST working solution was added to each of these wells
- Thereafter, 20 µl of dilution buffer was added to each blank 2 and blank 3 well; while 20 µl of enzyme working solution was added to each blank 1 and sample well (Table 7), with thorough mixing by pipetting.
- The reaction was incubated at 37 °C for 20 min in an incubator (SANYO, Milan, Italy). Absorbance was read at 450 nm in a microplate reader (ETI-System Fast Reader, SORIN Biomedica, Vicenza, Italy).

SOD activity of each sample was calculated as follows:

$$\text{SOD activity (\%)} = [(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})] \times 100 / (A_{\text{blank 1}} - A_{\text{blank 3}})$$

3.11 Estimation of the Protein Content of the Pancreas and Liver

Protein contents of the pancreas and liver were estimated by the BioRad Protein Assay Method (BioRad, Hercules, USA), according the method of Bradford (1976). Portions of the liver and pancreas were homogenised as described in section 3.10.1. The reagents required are shown in section 8. 16. Bovine serum albumin (BSA) (100 µg/ml) served as standard.

Standard Curve

For the standard curve, 0, 10, 20, 40, 80, and 160 µl of BSA (100 µg/ml) were placed in Eppendorf tubes (in duplicate) and the volume of each was made up to 800 µl with bidistilled water; followed by the addition of 200 µl of Bradford solution. The solutions were vortexed and absorbance of the coloured product was read at 595 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Samples

For each homogenate sample, the assay was done in duplicate. Bidistilled water (799 µl) was placed in an Eppendorf tube and to this was added 1 µl of the sample (homogenate), followed by 200 µl of Bradford solution. The solution was vortexed and absorbance was read at 595 nm. The blank reagent consisted of 800 µl of bidistilled water, into which 200 µl of Bradford solution was added. The amount of protein in each sample was estimated from the standard curve.

3.12 Tissue Processing for Light Microscopy

3.12.1 Paraffin Embedding

Paraffin embedding of the pancreas, liver, kidney and small intestine (jejunum) was done. At euthanasia, each organ was rinsed in PBS, trimmed free of adipose tissue, cut into smaller pieces (3 mm x 3 mm) and fixed in Bouin's fluid and 4% paraformaldehyde for a minimum

of 24 hours. For Bouin's-fixed samples, tissues were rinsed with PBS (2x/day) until the yellow coloration was removed. The following histological procedures were then observed:

- Dehydration of the sample

50% Ethanol, 1 hr at room temperature

70% Ethanol, 1 hr at room temperature

95% Ethanol, 1 hr at room temperature

100% Ethanol I and II, 30 min each, at room temperature

100% Ethanol III, 1 hr at room temperature

- Clearing of the sample

Xylene I and II, 20 min each

- Infiltration of the sample

Xylene : paraffin (1:1), 90 min at 59 °C

Paraffin I, 60 min at 59 °C

Paraffin II, 90 min at 59 °C

Paraffin III, overnight at 59 °C

- Embedding (Inclusion) of the sample

Embedding was done in paraffin wax (section 8.16)

Reagents were prepared as described in section 8.17.

3.12.2 Microtomy

For each treatment group, 3.5 µm-thick sections of the pancreas, liver, kidney and jejunum were cut on a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Germany). Sections were floated on the water bath at 50 °C, and mounted on pre-washed, sterilised, 25.4

mm x 76.2 mm glass slides (Pearls, China). Slides were washed with detergent, rinsed with deionised water, and sterilised with 70% ethanol.

3.12.3 Staining of Tissues

Haematoxylin and eosin stain, as well as special stains (Masson's trichrome, periodic acid-Schiff with diastase control, Alcian blue and Sudan black B) were employed in this study.

3.12.3.1 Haematoxylin and Eosin Staining

Paraffin sections of the pancreas, liver, kidney and jejunum were stained in haematoxylin and eosin as follows (Bancroft and Stefen, 1982):

- Sections were taken to water

Xylene I and II, 10 min each

100%, 95%, 70%, 50% and 30% Ethanol, about 1 min each

Bidistilled water

- Carazzi's haematoxylin, 15 min
- Tap water (for rinsing)
- Distilled water, a few dips
- Eosin Y (Sigma, MO, USA), 0.5%, 1-2 min
- 30%, 50%, 70%, 95%, and 100% Ethanol (a few dips each)
- Xylene, 15 min
- Mount in Eukit (Fluka, Buchs, Switzerland)

Reagents were prepared as shown in section 8.18.

3.12.3.2 Periodic Acid-Schiff (PAS) Staining

Paraffin sections of the kidney were stained in PAS (Bancroft and Stefen, 1982) to demonstrate renal tubule cells, microvilli of proximal tubules, basement membranes of renal tubules, and extracellular matrix of renal cortex. Liver sections were likewise stained to demonstrate glycogen content of hepatocytes.

Staining Procedure

- Sections were taken to water as in section 3.12.3.1
- Periodic acid, 0.8 % (Applichem, Germany) 10 min
- Ordinary water 10 min
- Schiff reagent (Sigma, MO, USA) 30 min
- Ordinary water 20 min
- Carazzi's haematoxylin 15 min
- Ordinary water 10 min
- Eosin Y (Sigma, MO, USA), 0.5% 1-2 min
- 30%, 50%, 70%, 95%, and 100% Ethanol, a few dips each
- Xylene 15 min
- Mount in Eukit (Fluka, Buchs, Germany)

Reagents were prepared as shown in section 8.19.

3.12.3.3 Periodic Acid-Schiff with Diastase Control (PAS-Diastase)

To test the presence of glycogen in hepatocytes, as well as glycogen and other periodate-reactive carbohydrates in the proximal tubule cells of untreated diabetic animals (at 50 d), PAS staining with diastase control was done on liver and kidney sections of these animals. In

the absence of diastase treatment, strong PAS-positive staining of hepatocytes and renal tissue was observed in this group.

- Sections were taken to water as described in section 3.12.3.1
- Liver sections were treated with human salivary amylase for 30 min at 37 °C in an incubator (SANYO, Milan, Italy)
- Thereafter, protocols described for PAS in section 3.12.3.2 were observed

3.12.3.4 Sudan Black B Staining for Lipid

Liver sections of metformin-treated diabetic rats and kidney sections of untreated diabetic rats were stained with Sudan black B (Bancroft and Stefen, 1982) to test the presence of lipid in vacuoles observed in hepatocytes and renal tubules of these animals at 50 d. Cryostat sections (8 µm) were obtained with a 975C Histocut Cryostat (Rankin Biomedical, MI, USA). Staining of the sections was done as follows:

- Rinse in 70% ethanol
- Sudan black B, 30 min at 70 °C
- Rinse in 70% ethanol and then in bidistilled water
- Mount in a glycerine medium

Preparation of reagents is described in section 8.20.

3.12.3.5 Masson's Trichrome Staining

Paraffin sections of the pancreas were stained in Masson's trichrome (Bancroft and Stefen, 1982) to demonstrate the degree of fibrosis and morphology of this organ with the progression of diabetes mellitus and its treatment. The staining protocol was as follows:

- Sections were taken to water as in section 3.12.3.1
- Carazzi's haematoxylin 15 min

- Rinse in tap water
- Ponceau 2R (Xylidine Ponceau) (Sigma, MO, USA) 3 min
- Rinse in 1% acetic acid
- Orange G (Merck, Germany) 5 min
- Rinse in 1% acetic acid
- Light green (Fluka, Steinheim, Germany) 5 min
- Rinse in 1% acetic acid
- Dehydrate in 100% ethanol 5 min
- Clear in xylene 15 min
- Mount in Eukit (Fluka, Buchs, Germany)

Preparation of reagents is described in section 8.21.

3.12.3.6 Alcian Blue Staining

To study the effect of *A. indica* administration on the epithelial lining of the small intestine (jejunum), paraffin sections of the jejunum were stained in Alcian blue (Bancroft and Stefen, 1982). The stain demonstrates the goblet cells, mucin coat and columnar cells of intestinal epithelium. Sections were stained as follows:

- Sections were taken to water as in section 3.12.3.1
- Alcian blue 20 min
- Rinse in tap water
- Carazzi's haematoxylin 5 min
- Rinse in tap water
- Eosin Y (Sigma, MO, USA), 0.5%, 1-2 min
- 30%, 50%, 70%, 95% and 100% Ethanol, a few dips each

- Xylene 15 min
- Mount in Eukit (Fluka, Buchs, Germany)

Preparation of reagents is described in section 8.22.

3.13 Photomicrography

Photomicrographs of sections of the pancreas, liver, kidney and jejunum were taken with a Nikon digital camera DXM1200F (Nikon, Tokyo, Japan) coupled with a Nikon Eclipse 80i light microscope (Nikon, Tokyo, Japan). Figures 41 K and 41 L, and figures 44 G–K were taken with a Kodak digital camera (Kodak, Hertfordshire, UK) mounted on an Olympus light microscope (Olympus, Tokyo, Japan).

3.14 Morphometric Analysis

3.14.1 Renal Glomerular Diameters

PAS-stained renal sections were observed under a Nikon Eclipse 80i light microscope (Nikon, Japan) equipped with a DXM1200F Nikon digital camera (Nikon, Japan), and connected to a desktop computer. Renal images were projected onto the computer monitor and glomerular diameters were taken at x60 (objective lens) using a calibrated ruler. Mean glomerular diameters were calculated for each treatment group.

3.14.2 Pancreas

Trichrome-stained sections of the pancreas were observed as described in section 3.14.1. For each treatment group, islet diameters were measured (at x40) with a calibrated ruler. The number of viable β -cell nuclei per unit area of the islet was also estimated in each group.

3.15 Statistical Analysis

Data were analysed using SPSS 15.0 (SPSS Inc, Chicago, USA) and Excel 2007 (Microsoft Corporation, USA). Data were expressed as mean $\pm$ standard error of the mean (mean $\pm$

SEM). Mean values were compared using one-way analysis of variance (SPSS Inc, Chicago, USA) and paired sample t test. P values less than 0.05 ($P < 0.05$) were taken to be statistically significant. All graphs were drawn with Excel 2007 (Microsoft Corporation, USA).

Chapter Four

Results

4.1 Total Flavonoid Content of *Azadirachta indica* Leaves

Total flavonoid content of the dry leaves of *A. indica*, as estimated by spectrophotometry, using rutin as standard, was 294.97 ± 0.46 μg flavonoids/g of dry *A. indica* leaf powder.

4.2 Mortality

Within the initial 72 hours of STZ injection, two of the STZ-treated animals died. Thus, mortality was 0.03%.

4.3 Blood Glucose

4.3.0 Blood Glucose Responses to Varied Neem Doses

Figure 8 shows blood glucose responses to the doses of neem leaf extract at 250 mg/kg bw/d, 500 mg/kg bw/d and 750 mg/kg bw/d. Normoglycemia was established and sustained only in the diabetic group treated with 500 mg/kg bw/d of the leaf extract. In the groups treated with 250 mg/kg bw/d and 750 mg/kg bw/d of the extract, normoglycemia was not established throughout the 21-day treatment period.

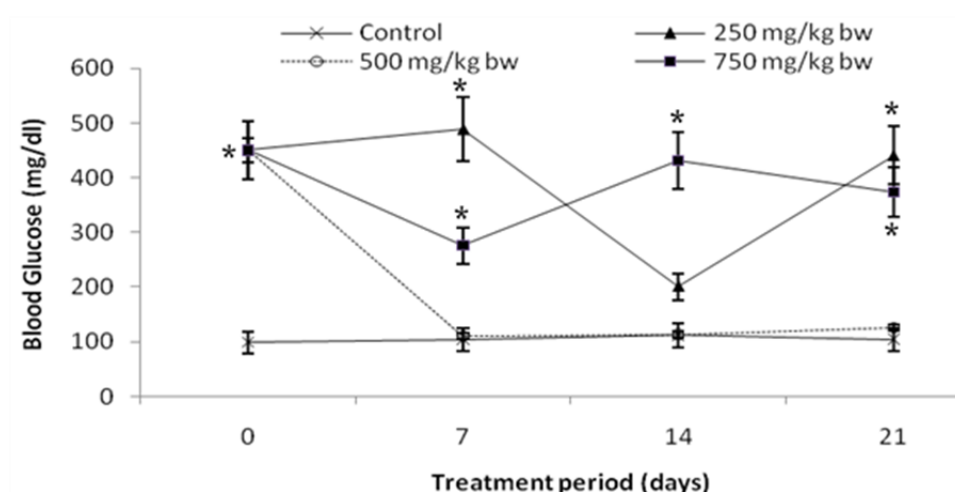


Figure 8. Blood glucose responses to varied doses of neem leaf extract. Bars represent mean $\pm$ SEM. *Significantly different from control, $P < 0.05$.

4.3.1 Blood Glucose at Day 0 of Treatment

Blood glucose levels of hyperglycemic rats at day 0 of treatment with ethanolic leaf extract of *A. indica* (500 mg/kg bw/d) and metformin (350 mg/kg bw/d), taken at alternate hours post-dose (0, 1, 3, 5, 7, 9 and 11 hours), are shown in figure 9. For each treatment, the highest reduction in blood glucose levels was recorded 5 hours post-dose. At this time, there was a 16.6% fall in blood glucose in the neem-treated rats and an 8.7% fall in blood glucose in the metformin-treated rats. Normoglycemia was not attained in any of these treatment groups during the initial 11 hours following the first dose of each treatment.

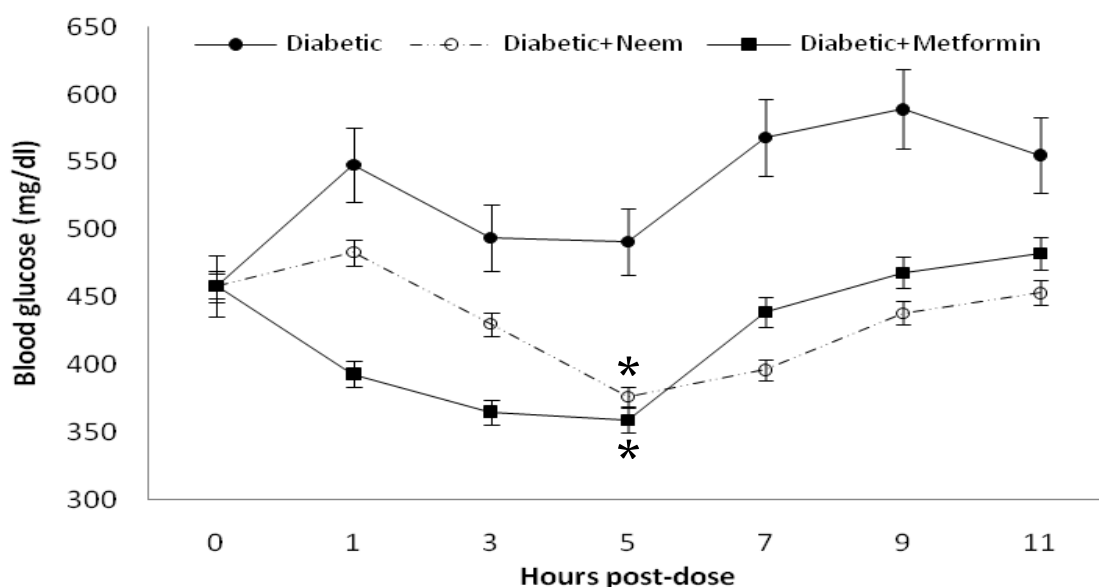


Figure 9: Blood glucose responses to neem and metformin treatment at day 0. Bar represents mean $\pm$ SEM. *Significantly different from diabetic group, $P < 0.05$.

4.3.2 Blood Glucose Levels on a Weekly Basis

Figure 10 shows blood glucose levels of the animals over the 7-week treatment period. At day 0 of neem and metformin treatment, all animals in the untreated diabetic, neem-treated diabetic and metformin-treated diabetic groups were hyperglycemic. By the end of week 1, 87.5 % of hyperglycemic rats treated with 500 mg/kg bw/d of neem had become

normoglycemic, and their blood glucose was similar to control ($P>0.05$). By the end of week 2, normoglycemia had been established in all neem-treated hyperglycemic rats ($P>0.05$); and this normoglycemic state was maintained till euthanasia at 50 d.

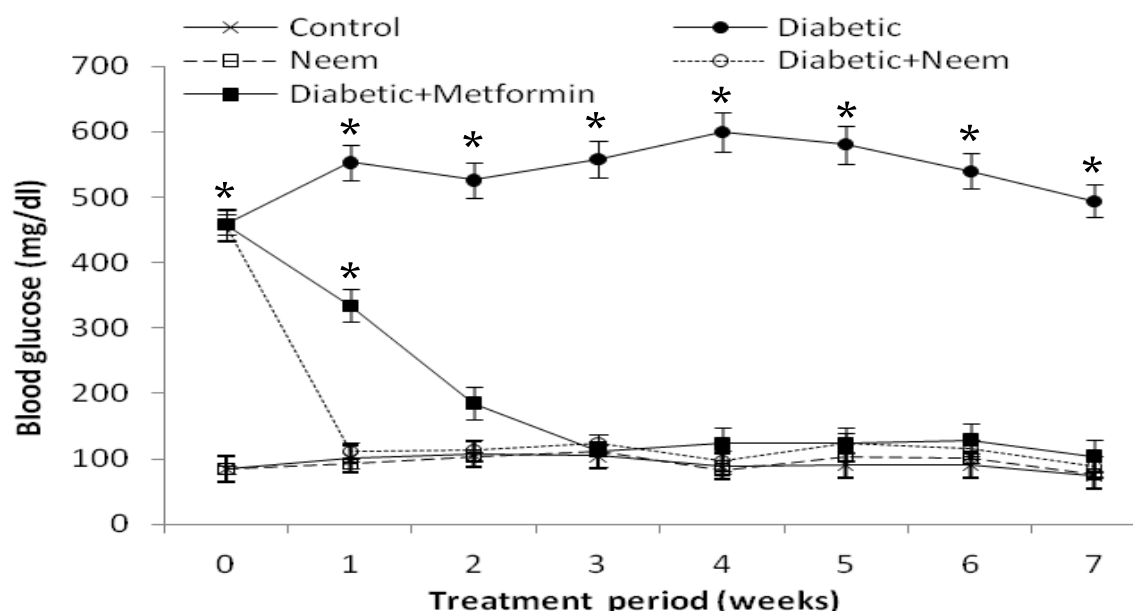


Figure 10: Weekly blood glucose of control and treated animals. Bar represents mean $\pm$ SEM. *Significantly different from control, $P<0.05$.

In metformin-treated hyperglycemic rats, 100% of these animals remained hyperglycemic at the end of week 1. Thus, their blood glucose was significantly higher than control ($P<0.05$). By the end of week 2, normoglycemia was attained in 66.7% of these animals, with 53% fall in blood glucose ($P>0.05$, metformin group vs. control). By week 3, all animals in this treatment group had become normoglycemic, with 71.6% fall in blood glucose. Continued metformin treatment maintained this normoglycemic state till euthanasia at 50 d. In contrast, hyperglycemia persisted in the untreated diabetic rats from week 0 to week 7 (Fig. 10).

4.4 Body Weight

Figure 11 shows changes in body weights of animals at 7 d, 21 d and 50 d. At the end of week 1 of treatment (7 d), 1.5% loss in body weight had occurred in neem-treated diabetic

animals, though this difference was not significant different from control ($P>0.05$). Similarly, body weight gain (0.3%) in neem-treated non-diabetic rats was negligible. However, relatively higher increases in body weight occurred in the control and metformin-treated diabetic rats (Fig. 11). By 21 d, neem-treated non-diabetic rats had the least gain in body weight (13%); while the highest gain (29.9%) was obtained in metformin-treated rats. This trend was maintained till 50 d. By this time, weight gain in neem-treated non-diabetic rats remained the least (19.9%) ($P>0.05$), while the highest weight increase of 49.9% occurred in metformin-treated rats ($P<0.05$).

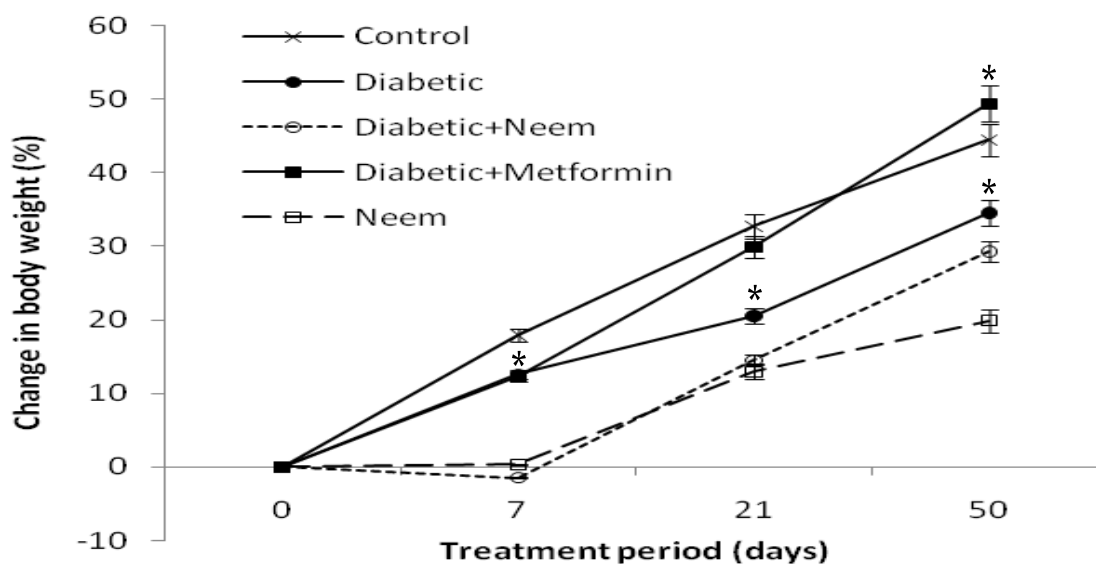


Figure 11: Change (%) in body weight of animals at 7 d, 21 d, and 50 d of treatment. Bar represents mean $\pm$ SEM. *Significantly different from control, $P<0.05$.

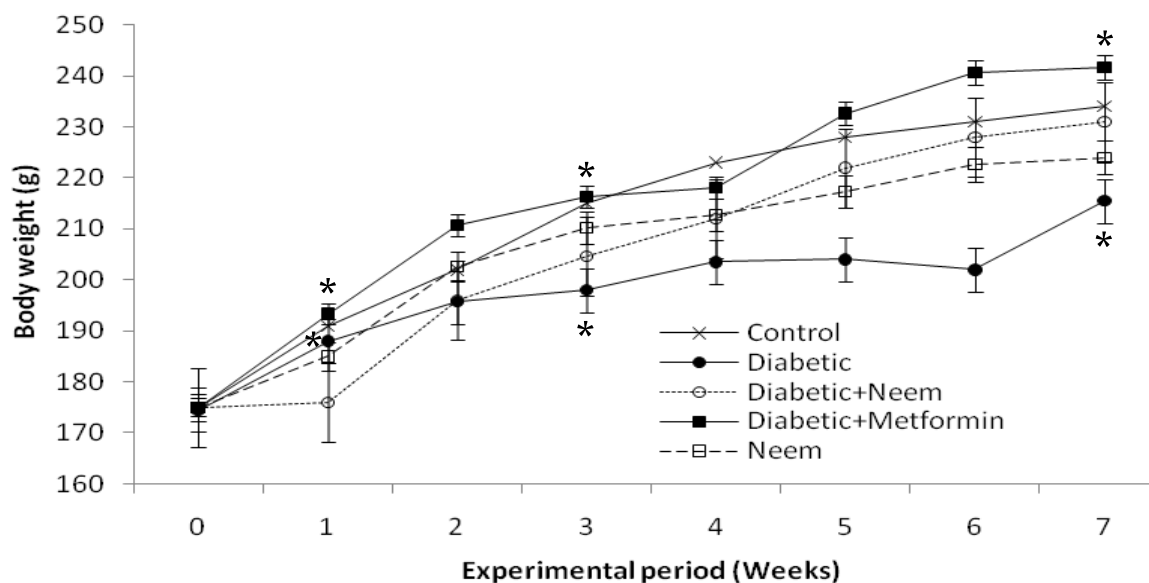


Figure 12: Weight of the animals over a 7-week period. Bar represents mean $\pm$ SEM. *Significantly different from control, $P < 0.05$.

Figure 12 gives details of the body weights of animals from week 0 to week 7. At week 0, animals weighed 161-190 g (average body weight = 175.5 g). At the end of week 1, body weights of untreated diabetic and metformin-treated diabetic rats were significantly different from sex-matched control ($P < 0.05$; Fig. 12). In contrast, body weights of neem-treated diabetic and neem-treated non-diabetic rats were not statistically different from sex-matched control ($P > 0.05$). This trend was maintained in all the treatment groups at 21 d and 50 d in relation to non-diabetic control rats.

4.5 Organ Weight

4.5.1 Liver Weight

Figure 13 shows liver weights of the different treatment groups relative to the body weight. By 50 d of treatment, significant increases in liver weight had occurred in untreated diabetic and metformin-treated diabetic rats compared with control ($P < 0.05$). Liver weights in neem-treated diabetic rats were not statistically different from control ($P > 0.05$).

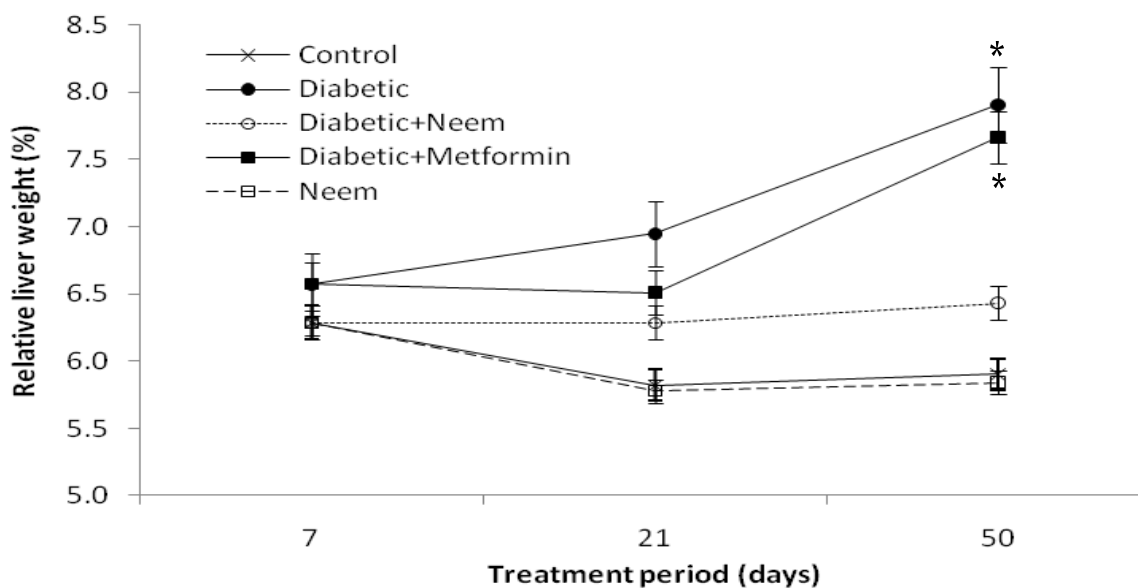


Figure 13: Relative weights of the liver. Bars represent mean $\pm$ SEM. *Significantly different from control, $P < 0.05$.

4.5.2 Kidney Weight

Figure 14 shows the weights of the kidney in the various treatment groups relative to the body weight. As obtained for the liver at 50 d, kidney weights had also increased significantly above control ($P < 0.05$) in the untreated diabetic and metformin-treated diabetic rats. Kidney weights in neem-treated diabetic rats were not significantly different from control ($P > 0.05$).

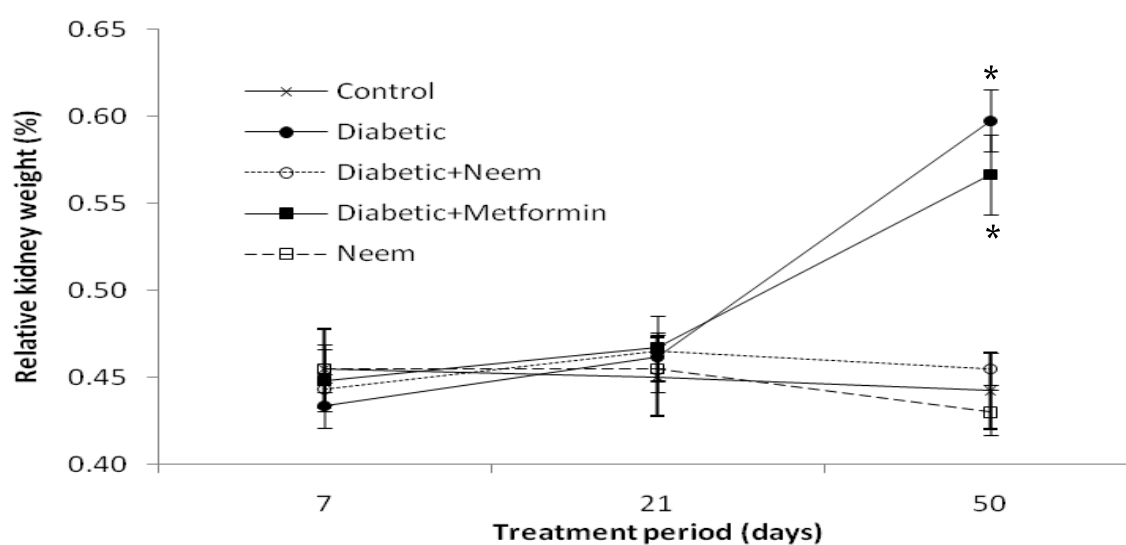


Figure 14: Relative weight of the kidney. Bar represents mean $\pm$ SEM. *Significantly different from control, $P < 0.05$.

4.5.3 Pancreas Weight

Figure 15 shows the weights of the pancreas in the various treatment groups relative to the body weight. At 50 d, significant decreases in pancreatic weights had occurred in the untreated diabetic and metformin-treated diabetic animals compared with control ($P<0.05$). Although the weights of the pancreas in neem-treated diabetic animals were less than control at 50 d, the difference was not significant ($P>0.05$).

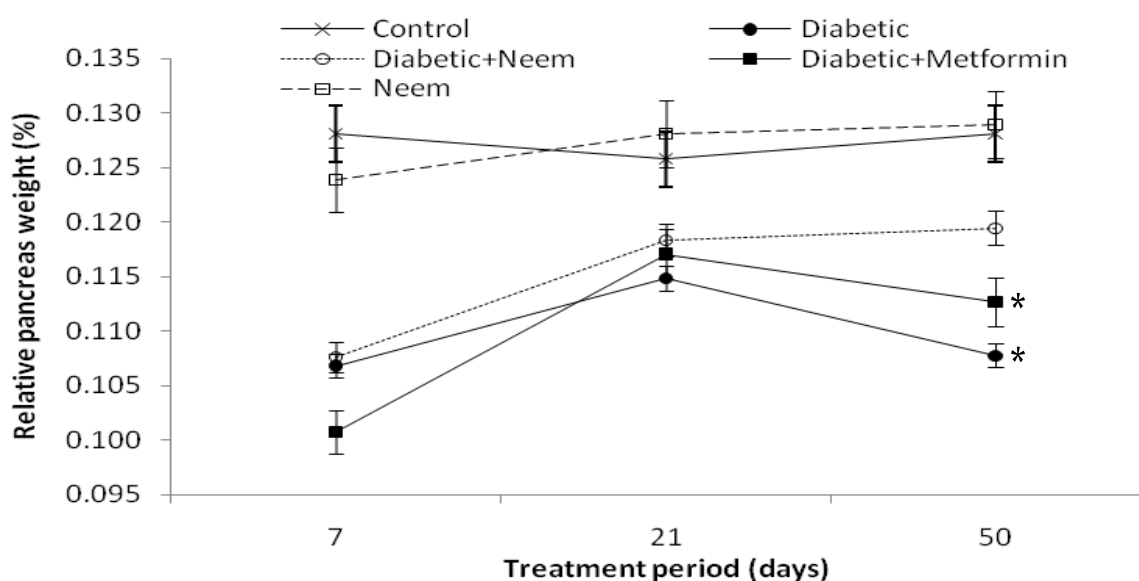


Figure 15: Relative weight of the pancreas. Bar represents mean $\pm$ SEM. *Significantly different from control, $P<0.05$.

4.6 Feed and Water Intake

4.6.1 Feed Intake

Figure 16 shows feed intake/g bw/d, while table 9 shows the amount of feed consumed/animal/d. At day 0, feed intake was comparable in all the groups. However, in the untreated diabetic group, 1.8-fold increase in feed consumption had occurred as early as 7 d, and by 50 d, feed intake had increased by 2.4 fold in this group compared with control ($P<0.05$). Neem-treated diabetic rats also had a slight increase (1.3 fold) in feed intake at 7 d; thereafter, feed consumption declined gradually and by 50 d, 1.3-fold decrease ($P>0.05$) in

feed intake had occurred compared with control. A similar trend was observed in the neem-treated non-diabetic rats. In these animals, more than 1.5-fold decrease in feed intake had occurred by 50 d compared with control ($P>0.05$). In metformin-treated rats, feed intake was 1.5-fold higher than non-diabetic control by 50 d ($P<0.05$).

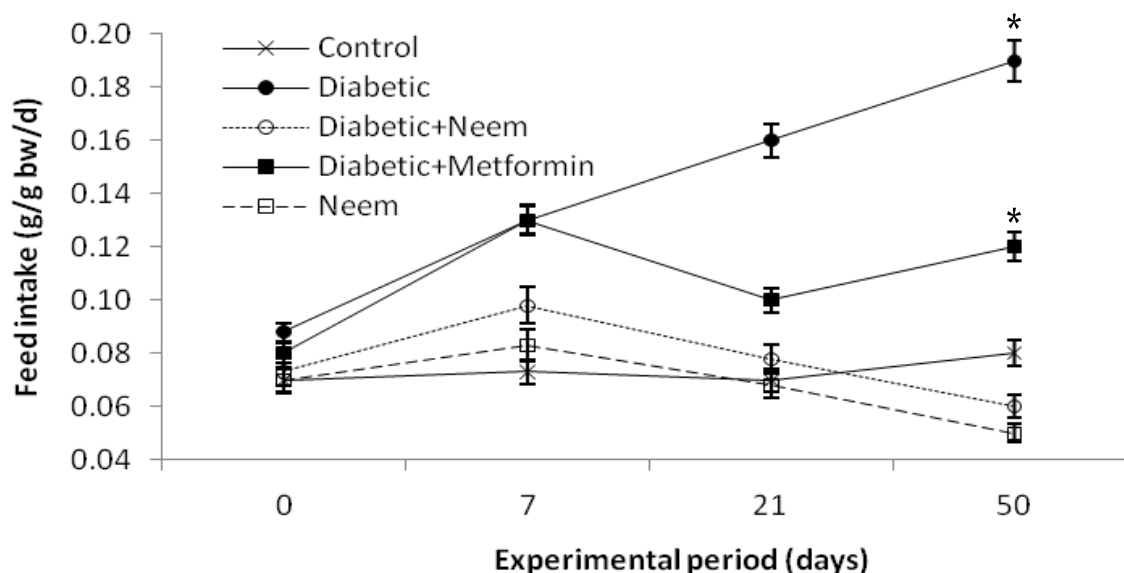


Figure 16: Feed intake per gram body weight of animals. Bar represents mean $\pm$ SEM. *Significantly different from control, $P<0.05$.

4.6.2 Water Intake

Figure 17 and table 9 show details of water intake of the animals. A 2-fold increase in water intake was evident in the untreated diabetic rats as early as 7 d ($P<0.05$). By 50 d of exposure, fluid intake in this group was 3-fold higher than control ($P<0.05$). In neem-treated diabetic rats, water intake in the first week of treatment was not significantly different from control ($P>0.05$). However, by the 3rd week, about 1.8-fold decrease in water intake had occurred. This trend continued till 50 d when water intake had dropped 2-fold compared with control ($P<0.05$) (Fig. 17). Similarly, a sustained gradual decrease in water intake began at the end of the first week in neem-treated non-diabetic rats; this continued till 50 d when fluid intake had decreased almost 3-fold compared with control ($P<0.05$). In the metformin-treated diabetic rats, a 1.8-fold increase in water intake had occurred by the end of the first week of treatment

($P < 0.05$). However, from the 3rd week onwards, water consumption in these animals was similar to control ($P > 0.05$).

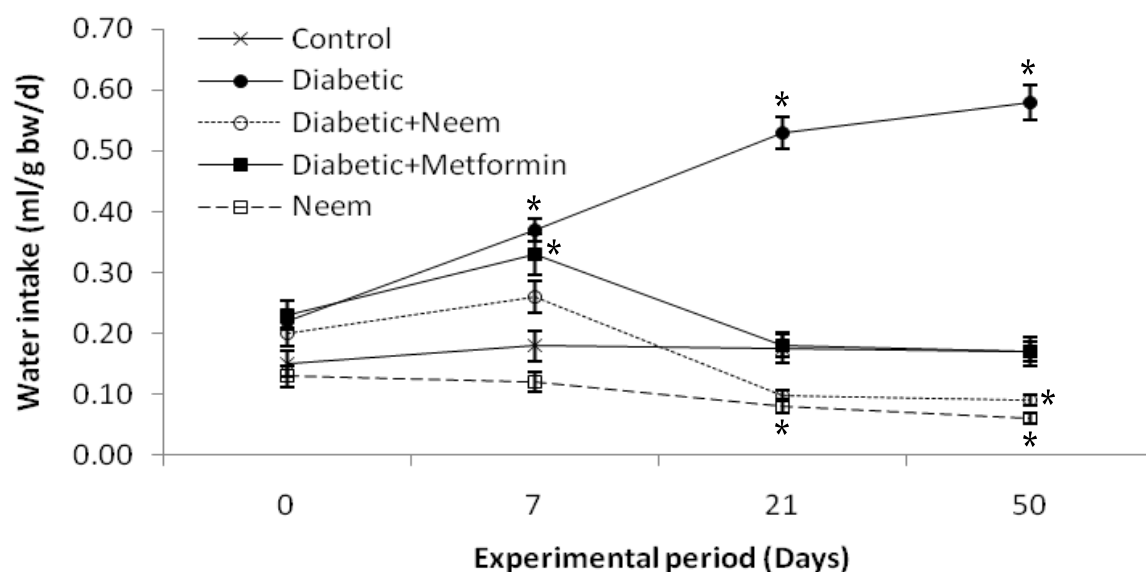


Figure 17: Water intake per gram body weight of animals. Bars represent mean $\pm$ SEM.

*Significantly different from control, $P < 0.05$.

Table 9. Feed and water intake of control and treated animals

Group/Day	Feed (g/animal/d)				Water (ml/animal/d)			
	0	7	21	50	0	7	21	50
Control	12.4 $\pm$ 2.3	14.2 $\pm$ 1.2	15.1 $\pm$ 0.6	18.0 $\pm$ 1.3	25.1 $\pm$ 2.9	35.2 $\pm$ 0.8	38.4 $\pm$ 1.3	41.1 $\pm$ 1.0
Diabetic	13.3 $\pm$ 2.4	18.0 $\pm$ 2.3	24.1 $\pm$ 1.2	32.3 $\pm$ 2.3*	28.0 $\pm$ 1.7	52.1 $\pm$ 4.6*	85.3 $\pm$ 5.8*	97.5 $\pm$ 4.5*
Diabetic+Neem	13.3 $\pm$ 0.3	17.1 $\pm$ 2.3	16.4 $\pm$ 2.3	14.0 $\pm$ 3.1	36.0 $\pm$ 2.3	44.7 $\pm$ 1.5	20.3 $\pm$ 5.1	21.0 $\pm$ 1.7*
Diabetic+Glib.	11.0 $\pm$ 0.3	18.8 $\pm$ 0.5	17.8 $\pm$ 0.9	22.7 $\pm$ 1.1*	30.0 $\pm$ 5.8	48.2 $\pm$ 2.3*	30.2 $\pm$ 1.9	34.0 $\pm$ 1.2
Neem	14.1 $\pm$ 2.3	16.9 $\pm$ 0.4	15.6 $\pm$ 0.2	12.3 $\pm$ 1.0	26.0 $\pm$ 1.2	25.4 $\pm$ 0.6	21.1 $\pm$ 2.2*	15.3 $\pm$ 0.9*

Data are means $\pm$ SEM; *significantly different from control, $P < 0.05$.

4.7 Plasma Analysis

4.7.1 Plasma Insulin Levels

Plasma insulin levels of the various treatment groups are shown in figure 18. By 7 d, a 3.5-fold decrease in plasma insulin had occurred in the untreated diabetic rats compared with control ($P<0.05$). A similar finding was obtained at 21 d and 50 d in this group. In neem-treated diabetic rats, plasma insulin levels increased gradually from a 3-fold decrease below control at 7 d ($P<0.05$) to a level not significantly different from control at 50 d ($P>0.05$). In metformin-treated diabetic rats, plasma insulin remained significantly low compared with control ($P<0.05$).

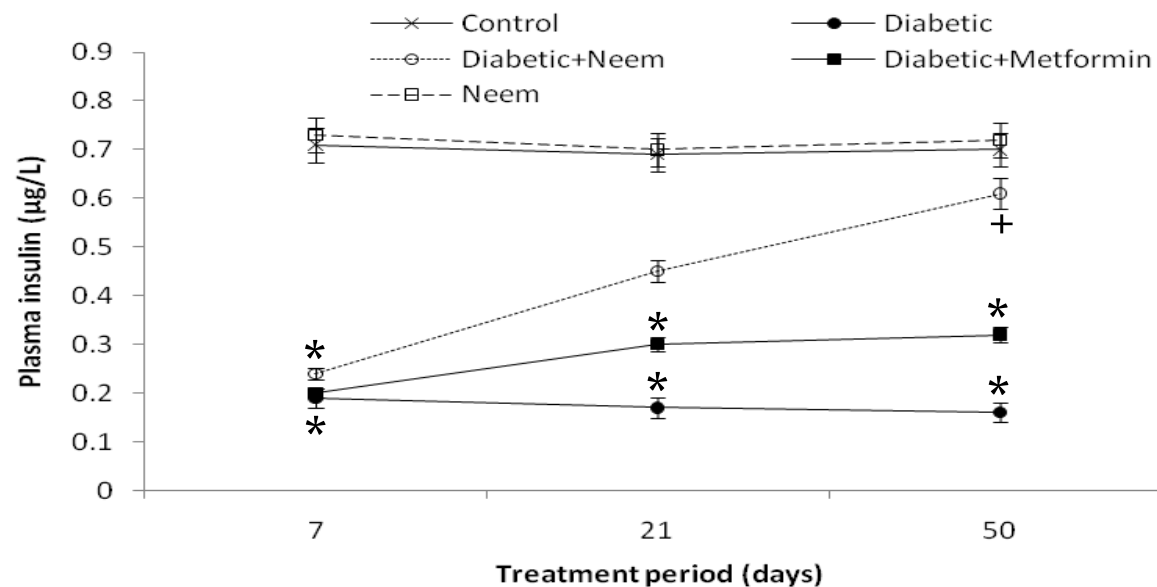


Figure 18: Plasma insulin levels. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$; +Significantly different from diabetic group, $P<0.05$.

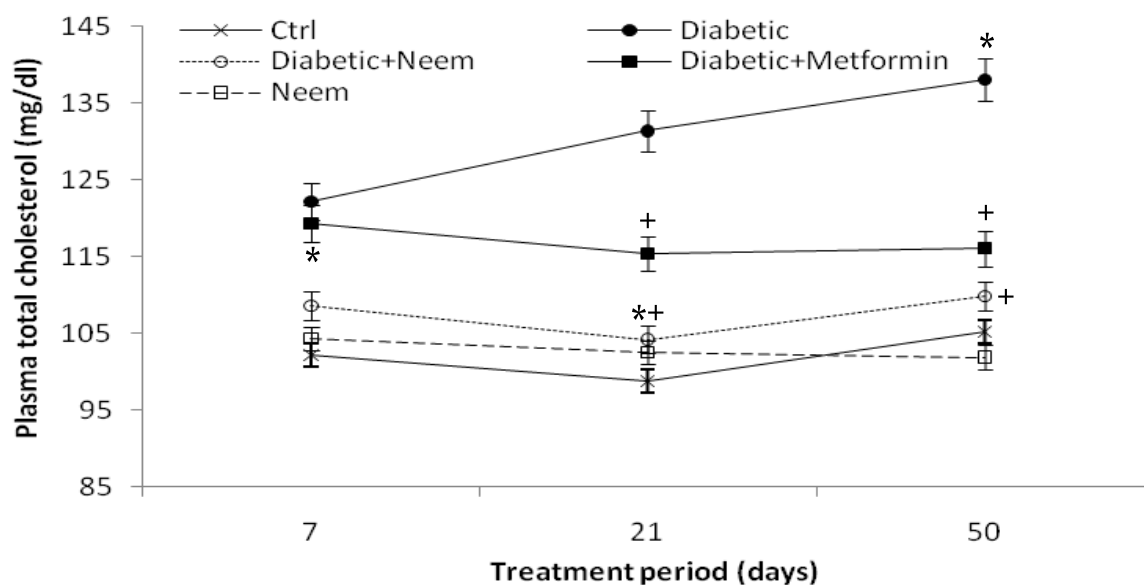


Figure 19: Plasma total cholesterol. Bars represent mean $\pm$ SEM; *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

4.7.2 Plasma Total Cholesterol

Plasma total cholesterol levels of the various treatment groups are shown in figure 19. At 7 d, significant increase in plasma total cholesterol had occurred in untreated diabetic animals compared with control ($P < 0.05$). By 50 d, contrary to what obtained in the untreated diabetic animals (Fig. 19), plasma cholesterol levels in the neem-treated diabetic rats were not significantly different from control. Similar findings were obtained for metformin-treated diabetic rats.

4.7.3 Plasma High Density Lipoprotein Cholesterol (HDL-c)

Plasma levels of HDL-c are shown in figure 20. Significant decreases in plasma HDL-c had occurred in the untreated diabetic, neem-treated diabetic and metformin-treated diabetic rats at 7 d compared with control ($P < 0.05$). By 50 d, plasma HDL-c levels remained low in the untreated diabetic rats ($P < 0.05$); while the levels had increased significantly in the neem-treated diabetic and metformin-treated diabetic rats compared with the untreated diabetic rats ($P < 0.05$; Fig. 20).

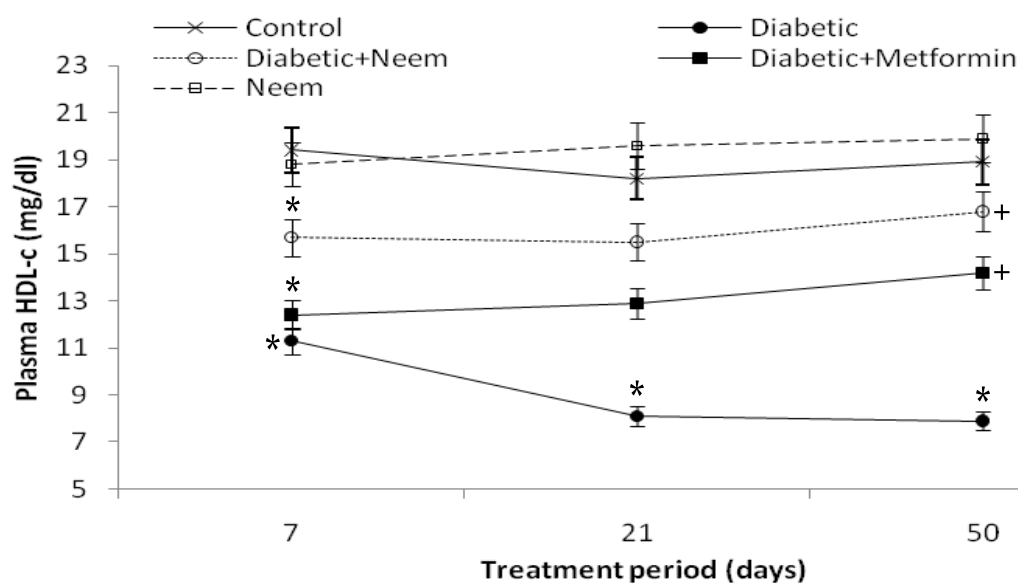


Figure 20: Plasma HDL-c levels. Bars represent mean $\pm$ SEM; *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

4.7.4 Plasma Low Density Lipoprotein Cholesterol (LDL-c)

Plasma LDL-c levels are shown in figure 21. In contrast to control, significant increases ($P < 0.05$) in plasma LDL-c occurred throughout the study period in the untreated diabetic animals. By 50 d, LDL-c levels in neem-treated and metformin-treated diabetic rats were not significantly different from control ($P > 0.05$).

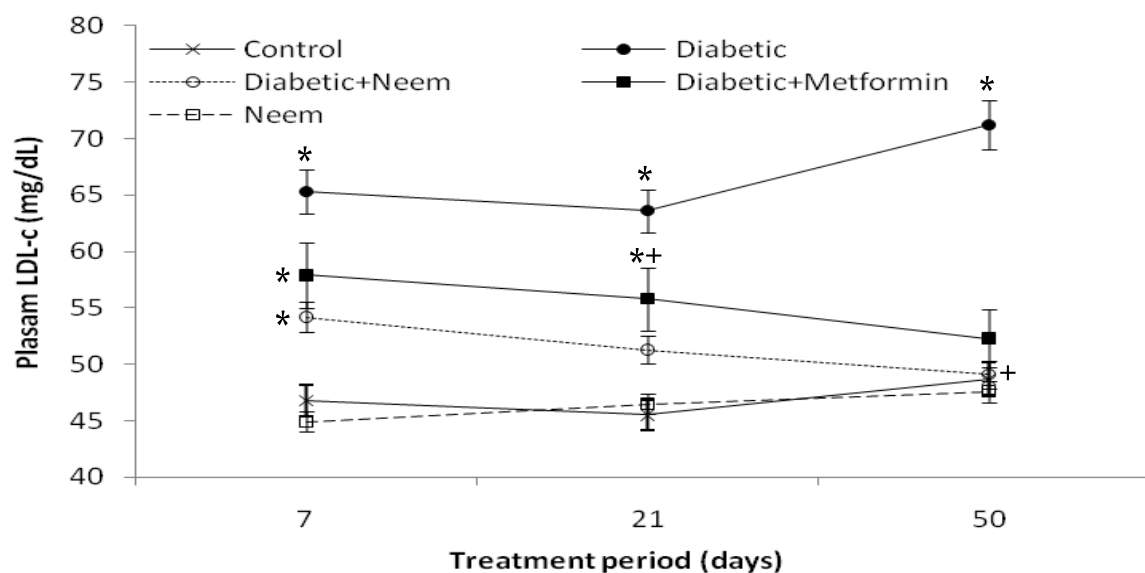


Figure 21: Plasma LDL-c levels. Bars represent mean $\pm$ SEM; *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

4.7.5 Plasma Very Low Density Lipoprotein Cholesterol (VLDL-c)

As shown in figure 22, plasma VLDL-c levels in the different treatment groups were similar to what obtained for LDL-c (Fig. 21). By 50 d, plasma VLDL-c levels were significantly high in the untreated diabetic rats compared with control ($P<0.05$). In contrast, VLDL-c levels in diabetic rats treated with neem and metformin were similar to control ($P>0.05$).

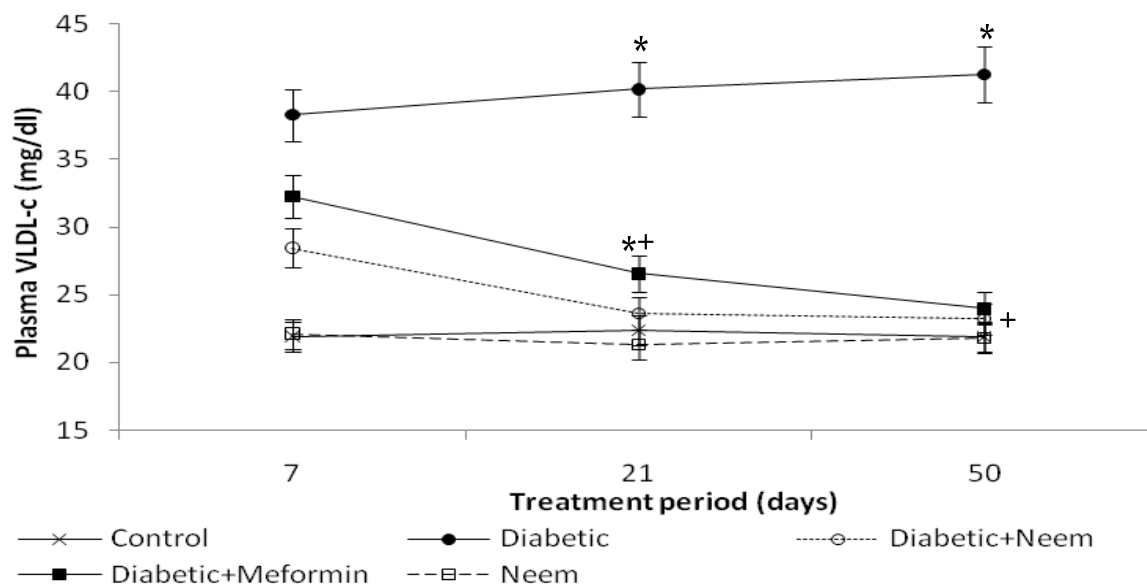


Figure 22: Plasma VLDL-c levels. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$; +Significantly different from diabetic group, $P<0.05$.

4.7.6 Plasma Total Triglyceride

Figure 23 shows plasma total triglyceride in the different treatment groups. In the untreated diabetic animals, sustained increase in plasma triglyceride levels were obtained at each time-point studied. At 50 d, plasma total triglyceride levels were similar to control in all the other treatment groups ($P>0.05$).

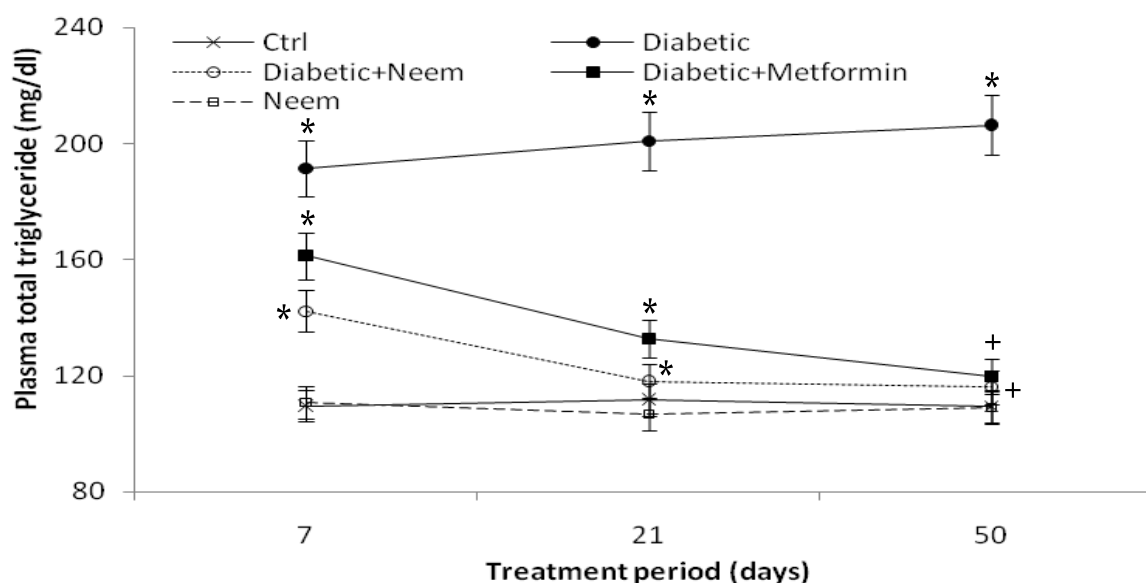


Figure 23: Plasma total triglyceride. Bars represent mean $\pm$ SEM; *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

4.7.7 Plasma Markers of Hepatic and Renal Function

Figures 24, 25 and 26 show changes in the plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea and creatinine at days 7, 21 and 50, respectively. At 7 d, significant increase ($P < 0.05$) in the plasma levels of AST had occurred in the untreated diabetic rats compared with control ($P < 0.05$; Fig. 24). In this same group, plasma levels of renal and hepatic markers (except urea) had increased significantly at 21 d compared with control ($P < 0.05$; Fig. 25). However, plasma levels of these markers were not significantly different from control in diabetic rats treated with neem and metformin. At 50 d, plasma levels of liver enzymes (ALT, AST and ALP) were significantly higher than control in the untreated diabetic rats ($P < 0.05$; Fig. 26). However, in neem-treated diabetic group, plasma levels of these enzymes did not differ significantly from control ($P > 0.05$; Fig. 26), except ALP, whose levels were significantly higher than control at this time. Furthermore, significant increases in plasma AST and ALT had occurred in the metformin-treated diabetic rats at 50 d ($P < 0.05$; Fig. 26).

Moreover, figures 24, 25 and 26 show changes in plasma urea and creatinine relative to control. In the untreated diabetic animals, significant increases in plasma urea and creatinine had occurred at day 50 ($P<0.05$). Similarly, plasma levels of creatinine had also increased significantly in metformin-treated rats at this time ($P<0.05$). In contrast, plasma urea and creatinine did not differ significantly from control in the neem-treated diabetic rats ($P>0.05$). In the latter group, plasma creatinine levels were significantly lower than the levels in the untreated diabetic rats ($P<0.05$; Fig. 26).

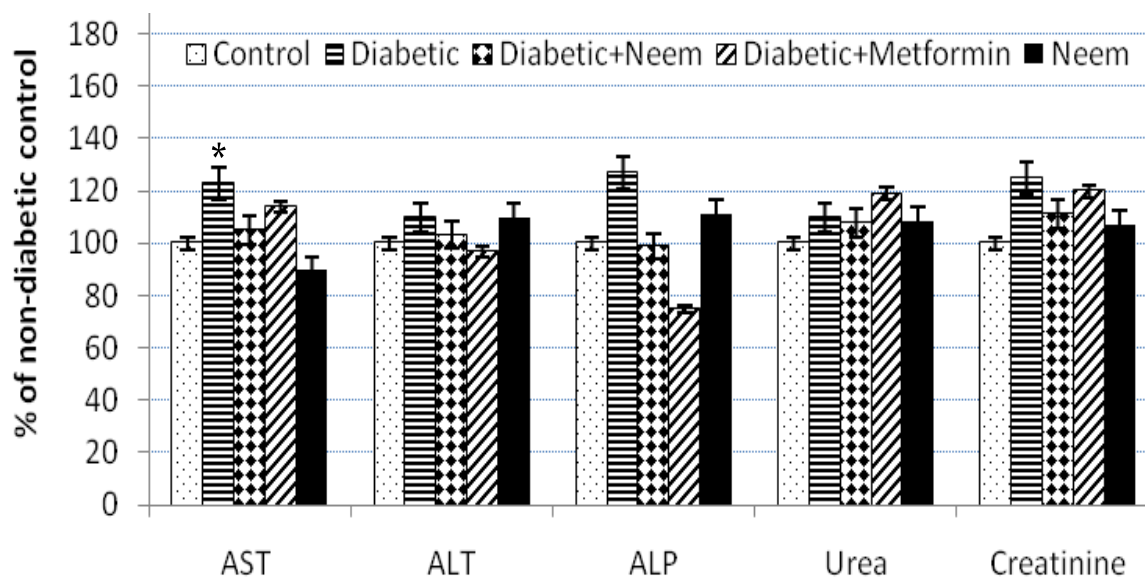


Figure 24. Plasma AST, ALT, ALP, urea and creatinine at 7 d. Bars represent mean $\pm$ SEM.

*Significantly different from control, $P<0.05$.

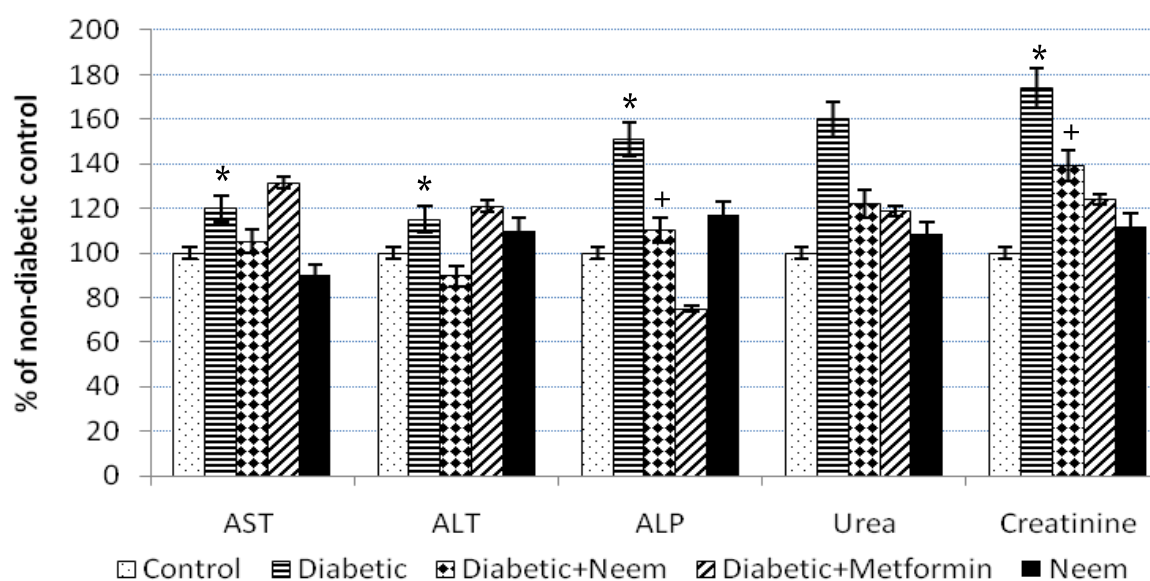


Figure 25. Plasma AST, ALT, ALP urea and creatinine at 21 d. Bars represent mean $\pm$ SEM. *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

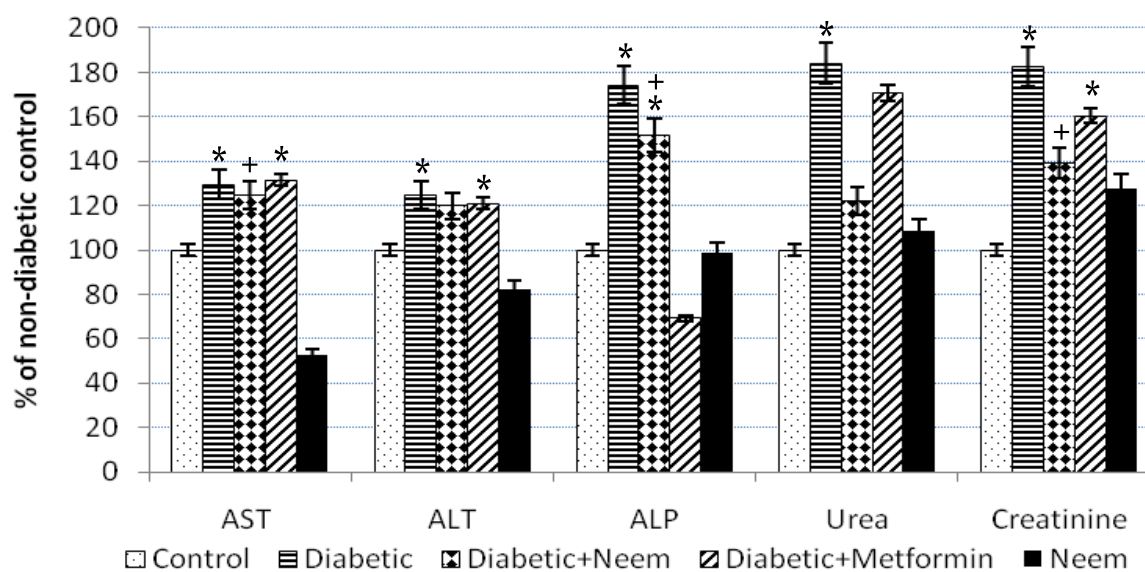


Figure 26. Plasma AST, ALT, ALP, urea and creatinine at 50 d. Bars represent mean $\pm$ SEM. *Significantly different from control, $P < 0.05$; +Significantly different from diabetic group, $P < 0.05$.

4.8 Biomarkers of Oxidative Stress

4.8.1 Aqueous Hydroperoxide Levels of the Pancreas

Figure 27 shows percentage changes in aqueous hydroperoxide (H_2O_2) levels in the various treatment groups compared with control. By 7 d of treatment, aqueous hydroperoxide levels of the pancreas were significantly lower ($P<0.05$) in all the treatment groups compared with control. However, by 21 d, aqueous hydroperoxide levels were highest in the metformin-treated diabetic rats, being more than 4-fold higher than non-diabetic control ($P<0.05$). Neem-treated diabetic rats had the lowest pancreatic aqueous hydroperoxide levels at this time (21 d). At 50 d, 29% rise in aqueous hydroperoxide levels had occurred in the pancreas of untreated diabetic rats compared with control ($P<0.05$). Moreover, treatment of diabetic rats with metformin appeared to exacerbate pancreatic aqueous hydroperoxide levels (125% higher than control). However, at this time, pancreatic aqueous hydroperoxide levels in neem-treated rats had fallen below control by 61% and 32%, respectively ($P<0.05$).

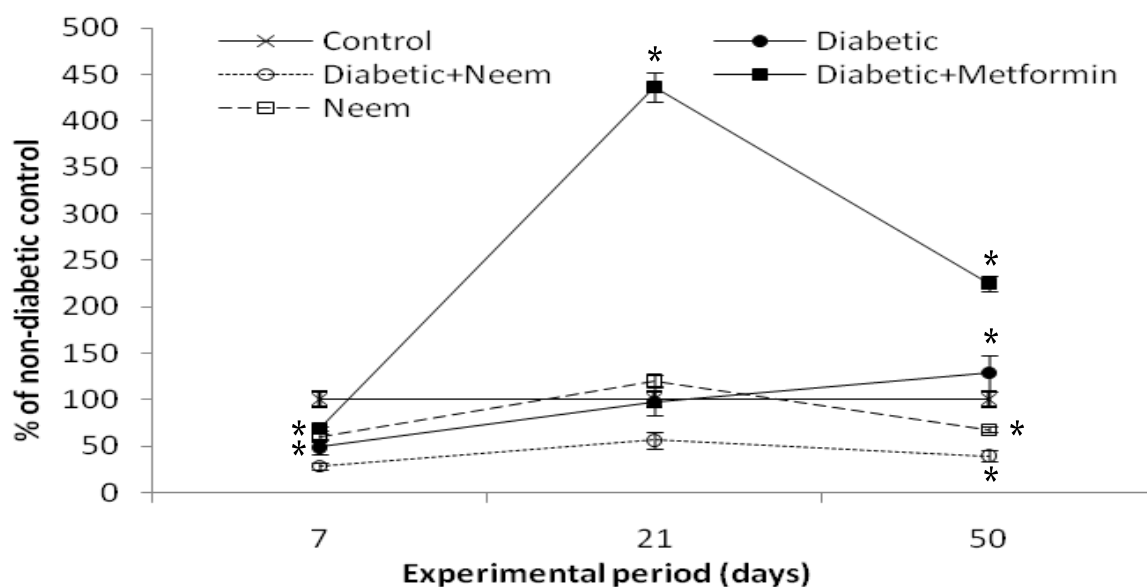


Figure 27: Aqueous hydroperoxide levels of the pancreas. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.8.2 Lipid Hydroperoxide Levels of the Pancreas

Figure 28 shows changes in lipid hydroperoxide levels of the pancreas in the various treatment groups compared control. As was obtained for aqueous hydroperoxides, pancreatic lipid hydroperoxide levels were significantly low in all the treatment groups at 7 d ($P<0.05$). Moreover, 5-fold increase ($P<0.05$) in pancreatic lipid hydroperoxides had occurred in the metformin-treated rats at 21 d (compared with control). At this time, lipid hydroperoxide levels had risen by 5% in the untreated diabetic animals ($P<0.05$) and by 37% in neem-treated normoglycemic rats ($P<0.05$) compared with control. In contrast, pancreatic lipid hydroperoxide had fallen by 35% in neem-treated diabetic animals compared with control ($P<0.05$). By 50 d, pancreatic lipid hydroperoxide levels in all treatment groups were similar to what obtained for aqueous hydroperoxide (Fig. 27).

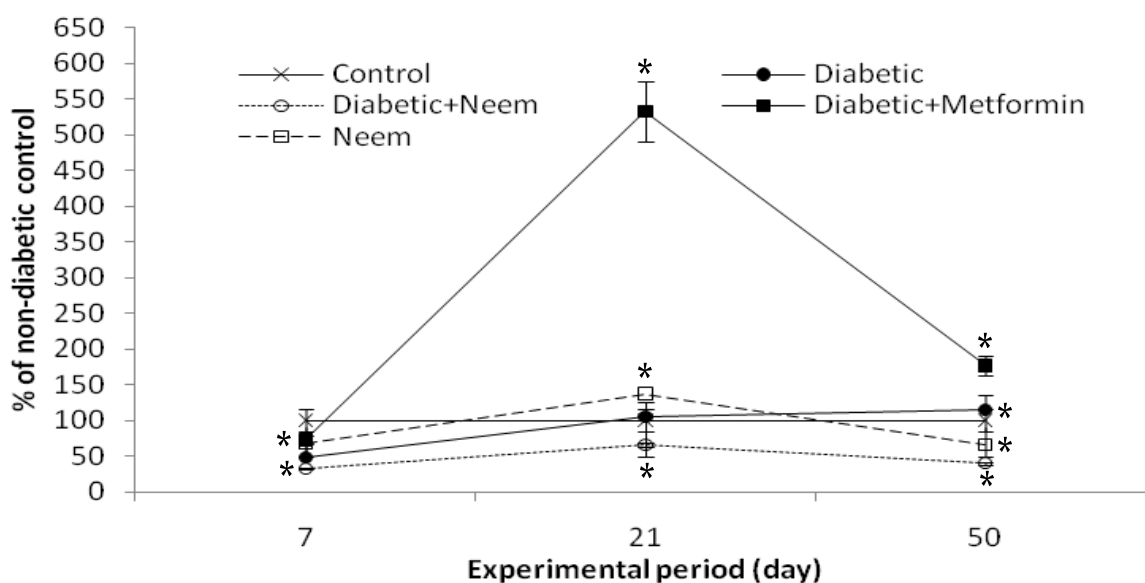


Figure 28: Lipid hydroperoxide levels of the pancreas. Bars represent mean $\pm$ SEM;

*Significantly different from control, $P<0.05$.

4.8.3 Superoxide Dismutase (SOD) Activity of the Pancreas

At 7 d of treatment, pancreatic SOD activities were similar to control in all the treatment groups (Fig. 29). The highest enzyme activity at this time (4.3% above control) was obtained in diabetic rats treated with metformin ($P>0.05$). This trend was also observed at 21 d, where SOD activity was highest in metformin-treated rats ($P<0.05$). Although SOD activity increased as well in the other treatment groups (both at 7 d and 21 d), the differences were not significant ($P>0.05$). At 50 d, SOD activity was not significantly different from control in all the treatment groups ($P>0.05$) (Fig. 29).

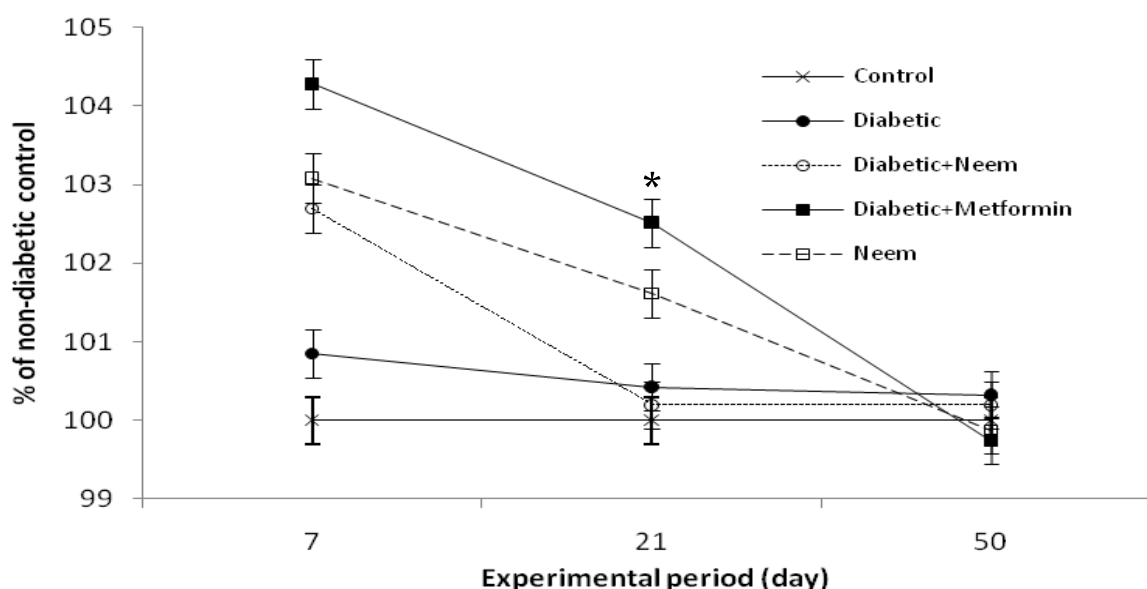


Figure 29: Superoxide dismutase activity of the pancreas. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.8.4 Lipid Hydroperoxide Levels of the Liver

Figure 30 shows percentage changes in lipid hydroperoxide levels of the different treatment groups compared with control. At days 21 and 50, hepatic lipid hydroperoxide levels had increased by 60% and 14%, respectively, in the untreated diabetic rats compared with control ($P<0.05$). Moreover, in neem-treated diabetic rats, hepatic oxidative stress was exacerbated (55% above control; $P<0.05$) in the early phase of treatment. However, in the latter phase of

neem treatment, hepatic lipid hydroperoxide levels had decreased markedly compared with untreated diabetic group, but these levels were still significantly higher than control ($P<0.05$). In non-diabetic rats treated with neem, lipid hydroperoxide levels were significantly higher than control in the early phase of treatment (7d and 21) ($P<0.05$). However, a significant reduction in lipid peroxidation had occurred at 50 d in this group (Fig. 30).

Furthermore, metformin treatment did not prevent the rise in hepatic lipid peroxide levels in the diabetic rats. In these animals, lipid hydroperoxide levels increased gradually from 31% above control at 7 d, to 44% at 50 d. Thus, at the latter phase of treatment, hepatic lipid hydroperoxide levels were significantly higher than control in all the groups except neem-treated non-diabetic rats (Fig. 30).

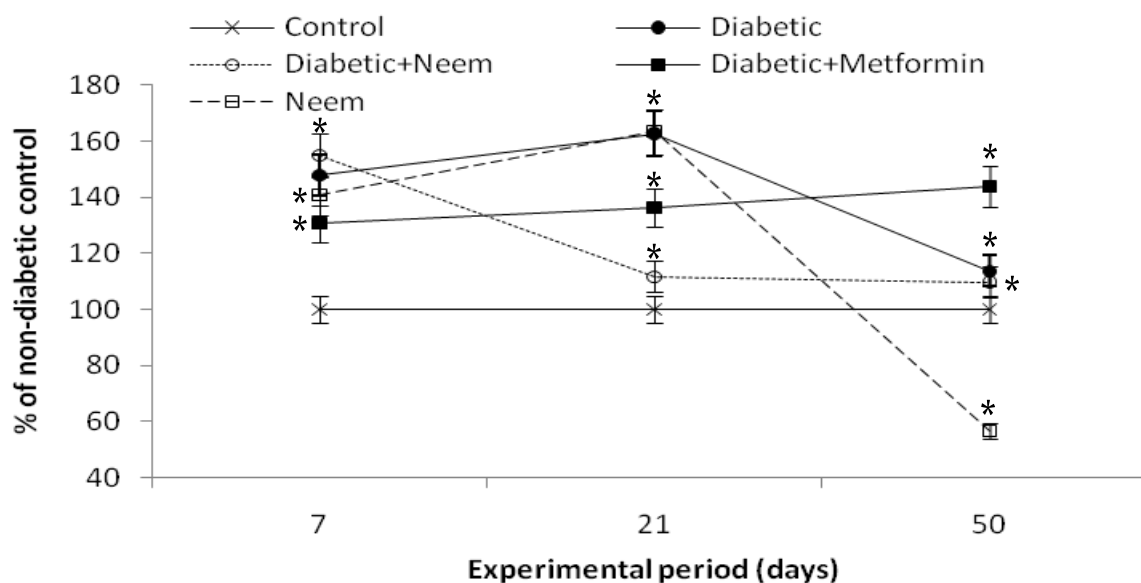


Figure 30: Lipid hydroperoxide levels of the liver. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.8.5 Aqueous Hydroperoxide Levels of the Liver

Hepatic aqueous hydroperoxide levels are shown in figure 31. In the untreated diabetic group, aqueous hydroperoxide levels were similar to control at 7 d. By days 21 and 50, however, the levels had increased significantly above control (by 41% and 11%, respectively; $P<0.05$).

Furthermore, in neem-treated diabetic rats, hepatic aqueous hydroperoxide levels did not differ significantly from control early in the treatment ($P>0.05$) (Fig. 31). However, by 50 d, the levels had increased, and was nearly 60% above control ($P<0.05$). Similarly, metformin treatment could not prevent the rise in aqueous hydroperoxide levels. In these animals, aqueous hydroperoxide levels rose from 15% above control at 7 d, to nearly 50% at 50 d. Moreover, hepatic aqueous hydroperoxide levels in the neem-treated non-diabetic rats showed a trend similar to hepatic lipid hydroperoxide levels (Fig. 30 and 31).

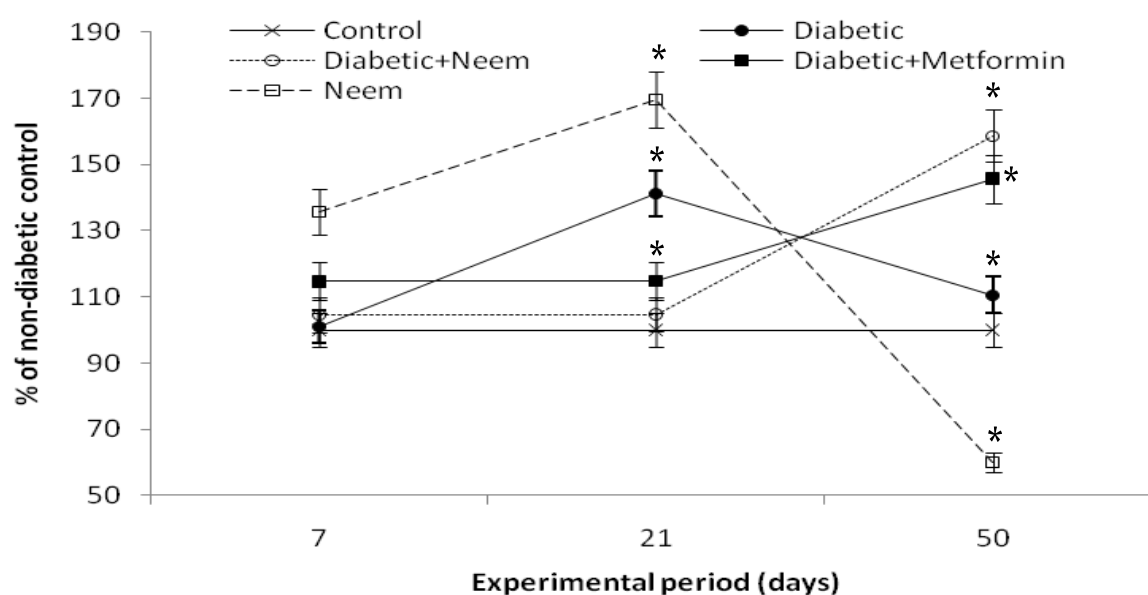


Figure 31: Aqueous hydroperoxide levels of the liver. Bars represent mean $\pm$ SEM;

*Significantly different from control, $P<0.05$.

4.8.6 Superoxide Dismutase Activity of the Liver

Hepatic SOD activities are shown in figure 32. By 7 d, liver SOD activity had decreased significantly by 42% in the untreated diabetic rats compared with control ($P < 0.05$). Similarly, significant decreases ($P < 0.05$) in SOD activity had occurred in metformin-treated diabetic and neem-treated non-diabetic rats by this time. SOD activities in hyperglycemic rats treated with neem were however not significantly different from control ($P > 0.05$). In the latter part of treatment, SOD activities in all the groups did not differ statistically from control ($P > 0.05$), except for the neem-treated non-diabetic rats, where a significant decrease had occurred.

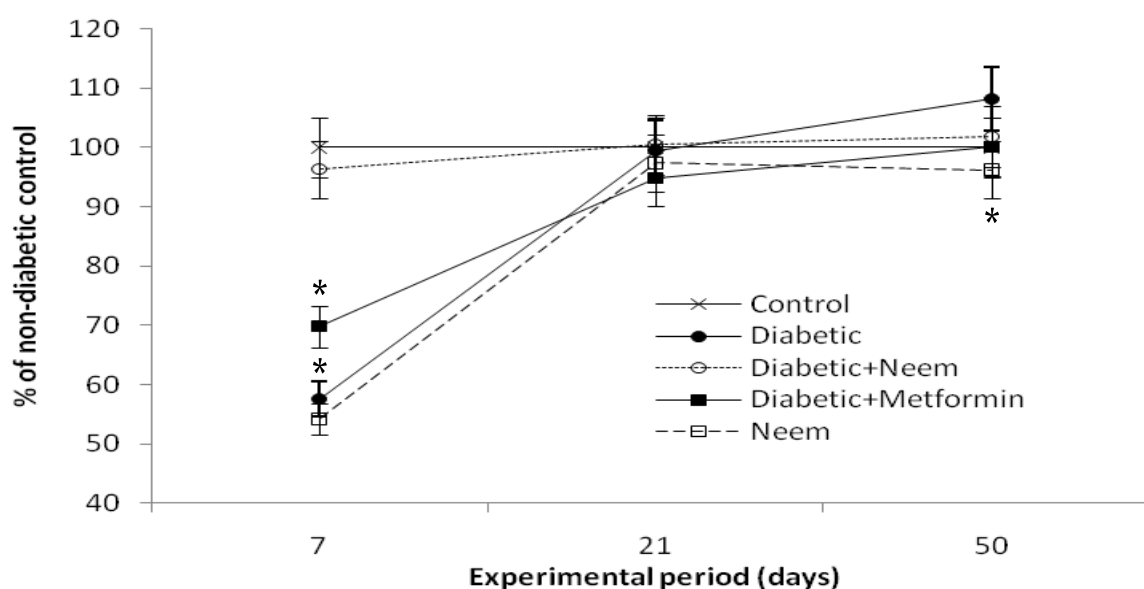


Figure 32: SOD activity of the liver. Bars represent mean $\pm$ SEM; *Significantly different from control, $P < 0.05$.

4.8.7 Reduced Glutathione (GSH) Levels of the Liver

Hepatic GSH levels are shown in figure 33. Cellular GSH levels decreased progressively in the untreated diabetic rats from a 3-fold increase ($P < 0.05$) above control at 7 d, to a level 5% below control at 50 d. Similarly, hepatic GSH levels in the neem-treated diabetic rats decreased from a level 58% above control at 7 d to 20% below control at 50 d ($P > 0.05$). In diabetic rats treated with metformin, hepatic GSH increased significantly above control at 7 d

($P<0.05$); dropped slightly at 21 d; and peaked again at 50 d (20% above control; $P<0.05$). Treatment of non-diabetic rats with neem leaf extract resulted in significant increases in hepatic GSH by 21 d ($P<0.05$). However, by 50 d, GSH levels in these animals had decreased to a level 20% below control ($P<0.05$).

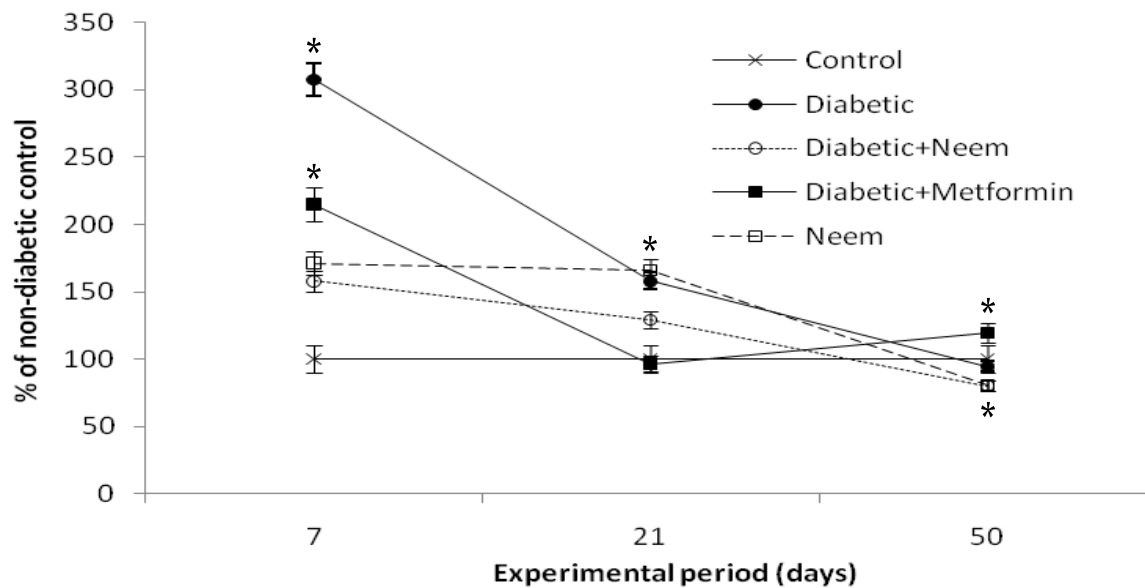


Figure 33: GSH levels of the liver. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.9 Morphometric Analysis

4.9.1 Morphometric Analysis of Pancreatic Islets

Figure 34 shows the morphometric analysis of the pancreatic islets. At 7 d of treatment, islet β -cell density (number of β -cells/ mm^2 of the islet) had decreased significantly in the untreated diabetic, neem-treated diabetic, and metformin-treated diabetic rats compared with control ($P<0.05$). Only 24.4%, 29.8% and 24.0%, respectively, of β cells remained viable in these groups. Islet β -cell density in neem-treated non-diabetic rats did not differ significantly from control ($P>0.05$).

However, by 21 d of treatment, increases in the number of viable islet β cells had occurred in the neem-treated diabetic and metformin-treated diabetic rats (Fig. 34). In contrast, viable β cells in the untreated diabetic rats remained relatively low compared with control ($P<0.05$). By 50 d, neem-treated diabetic rats had the highest percentage (50%) of viable β cells (relative to the untreated diabetic and metformin-treated diabetic groups (Fig. 34).

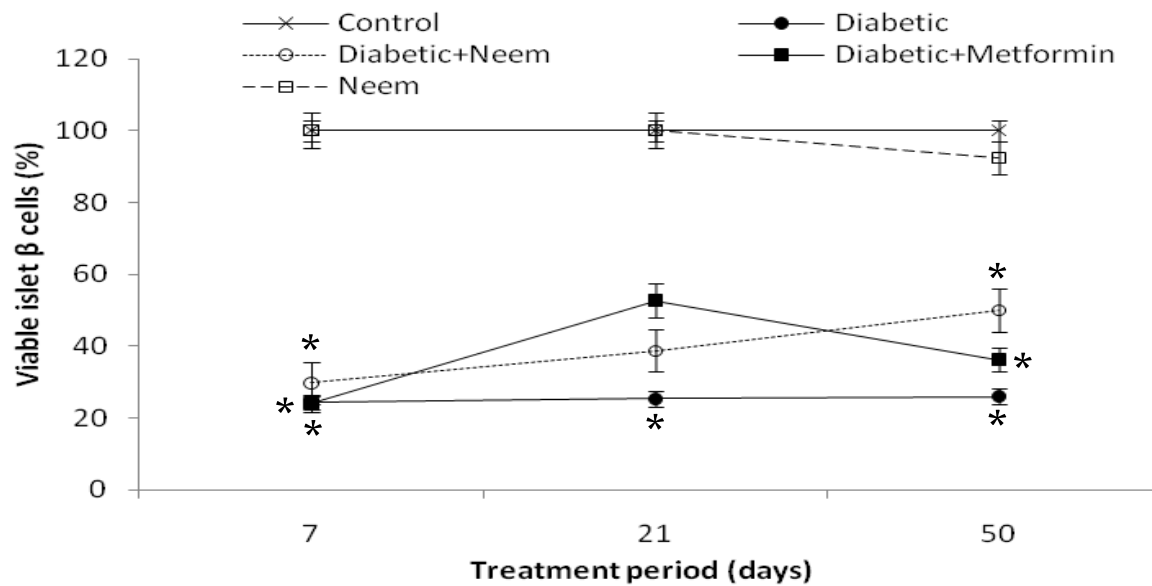


Figure 34: Percentage of viable islet β cells relative to control. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.9.2 Morphometric Analysis of the Kidney

Figure 35 shows the morphometric analysis of renal glomeruli. At 7 d of treatment, glomerular size (diameter) in all the treatment groups was not significantly different from control ($P>0.05$), with an average value of $179.3\pm5.8\ \mu\text{m}$ in the latter group. By 21 d, 19% increase in glomerular size had occurred in the neem-treated diabetic animals ($P<0.05$). Glomerular size in each of the other treatment groups was not significantly different from control ($P>0.05$).

Meanwhile, at 50 d, virtually all glomeruli in the untreated diabetic rats had become sclerotic (Fig. 44 E), with a 5% decrease in glomerular size compared with control (Fig. 35). In

contrast, 30% increase in glomerular size was observed in the metformin-treated diabetic rats compared with control ($P<0.05$). Glomerular diameter in each of the remaining treatment groups was not different from control at this time ($P>0.05$).

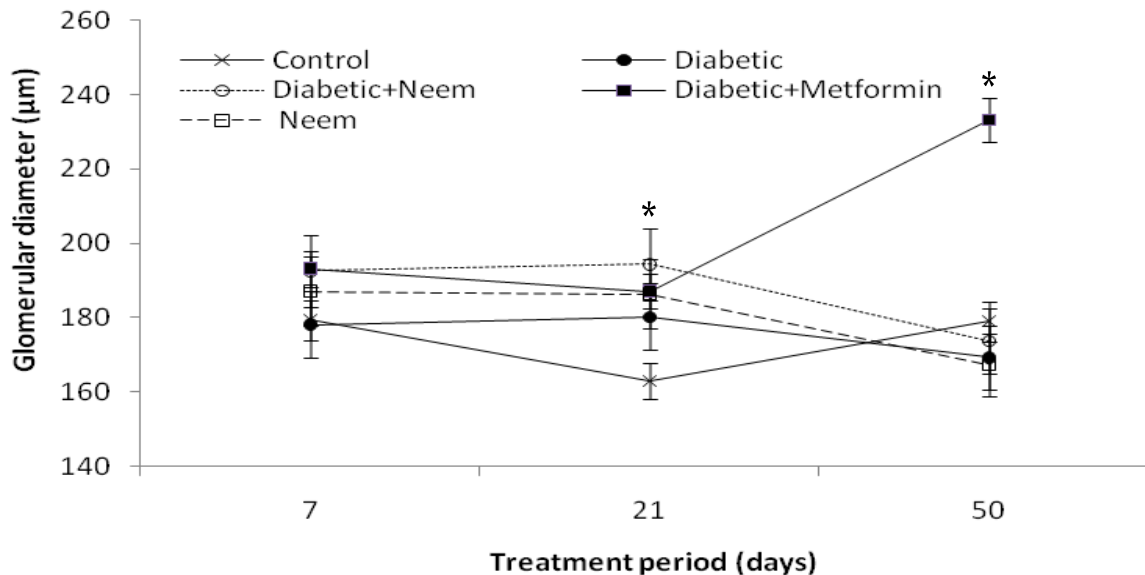


Figure 35: Glomerular diameters at treatment days 7, 21 and 50. Bars represent mean $\pm$ SEM; *Significantly different from control, $P<0.05$.

4.10 Histological Findings

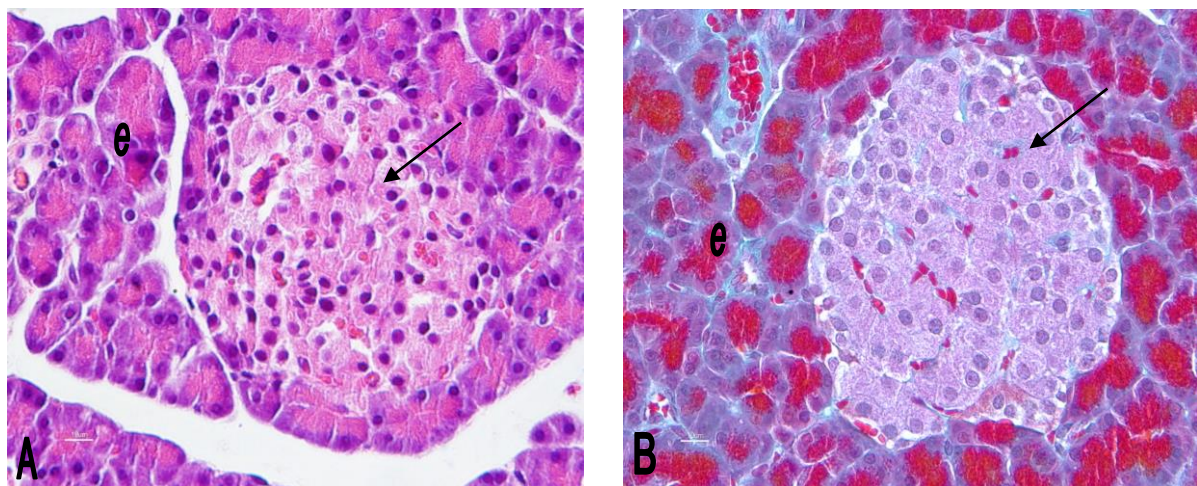
4.10.1 Histology of the Pancreas

At 7 d of treatment, the pancreas of untreated diabetic and neem-treated diabetic rats were characterised by islet necrosis and β -cell loss (Fig. 36 C-F). Such necrotic changes were absent in the control and neem-treated non-diabetic rats. In the islets of metformin-treated diabetic rats, necrotic β cells were observable (Fig. 36 G). Furthermore, trichrome staining showed absence of islet fibrosis in all the groups at 7 d.

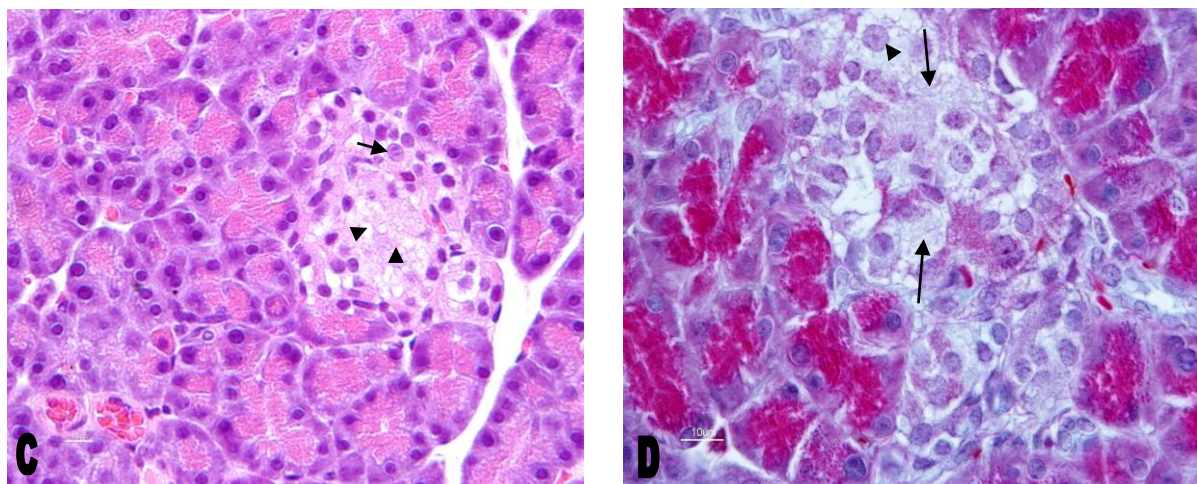
By 21 d of treatment, islet necrosis characteristic of the untreated diabetic and neem-treated diabetic rats at 7 d were still observable (Fig. 37 C-F). Besides, metformin-treated diabetic rats had developed appreciable necrotic changes at 21 d. Hypertrophic β cells and those undergoing necrosis were also observable in the untreated diabetic, neem-treated diabetic and

metformin-treated diabetic rats (Fig. 36 and 37). Such changes were absent in the control and neem-treated non-diabetic rats.

Furthermore, some fibrous deposits were observable in the islets of neem-treated diabetic rats at 21 d (Fig. 37 F). However, fibrosis was absent in the islets of the control, neem-treated non-diabetic, and metformin-treated diabetic rats at this time (21 d).

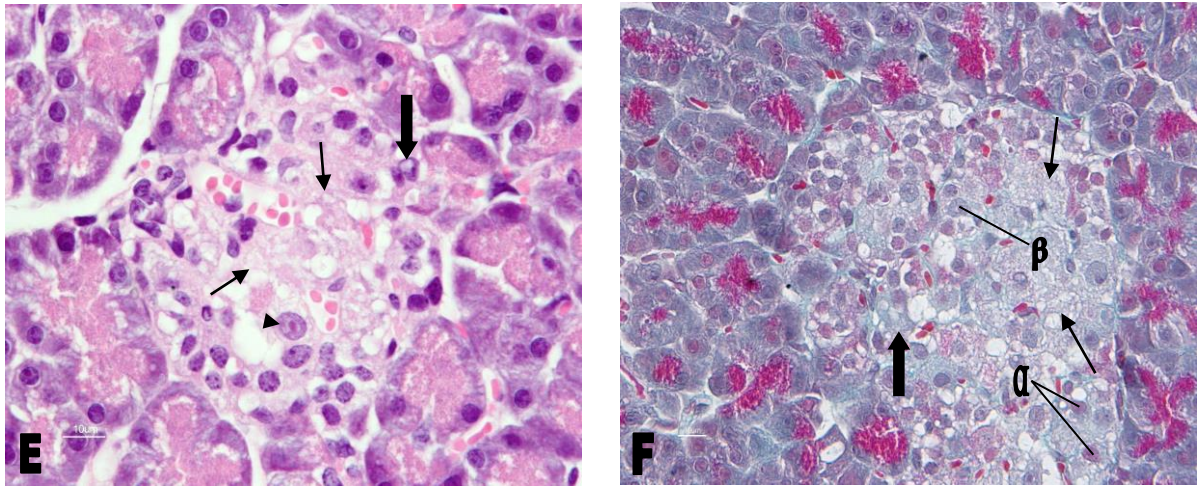


Figures 36 A and B. Pancreatic islets of control rats at day 7. **(A)** Necrosis is absent in the islet (arrow); H&E, x400. **(B)** Fibrosis is absent in the islet (arrow); Masson's trichrome, x400. *e* indicates exocrine pancreas. (Scale bar = 10 μm).

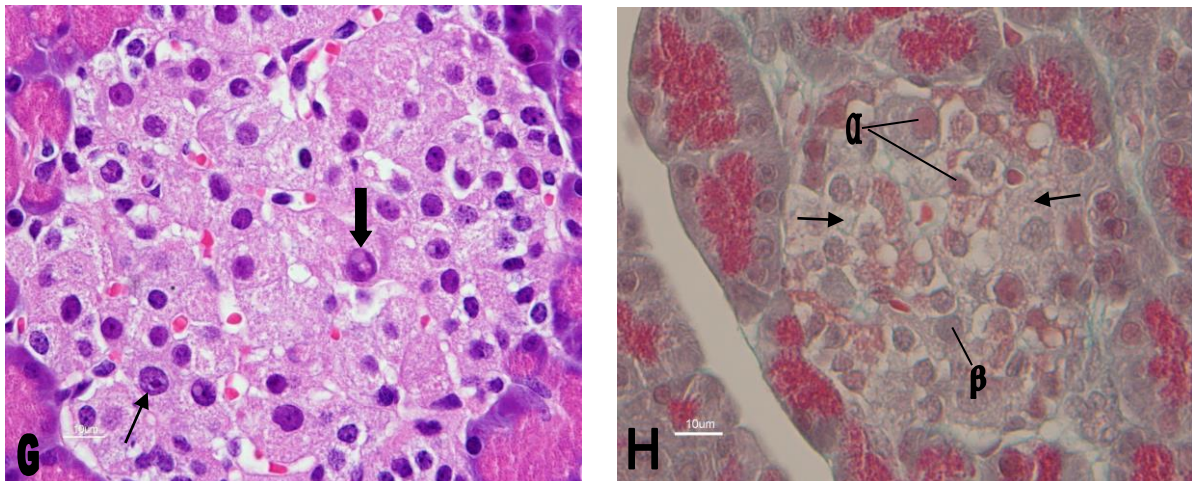


Figures 36 C and D. Pancreatic islets of untreated diabetic rats at day 7. **(C)** Intra-islet necrosis is observable (arrowhead), while some cells appear hypertrophic (arrow). H&E,

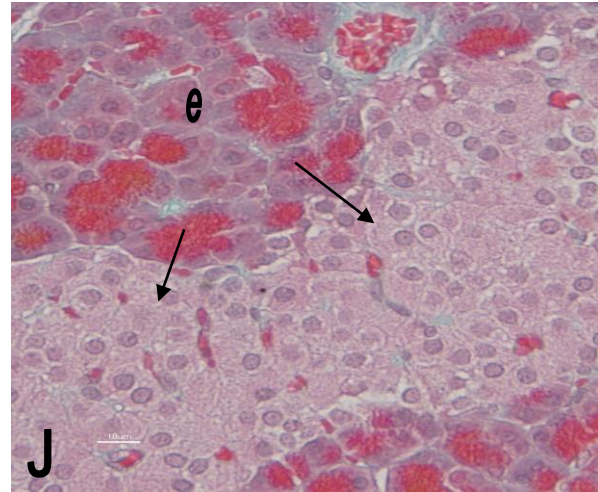
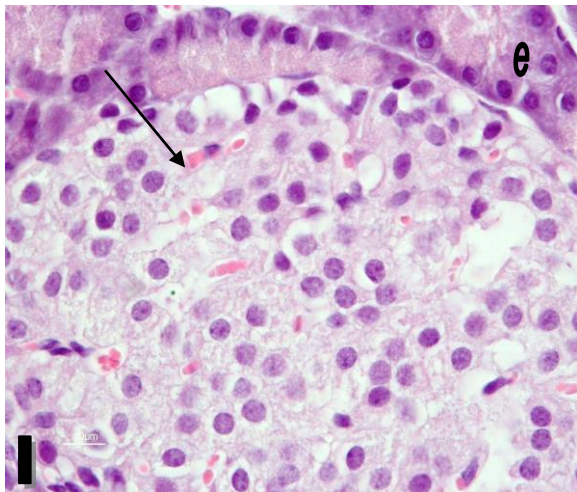
x600. (D) Fibrosis is absent; β cells (arrowhead) and necrotic areas (arrows) are observable. Masson's trichrome, x600. (Scale bar = 10 μ m).



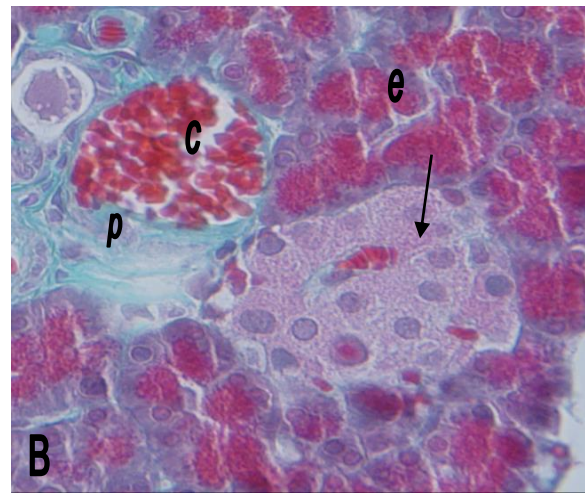
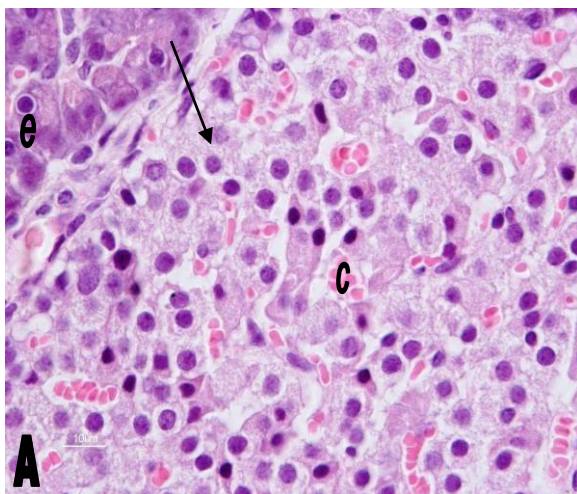
Figures 36 E and F. Pancreatic islets of neem-treated diabetic rats at day 7. (E) Islet necrosis is observable (thin arrows); some cells are hypertrophic (arrowhead), while others show necrotic spots (thick arrow); H&E, x600. (F) Necrosis (thin arrows) and some collagen fibres (thick arrow) are observable; α indicates alpha cells; β indicates beta cells. Masson's trichrome, x400. (Scale bar = 10 μ m).



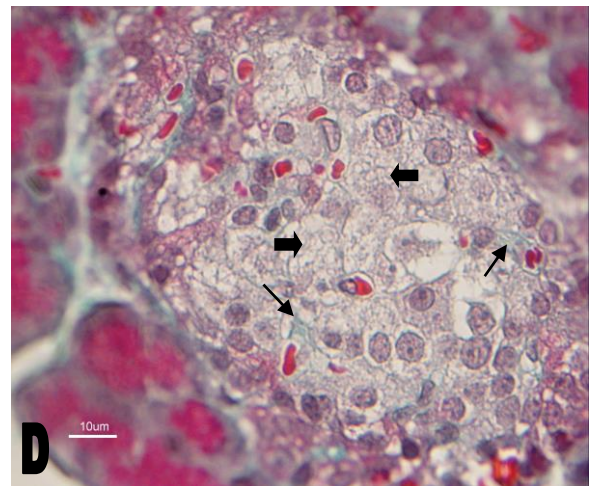
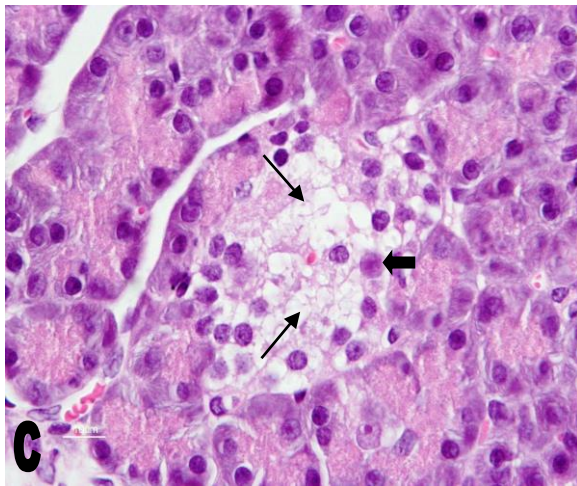
Figures 36 G and H. Pancreatic islets of metformin-treated diabetic rats at day 7. (G) Hypertrophic nuclei (thin arrow) and nuclei with necrotic spots (thick arrow) are observable; H&E, x600. (H) Fibrosis is absent in the islet, but necrosis is observable (thin arrows); α indicates α cells; β indicates β cells; Masson's trichrome, x600. (Scale bar = 10 μ m).



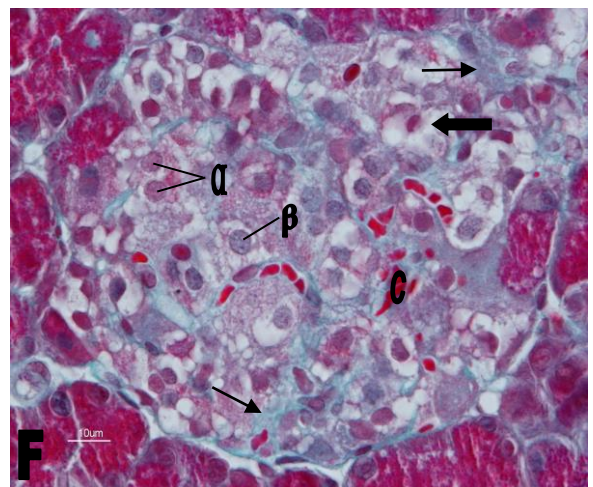
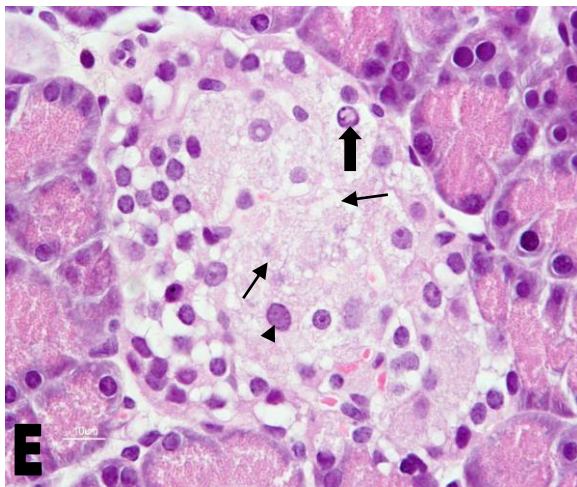
Figures 36 I and J. Pancreatic islets of neem-treated non-diabetic rats at day 7. **(I)** Islet appears normal (arrow). H&E, x600. **(J)** Fibrosis is absent in the islet (arrows). *e* indicates exocrine pancreas; Masson's trichrome, x400. (Scale bar = 10 μ m).



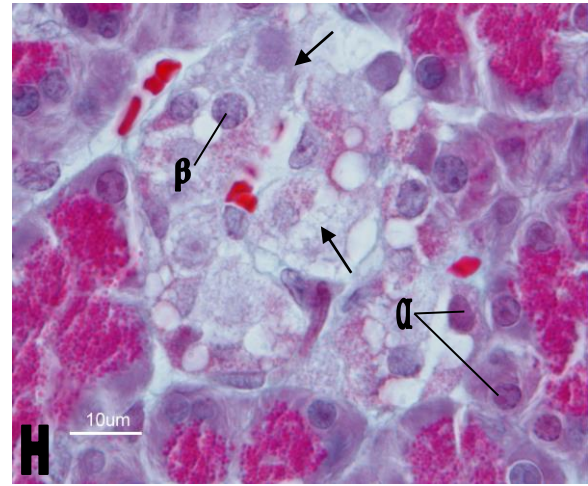
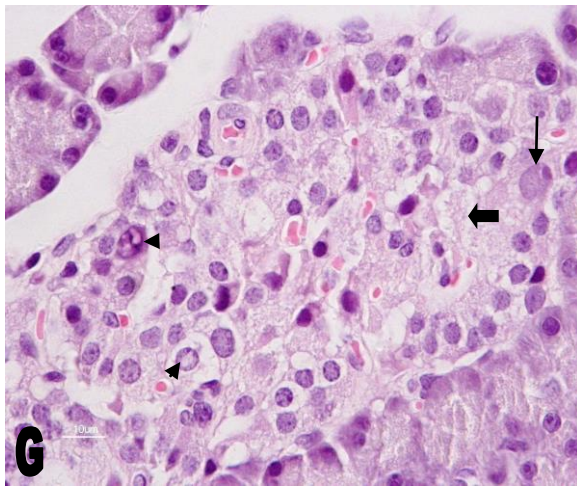
Figures 37 A and B. Pancreatic islets of control rats at day 21. **(A)** Islet (arrow) is devoid of necrosis; H&E, x600. **(B)** Fibrosis is absent in the islet (arrow). Masson's trichrome, x600; *e* indicates exocrine pancreas; *c* indicates capillary; *p* indicates perivascular collagen. (Scale bar = 10 μ m).



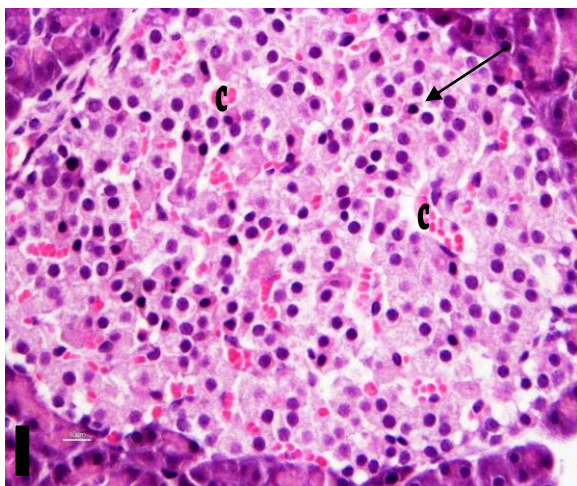
Figures 37 C and D. Pancreatic islets of untreated diabetic rats at day 21. **(C)** Islet necrosis (thin arrows) and hypertrophic cells (thick arrow) are observable. H&E, x600. **(D)** Fibrous spots (thin arrows) and necrotic changes (thick arrows) are observable. Masson's trichrome, x600. (Scale bar = 10 μ m).



Figures 37 E and F. Pancreatic islets of neem-treated diabetic rats at day 21. **(E)** Necrosis (thin arrows), hypertrophic cell (arrowhead) and necrotic cell (thick arrow) are observable. H&E, x600. **(F)** Fibrous deposits (thin arrows) and necrotic changes (thick arrow) are observable; *c* indicates capillary; α indicates alpha cells; β indicates beta cells. Masson's trichrome, x600. (Scale bar = 10 μ m).



Figures 37 G and H. Pancreatic islets of metformin-treated diabetic rats at day 21. **(G)** Hypertrophic cell (thin arrow), necrotic area (thick arrow) and necrotic nuclei (arrowheads) are observable. H&E, x600. **(H)** Necrosis is extensive (arrows) while fibrosis is absent; α indicates alpha cells; β indicates beta cells. Masson's trichrome, x600. (Scale bar = 10 μ m).

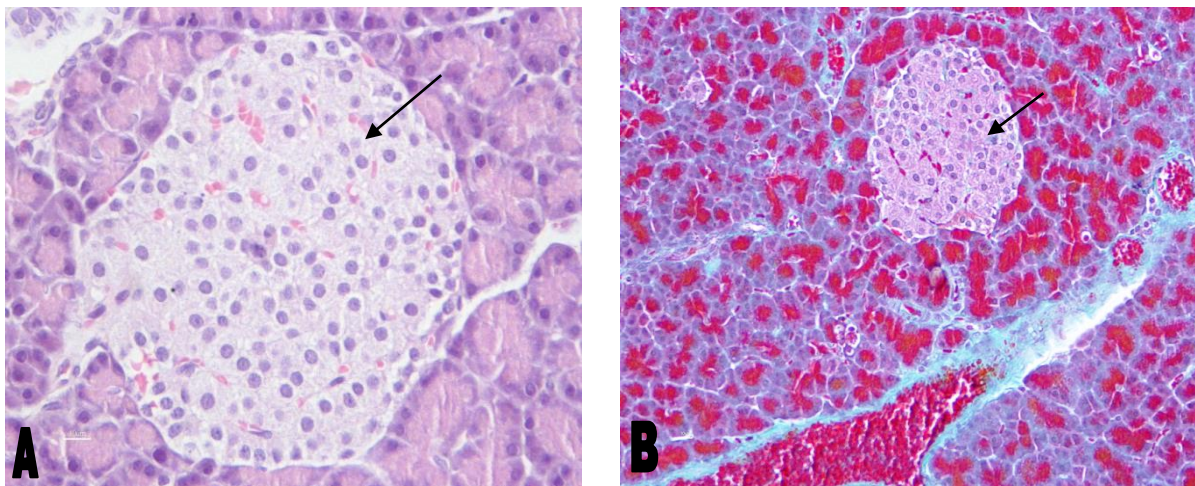


Figures 37 I and J. Pancreatic islets of neem-treated non-diabetic rats at day 21. **(I)** Islet is well vascularised and its cells are intact (arrow); c indicates capillaries. H&E, x400. **(J)** Fibrosis and necrosis are absent in the islet (arrow); α indicates alpha cells; β indicates beta cells. Masson's trichrome, x600. (Scale bar = 10 μ m).

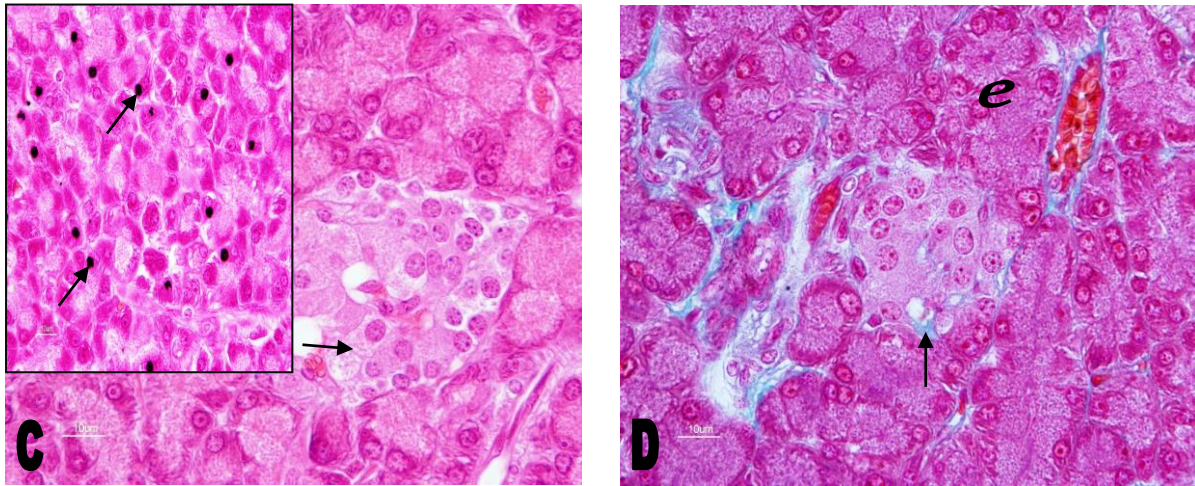
At 50 d of treatment, islets of untreated diabetic rats were markedly diminished, with necrosis and paucity of cells (Fig. 34 and 38 C). In addition to these changes, marked eosinophilia was observed in both the exocrine and endocrine pancreas (Fig. 38 C). Moreover, characteristic

darkly-pigmented intracytoplasmic bodies were observable in the acinar cells of exocrine pancreas (Fig. 38 C, inset). Furthermore, the islet-wide necrotic changes seen in the neem-treated diabetic rats at 7 d and 21 d were largely reduced at 50 d. By this time, necrosis was restricted to islet periphery, while the central part of the islet was well vascularised (Fig. 38 E) and possessed viable β cells (Fig. 38 E and F). In contrast, diffuse necrosis that characterised the islets of metformin-treated diabetic rats at 21 d was also noted at 50 d (Fig. 38 G).

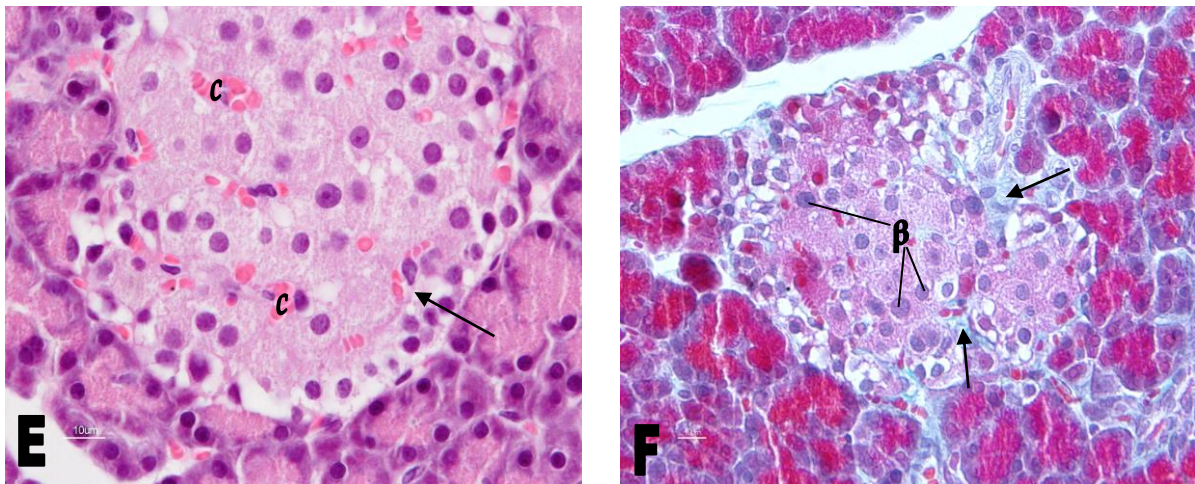
Fibrosis and necrosis were absent in the islets of control and neem-treated normoglycemic rats at 50 d (Fig. 38 A, 38 B, 38 I and 38 J). In neem-treated diabetic animals, fibrosis was restricted to islet periphery by this time (50 d) (Fig. 38 F). Furthermore, islets of untreated diabetic rats showed fibrotic spots at the periphery (Fig. 38 D); but fibrosis was absent in the islet of metformin-treated diabetic rats (except for extensive necrosis) (Fig. 38 H).



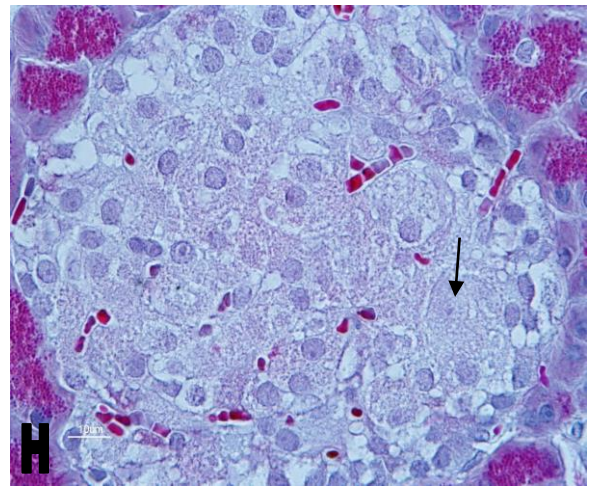
Figures 38 A and B. Pancreatic islets of control rats at day 50. **(A)** Islet is devoid of necrotic changes (arrow); H&E, x400. **(B)** Fibrosis and necrosis are absent in the islet (arrow). Masson's trichrome, x200. (Scale bar = 10 μ m).



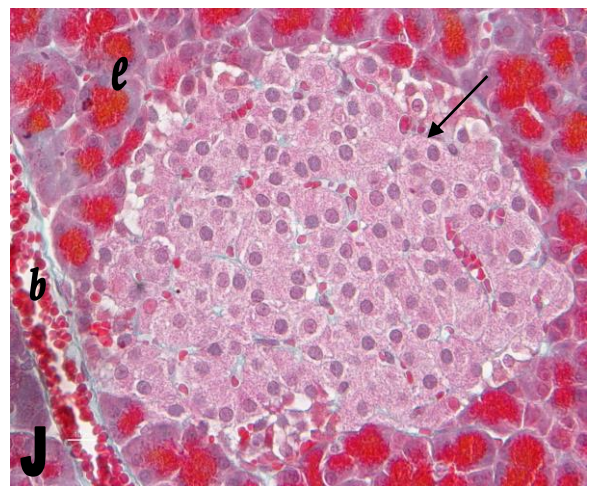
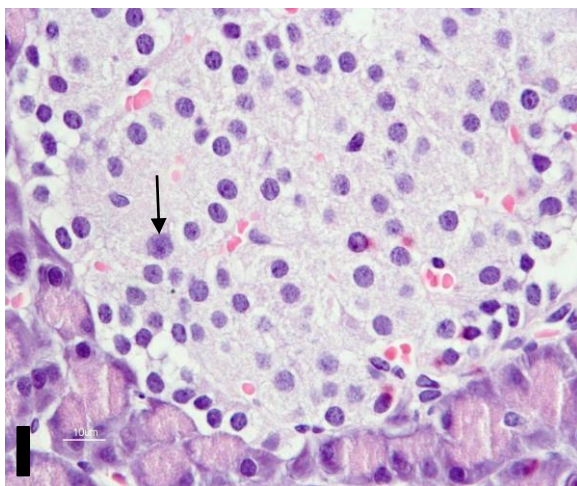
Figures 38 C and D. Pancreatic islets of untreated diabetic rats at day 50. **(C)** Islet (arrow) is diminished, with paucity of cells and necrosis; **(Inset)** exocrine pancreas of diabetic rats; darkly-pigmented bodies are seen in many acinar cells. Increased eosinophilia of exocrine pancreas is also evident. H&E, x600. **(D)** There is paucity of islet cells and presence of fibrous spots at islet periphery (arrow); **e** indicates exocrine pancreas. Masson's trichrome, x600. (Scale bar = 10 μ m).



Figures 38 E and F. Pancreatic islets of neem-treated diabetic rats at day 50. **(E)** Islet is well-vascularised (arrow), and viable cells are observable; **c** indicates capillary. H&E, x600. **(F)** Viable beta cells (β) are observable in the islet; necrosis and fibrosis are confined to islet periphery (arrows). Masson's trichrome, x600. (Scale bar = 10 μ m).



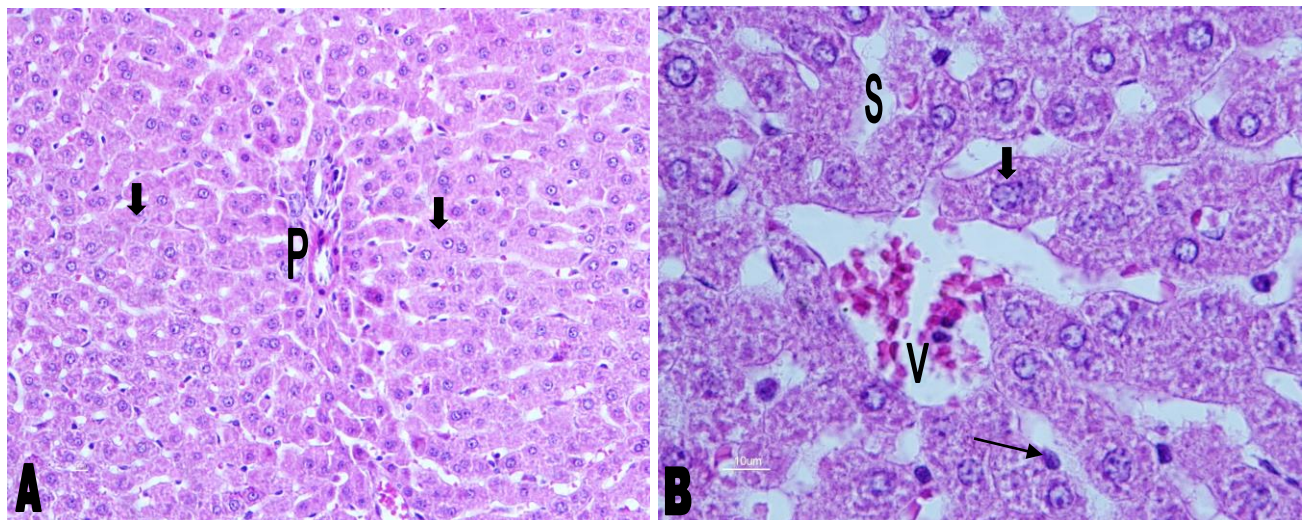
Figures 38 G and H. Pancreatic islets of metformin-treated diabetic rats at day 50. **(G)** Necrosis is observable in the islet (arrow). H&E, x600. **(H)** Islet fibrosis is negligible, but islet-wide necrosis is observable (arrow). Masson's trichrome, x600. (Scale bar = 10 μ m).



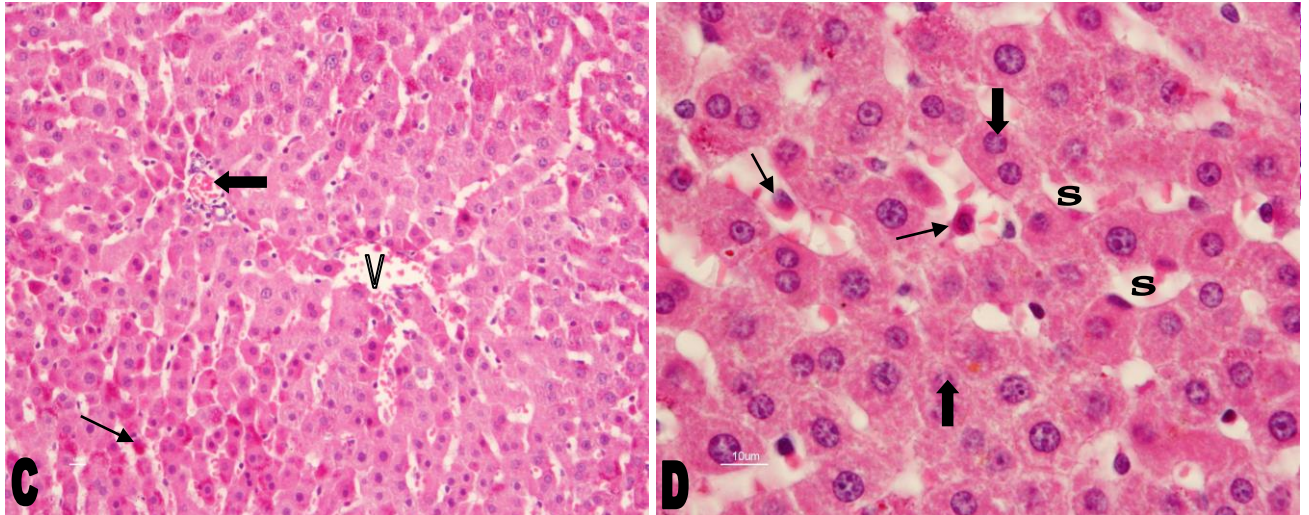
Figures 38 I and J. Pancreatic islets of neem-treated non-diabetic rats at day 50. **(I)** Necrosis is absent; and islet cells (arrow) remain viable. H&E, x600. **(J)** Fibrosis and necrosis are absent in the islet (arrow); *e* indicates exocrine pancreas; *b* indicates blood vessel. Masson's trichrome, x400. (Scale bar = 10 μ m).

4.10.2 Hepatic Histology

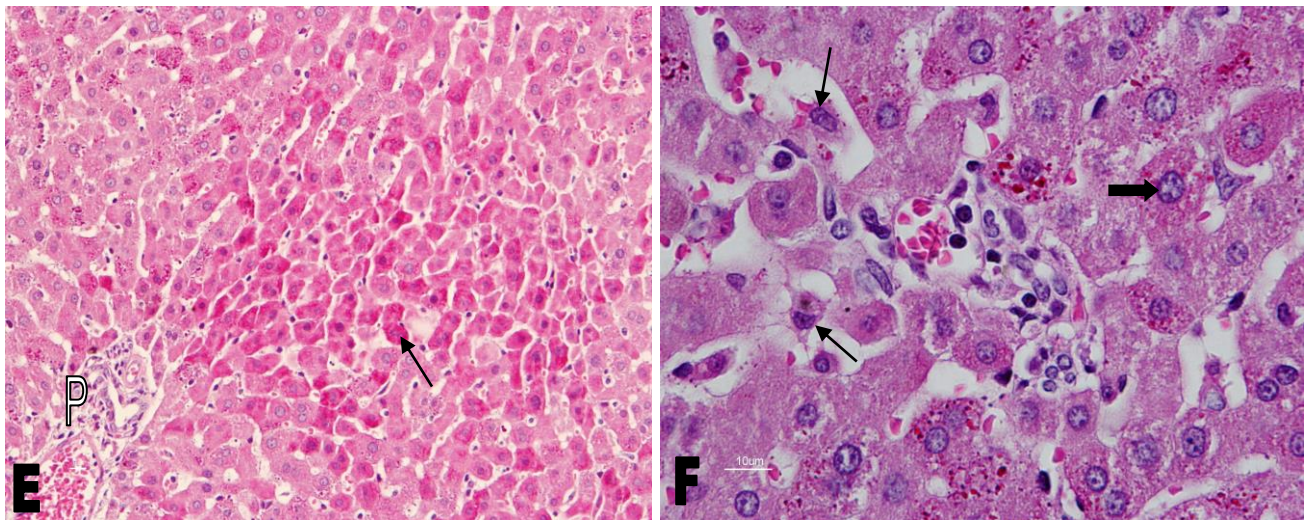
At 7 d of treatment, hepatic sections of overnight-fasted untreated diabetic rats were characterised by the presence of PAS-positive hepatocytes in all hepatic zones (Fig. 39 C). These finding was also observed in the neem-treated diabetic rats at 7 d (Fig. 39 E and 39 F), but were absent in the control (Fig. 39 A and 39 B) and metformin-treated diabetic group (Fig. 39 G and 39 H). In non-diabetic rats treated with neem, PAS-positive hepatocytes were absent in the pericentral area (Fig. 39 J) but present in the periportal zone (Fig. 39 I).



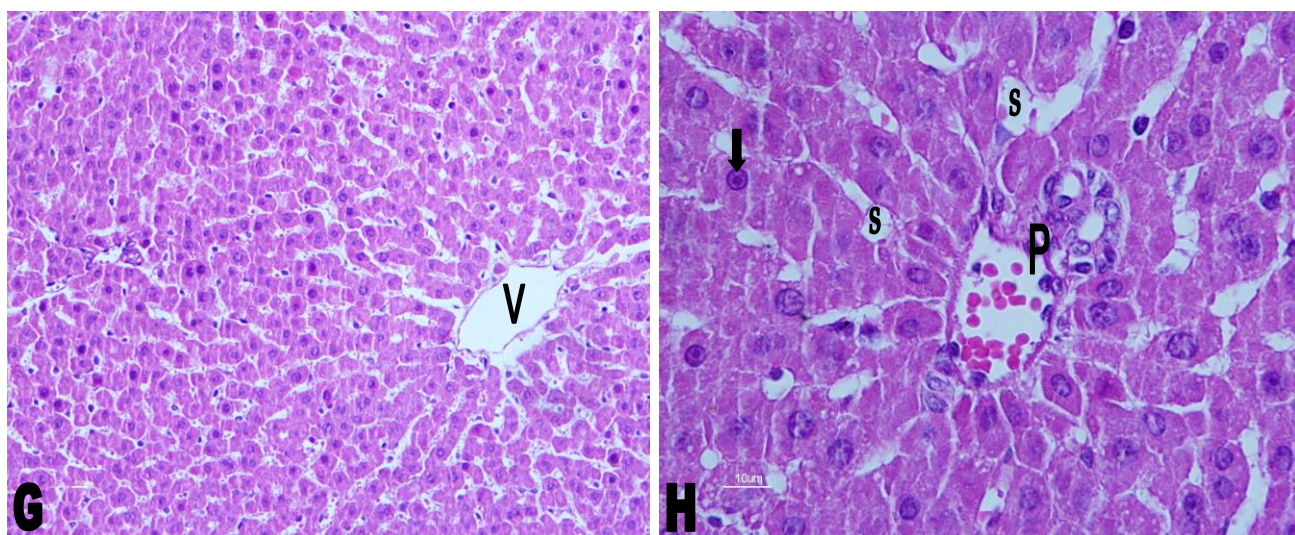
Figures 39 A and B. Hepatic sections of control rats at day 7. **(A)** Hepatocytes are PAS-negative (arrows); *p* indicates portal area; x200. **(B)** Sinusoids (*s*), central vein (*v*) and Kupffer cells (thin arrow) are observable; thick arrow indicates hepatocytes. x600. Periodic acid-Schiff. (Scale bar = 10 μ m).



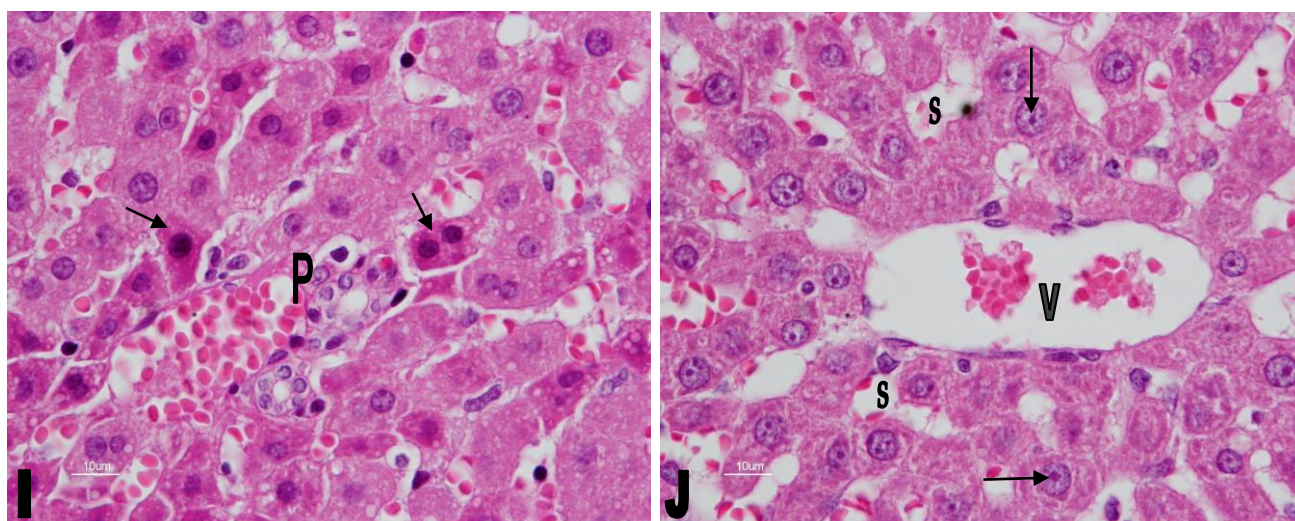
Figures 39 C and D. Hepatic sections of untreated diabetic rats at day 7. **C** shows PAS-positive hepatocytes in the periportal area (thick arrow), midzonal area (thin arrow) and around the central vein (v); x200. **D** shows some degenerating hepatocytes (thin arrows) and intact hepatocytes (thick arrows); s indicates sinusoids. x600. Periodic acid-Schiff. (Scale bar = 10 µm).



Figures 39 E and F. Hepatic sections of neem-treated diabetic rats at day 7. **(E)** PAS-positive hepatocytes are observable (arrow); p indicates periportal area; x200. **(F)** Degenerating hepatocytes are observable (thin arrows); intact hepatocytes can also be seen (thick arrow); x600. Periodic acid-Schiff. (Scale bar = 10 µm).



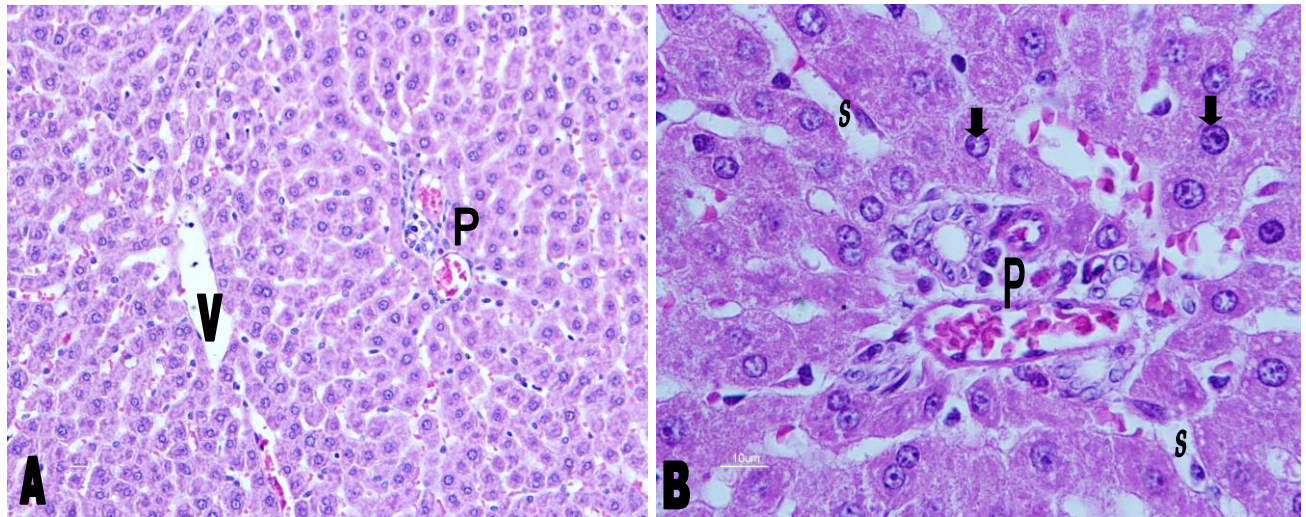
Figures 39 G and H. Hepatic sections of metformin-treated diabetic rats at day 7. Liver structure is normal. **G**, x200; **H**, x600. Periodic acid-Schiff. (Scale bar = 10 µm).



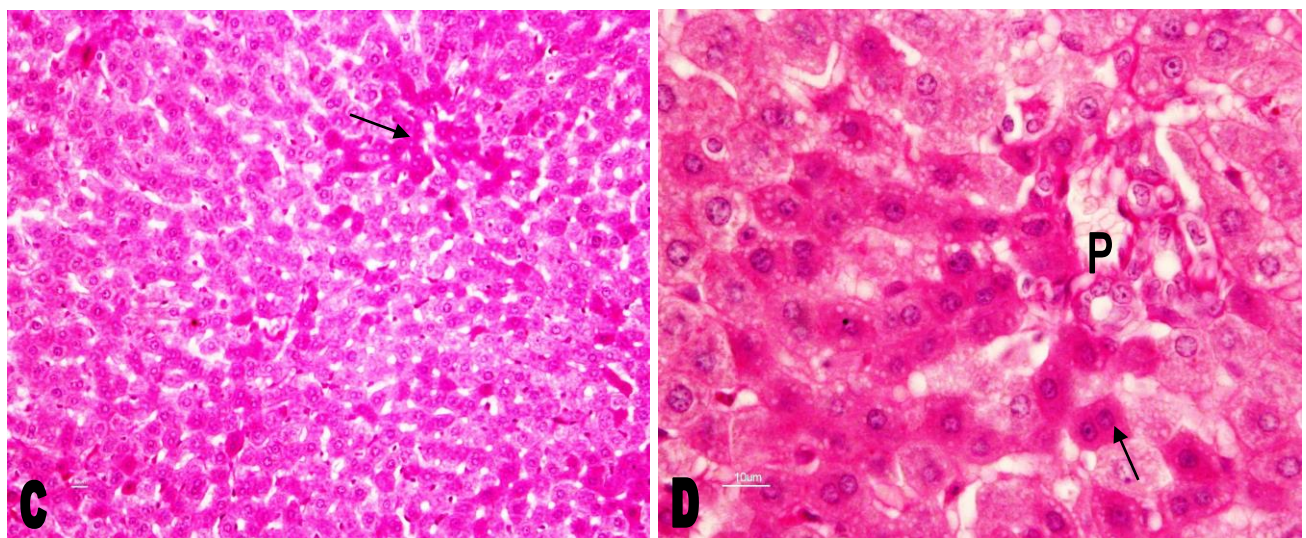
Figures 39 I and J. Hepatic sections of neem-treated non-diabetic rats at day 7. **(I)** PAS-positive hepatocytes are seen in the periportal area (p); x600. **(J)** Pericentral hepatocytes (arrows) are PAS-negative; v indicates central vein; s indicates sinusoids; x600. Periodic acid-Schiff stain. (Scale bar = 10 µm).

At 21 d of treatment, hepatic sections of untreated diabetic rats showed histological changes similar to those seen at 7 d (Fig. 40 C and 40 D). PAS-positive (perhaps glycogen-laden) hepatocytes were observed in all hepatic zones; and hepatocytes in the periportal areas were partially obstructing the sinusoids, as seen in figure 40 D. Hepatic sections of control and

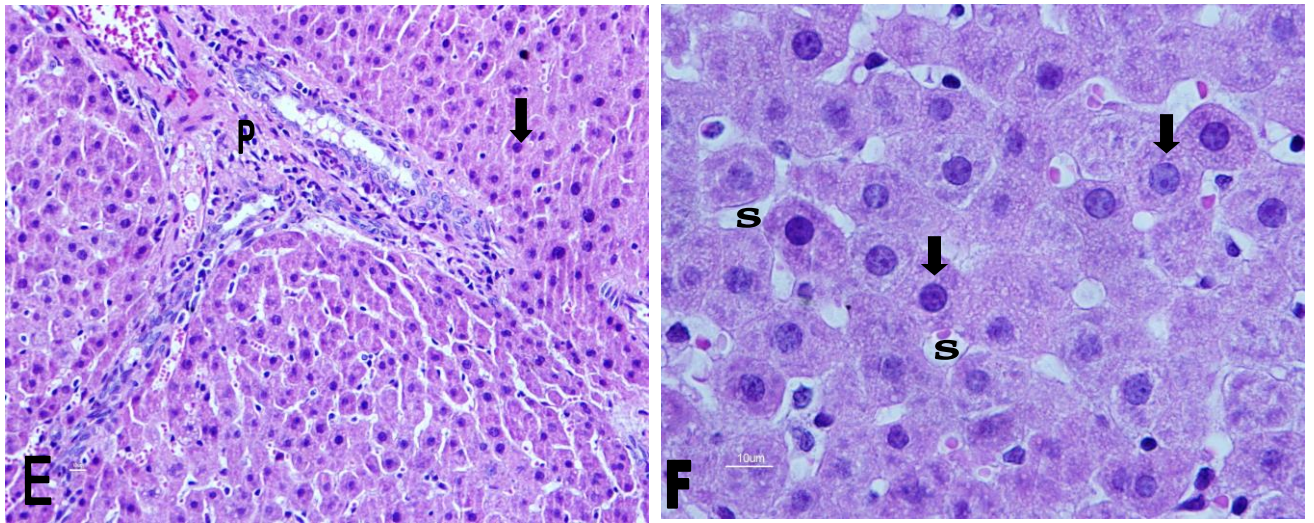
neem-treated diabetic animals were largely similar (Fig. 40 A, 40 B, 40 E and 40 F). Most hepatocytes of metformin-treated diabetic and neem-treated non-diabetic animals were PAS-negative, and their morphology was largely normal (Fig. 40 G-I).



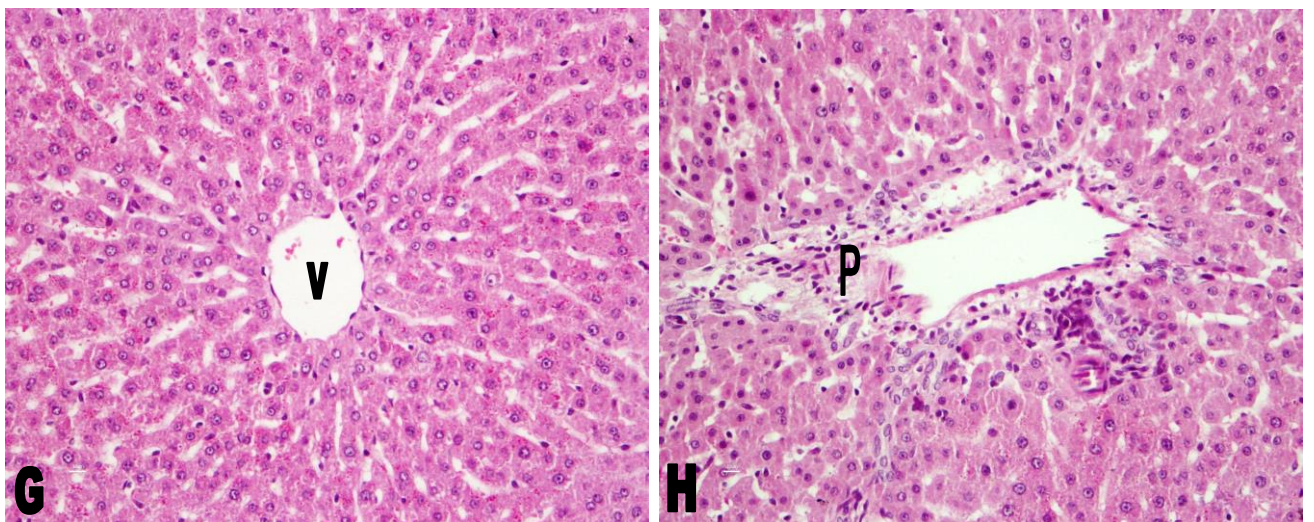
Figures 40 A and B. Hepatic sections of control rats at day 21. The sections appear normal; **v** indicates central vein; **p** indicates portal area; **s** indicates sinusoids; thick arrows indicate hepatocytes. (A, x200; B, x600). Periodic acid-Schiff. (Scale bar = 10 μ m).



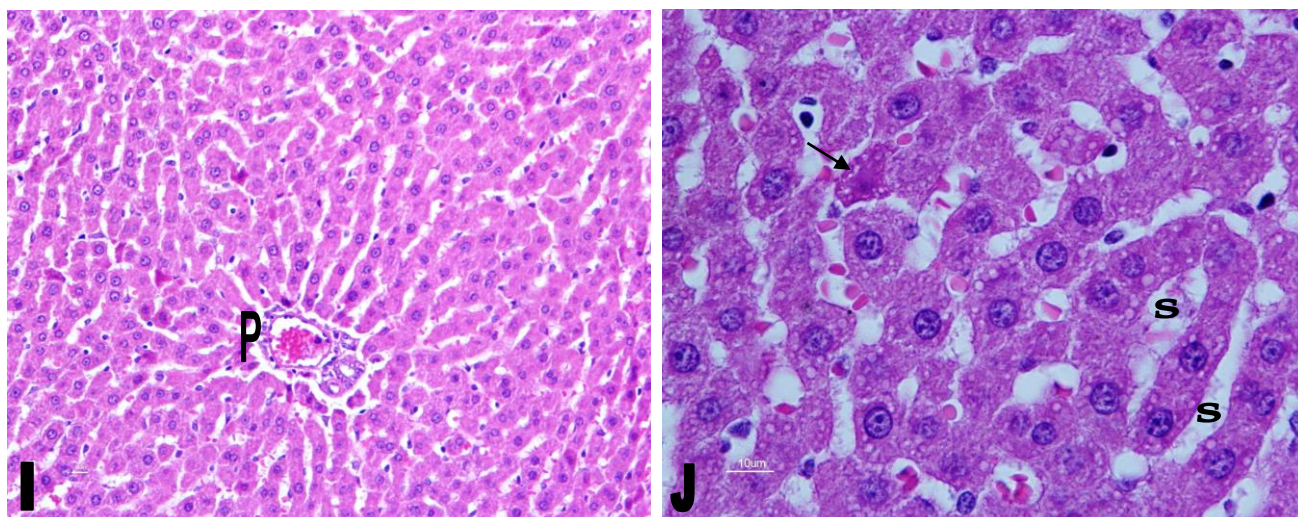
Figures 40 C and D. Hepatic sections of untreated diabetic rats at day 21. PAS-positive hepatocytes (arrows) are seen in all hepatic zones. Some sinusoids adjacent to portal area (p) are being obstructed, as shown in section **D**. (C, x200; D, x600). Periodic acid-Schiff. (Scale bar = 10 μ m).



Figures 40 E and F. Hepatic sections of neem-treated diabetic rats at day 21. Hepatic morphology is normal; thick arrows indicate hepatocytes; *s* indicates sinusoid; *p* indicates portal area (*E*, x200; *F*, x600). Periodic acid-Schiff. (Scale bar = 10 μ m).



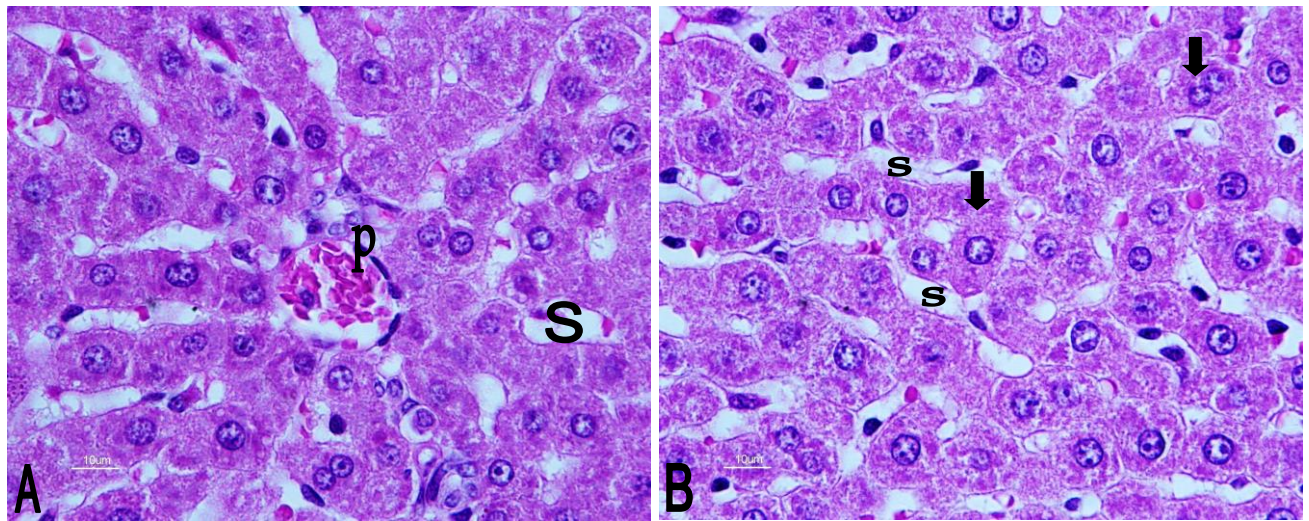
Figures 40 G and H. Hepatic sections of metformin-treated diabetic rats at day 21. Hepatic morphology is largely normal; x200; periodic acid-Schiff. (Scale bar = 10 μ m).



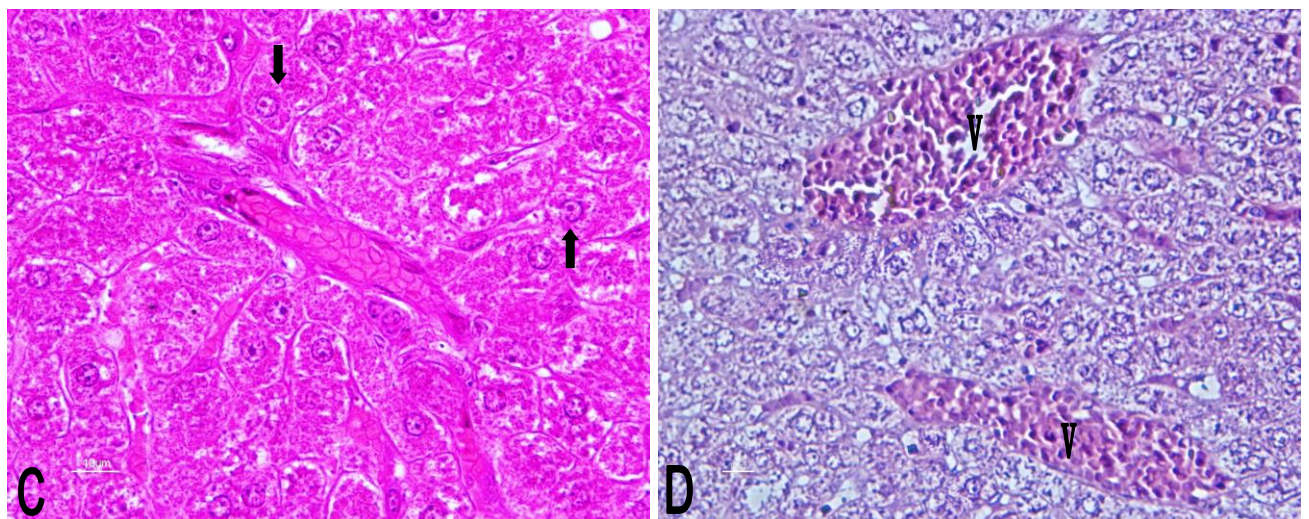
Figures 40 I and J. Hepatic histology of neem-treated non-diabetic rats at day 21. A few PAS-positive hepatocytes are observable (as shown in J; arrow); (I, x200; J, x600). Periodic acid-Schiff. (Scale bar = 10 μ m).

At 50 d of treatment, hepatic sections of untreated diabetic rats showed changes characteristic of hepatic glycogenosis (glycogenic hepatopathy) (Fig. 41 C-F). Hepatocytes had become swollen and strongly PAS-positive. Such PAS-positive staining was observed in the cytoplasm and nuclei of hepatocytes (Fig. 41 C and 41 E), and this suggests glycogen accumulation in both. Diastase digestion of the sections resulted in loss of the PAS-positive staining (Fig. 41 D and 41 F), thereby confirming the presence of glycogen in hepatocytes of untreated diabetic rats. Besides, the central veins were compressed and congested, while sinusoids were either occluded or compressed and congested (Fig. 41 C). Portal areas were rarely discernible, and so were Kupffer cells. In contrast, such histological findings were absent in neem-treated diabetic rats. The major finding in these animals was the presence of a few aggregates of leucocytes (Fig. 41 G and 41 H). Other than this, hepatic morphology in this group was similar to control and neem-treated non-diabetic rats (Fig. 41 A, 41 B, 41 M and 41 N). In the metformin-treated diabetic group, hepatocytes were characterised by the

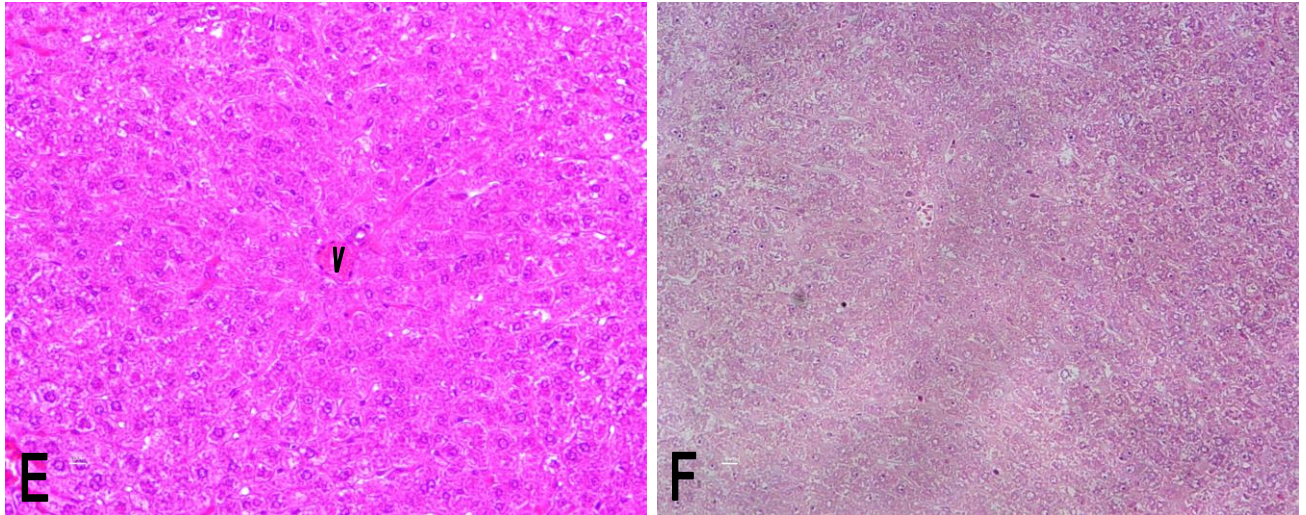
presence of multivesicular intracytoplasmic vacuoles in all hepatic zones (Fig. 41 I and 41 J). These vacuoles stained negative for Sudan black B (Fig. 41 K and L).



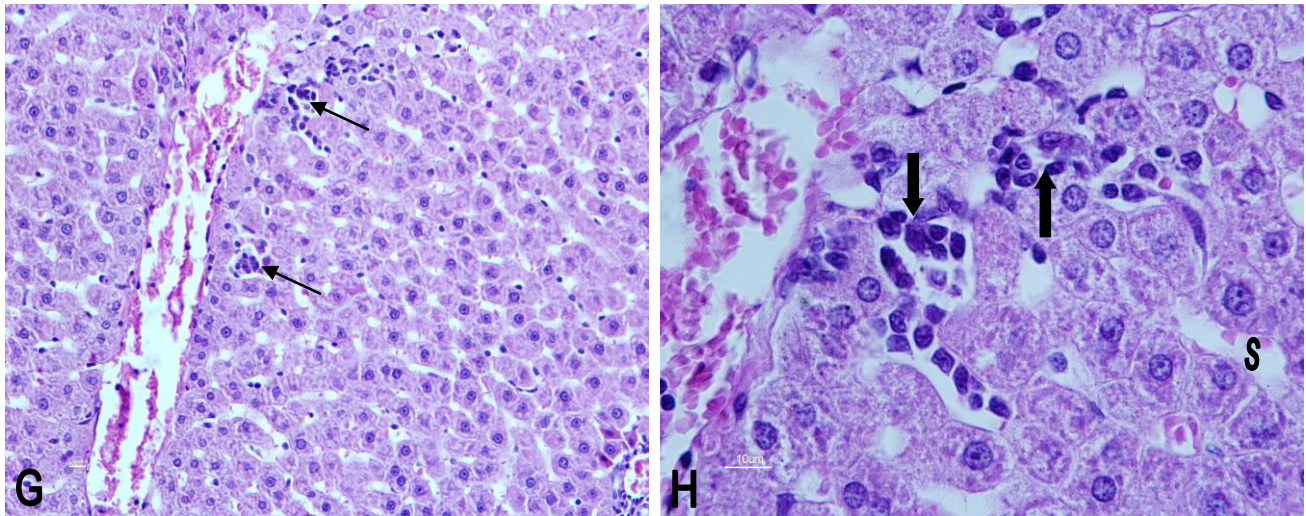
Figures 41 A and B. Hepatic sections of control rats at day 50. Cords of hepatocytes appear distinct (arrows) and sinusoids (s) remain patent. Hepatocytes are PAS-negative (arrows); x600. Periodic acid-Schiff. (Scale bar = 10 µm).



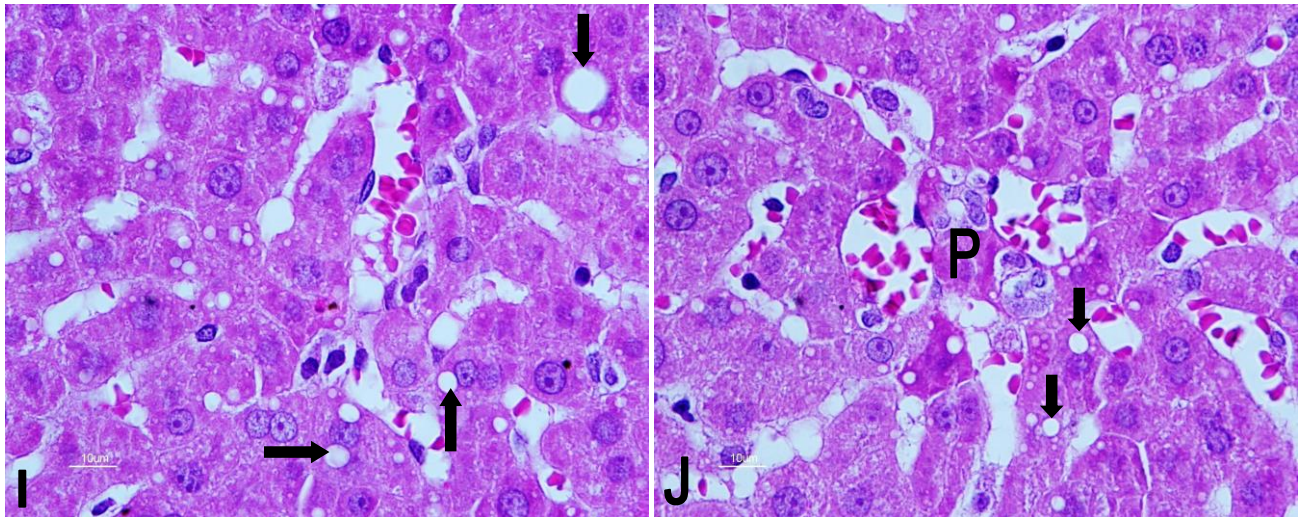
Figures 41 C and D. Hepatic sections of untreated diabetic rats at day 50. **C** shows swollen and strongly PAS-positive hepatocytes (arrows). Sinusoids are either occluded or are compressed and congested; x600. **(D)** The same tissue as in section **C**, after diastase digestion; the section shows PAS-negative staining that confirms the presence of glycogen in hepatocytes of section **C**. x600; Periodic acid-Schiff. (Scale bar = 10 µm).



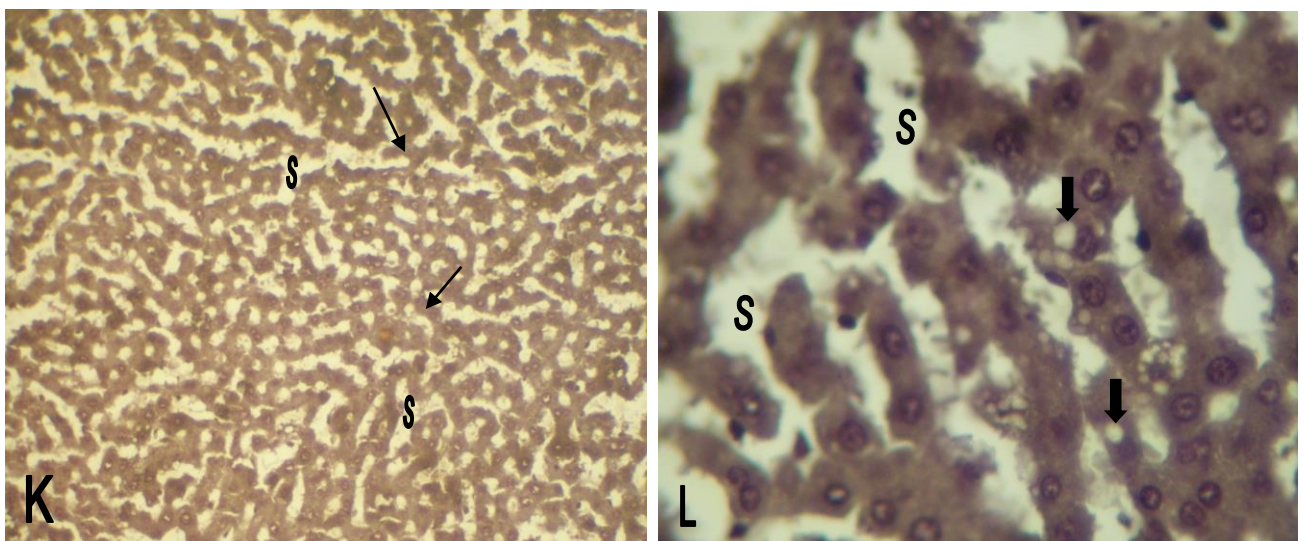
Figures 41 E and F. Hepatic histology of untreated diabetic rats at day 50. **(E)** The same section as figure 23 C; x200; v indicates central vein. **(F)** The same section as figure 23 D; x200. Periodic acid-Schiff. (Scale bar = 10 μ m).



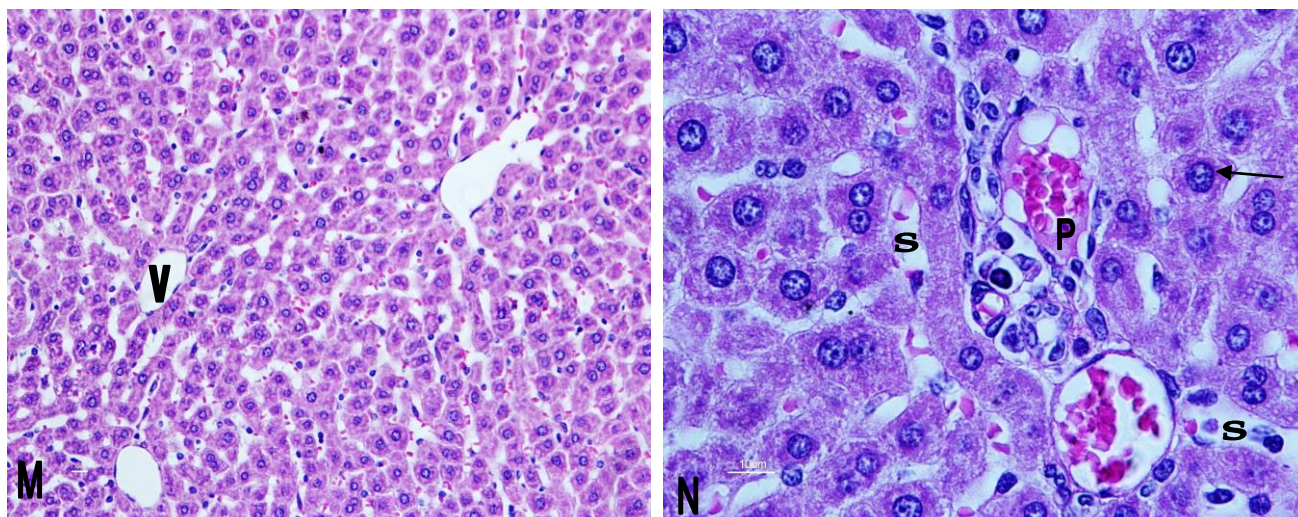
Figures 41 G and H. Hepatic histology of neem-treated diabetic rats at day 50. Lobular inflammation (arrows) is observable; s indicates sinusoids. *G*, x200; *H*, x600. Periodic acid-Schiff. (Scale bar = 10 μ m).



Figures 41 I and J. Hepatic histology of metformin-treated diabetic rats at day 50. Multivesicular intracytoplasmic vacuoles are observable in hepatocytes (arrows); *p* indicates portal area; x600. Period acid-Schiff. (Scale bar = 10 μ m).



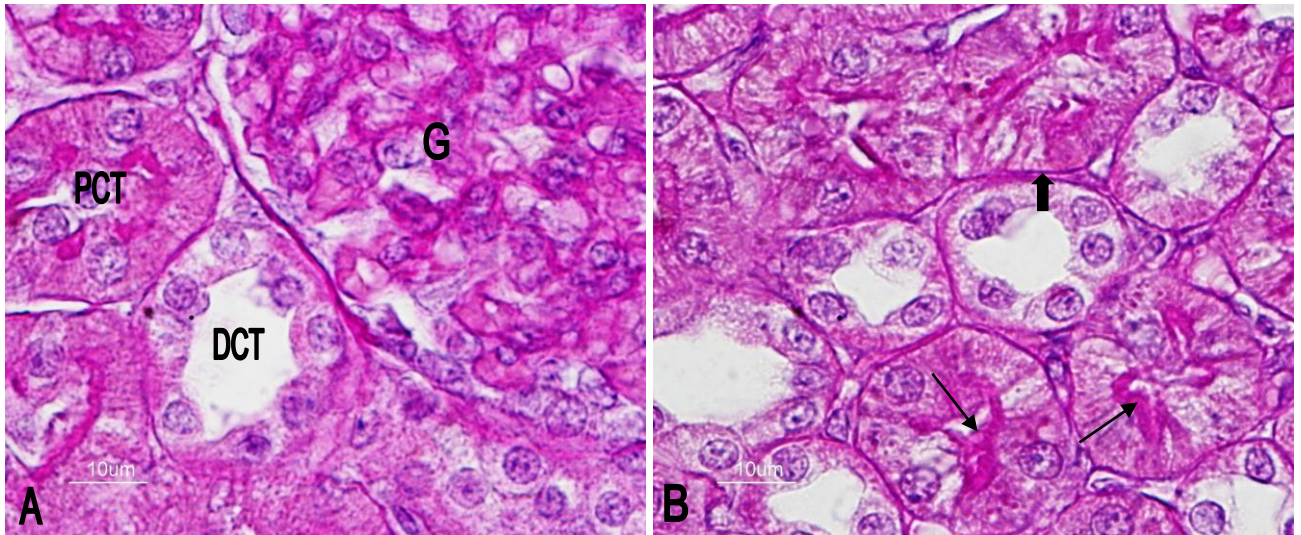
Figures 41 K and L. Hepatic sections of metformin-treated diabetic rats at day 50. The intracytoplasmic vacuoles (thick arrows) seen in figures 41 I and J are negative for Sudan black B staining. *S* indicates sinusoids; thin arrows indicate hepatocytes. *K*, x100; *L*, x400. Sudan black B stain.



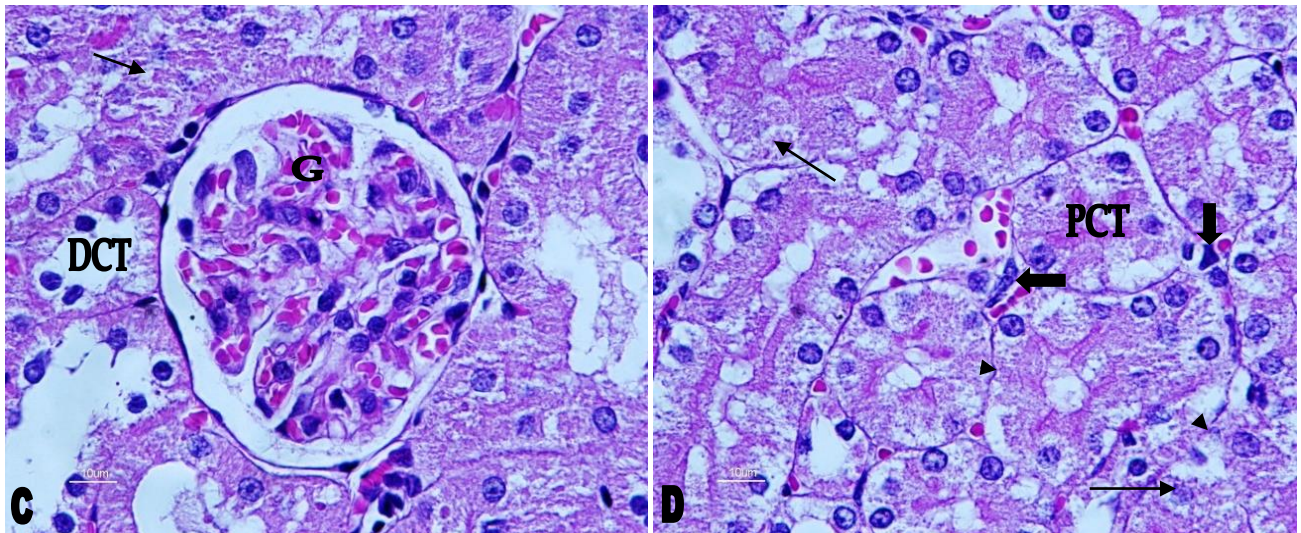
Figures 41 M and N. Hepatic histology of neem-treated non-diabetic rats at day 50. Hepatic morphology remains normal; **v** indicates central vein; **p** indicates portal area; **s** indicates sinusoids; arrow shows hepatocyte. **M**, x200; **N**, x600. Periodic acid-Schiff. (Scale bar = 10 μ m).

4.10.3 Renal Histology

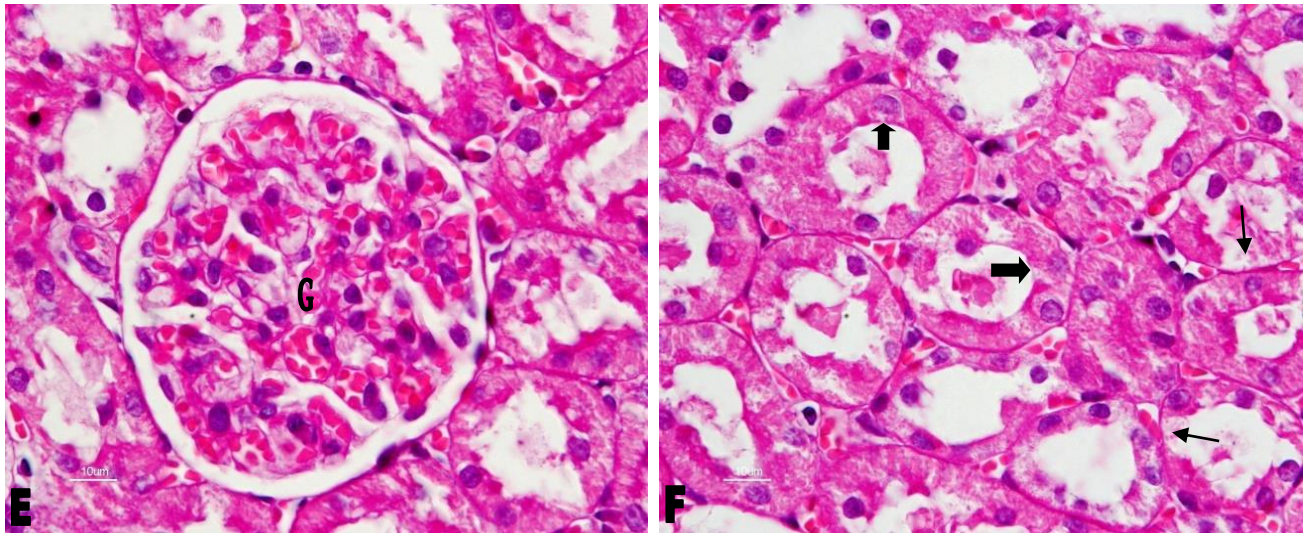
At 7 d, renal sections of control and metformin-treated diabetic animals appeared normal (Fig. 42 A, 42 B, 42 G and 42 H). However, morphologic changes suggestive of renal lesions were observed in the untreated diabetic, neem-treated diabetic and neem-treated non-diabetic rats (Fig. 42 C-F and 42 I-J). There was loss of some brush border and swelling of tubular cells. Such changes were more marked in the untreated diabetic rats (Fig. 42 D). Glomeruli were normal except for neem-treated non-diabetic animals where some appeared atrophic (Fig. 42 J, inset).



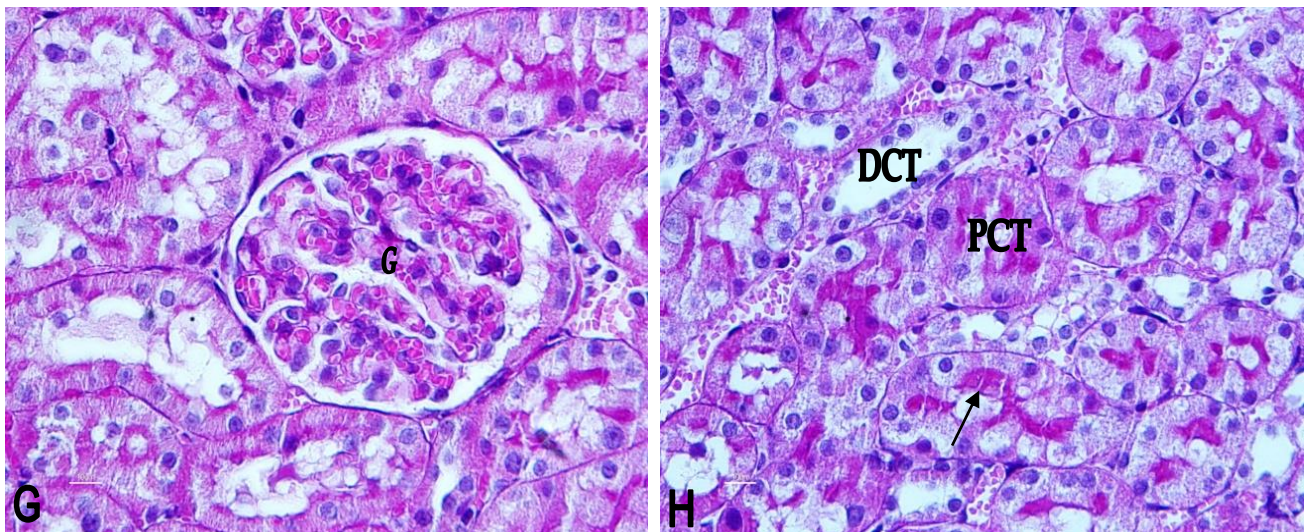
Figures 42 A and B. Renal cortex of control rats at day 7. The glomerulus (G), proximal tubules (PCT), distal tubules (DCT), microvilli of PCT (thin arrows), and tubular basement membrane (thick arrow) appear normal. Periodic acid-Schiff; x600. (Scale bar = 10 μ m).



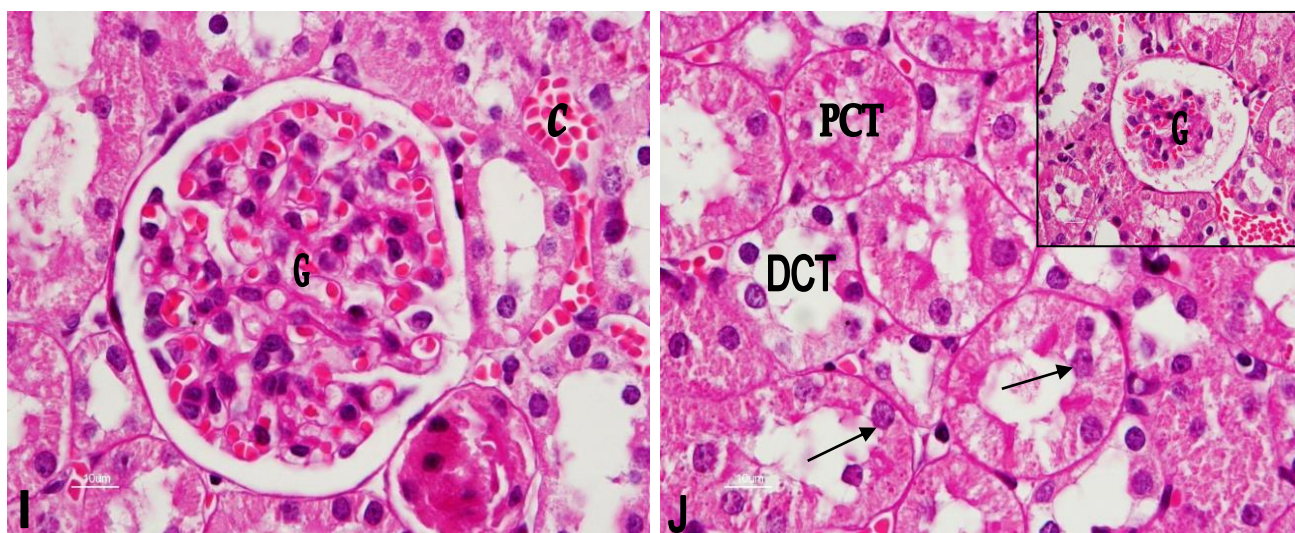
Figures 42 C and D. Renal cortex of untreated diabetic rats at day 7. Many proximal tubules appear distorted (thin arrows). Some leucocytes are seen in the interstitium (thick arrows), while tubular basement membrane is attenuated (arrowhead); glomerulus (G) is normal; DCT indicates distal tubule; PCT indicates proximal tubule. Periodic acid-Schiff; x600. (Scale bar = 10 μ m).



Figures 42 E and F. Renal cortex of neem-treated diabetic rats at day 7. *F* shows loss of brush border (thick arrow) and distortion of some tubular cells (thin arrows). Glomerulus (*G*) appears normal. Periodic acid-Schiff; x600. (Scale bar = 10 μm).

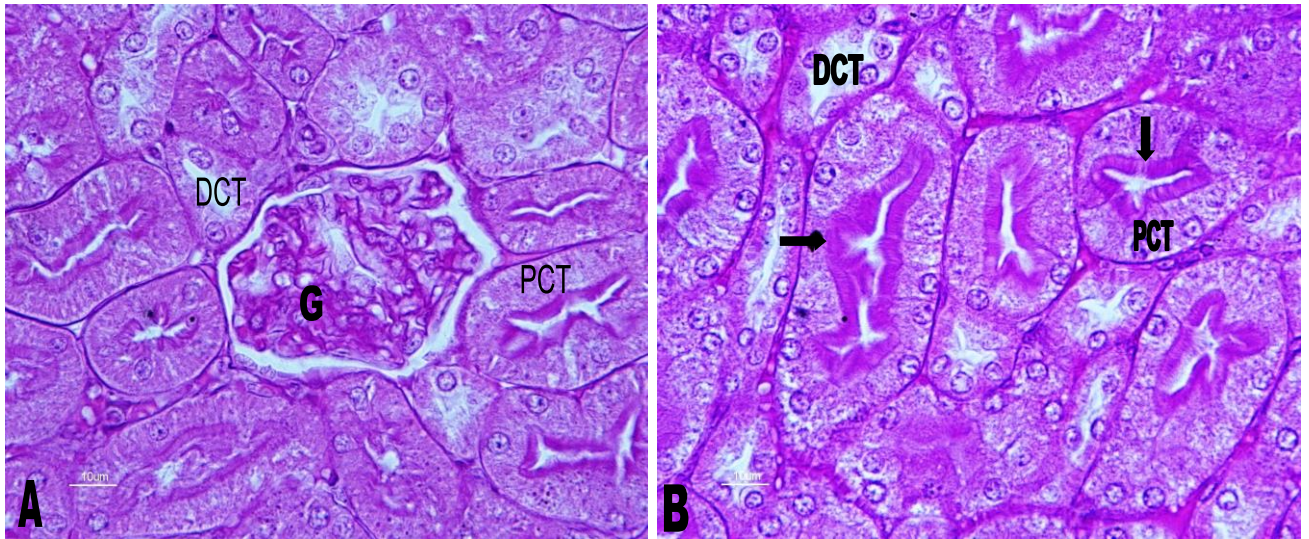


Figures 42 G and H. Renal cortex of metformin-treated diabetic rats at day 7. Renal morphology is largely normal. *G* indicates glomerulus; DCT indicates distal tubule; PCT indicates proximal tubule. Periodic acid-Schiff; x600. (Scale bar = 10 μm).

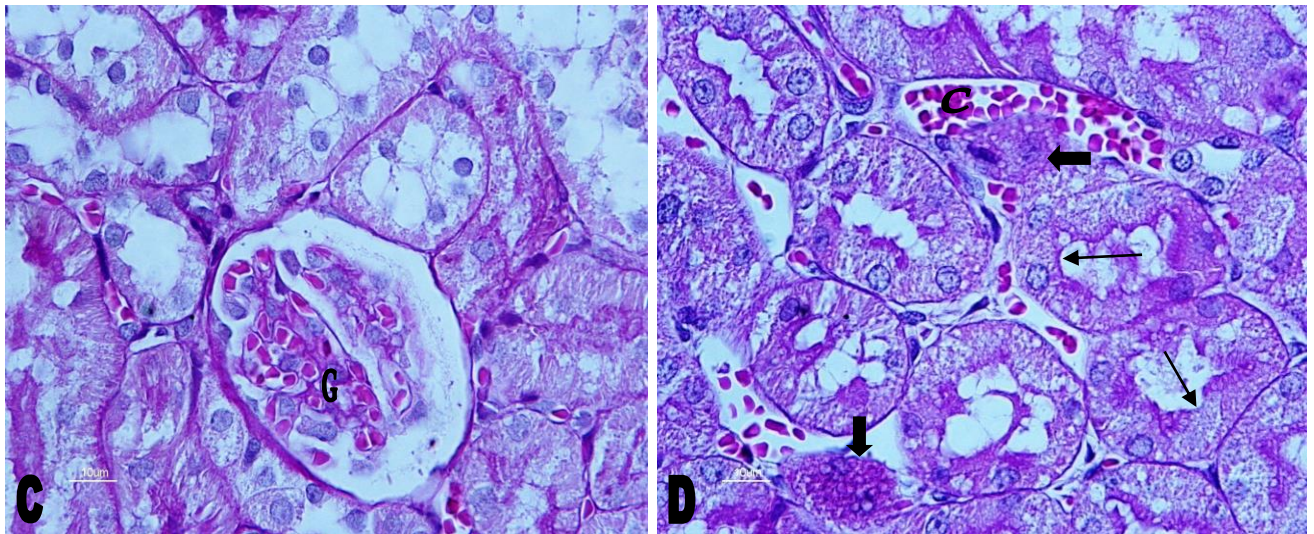


Figures 42 I and J. Renal cortex of neem-treated non-diabetic rats at day 7. There is loss of some brush border (thin arrows). Some glomeruli are partially atrophic (Fig. 42 J, inset), while others are normal (Fig. 42 I). DCT indicates distal tubule; PCT indicates proximal tubule; G indicates glomerulus. Periodic acid-Schiff; x600. (Scale bar = 10 μ m).

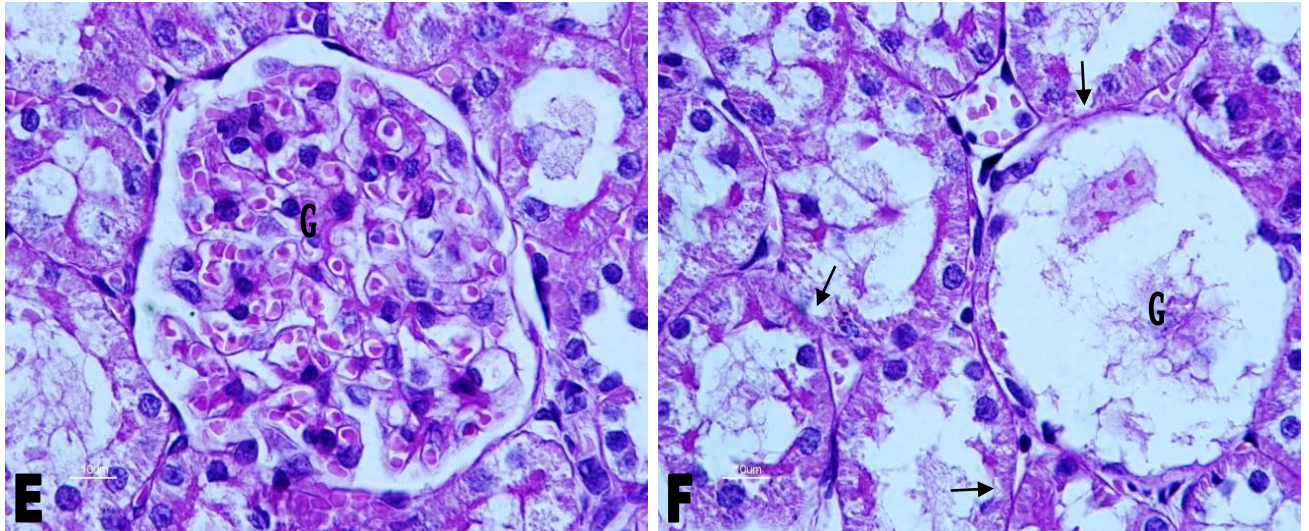
At 21 d of treatment, histological changes suggestive of renal lesions were observed in all treatment groups except control (Fig. 43 C-J). Such changes were most marked in neem-treated diabetic rats. They included distorted tubular cells, with attenuated basement membranes (BM), and loss of brush border. Some degenerating/partly atrophic glomeruli were also observable (Fig. 43 F, 43 J). Besides, many glomeruli in the neem-treated diabetic group appeared hypertrophic (Fig. 43 E), and this was confirmed by histomorphometric data (Fig. 35).



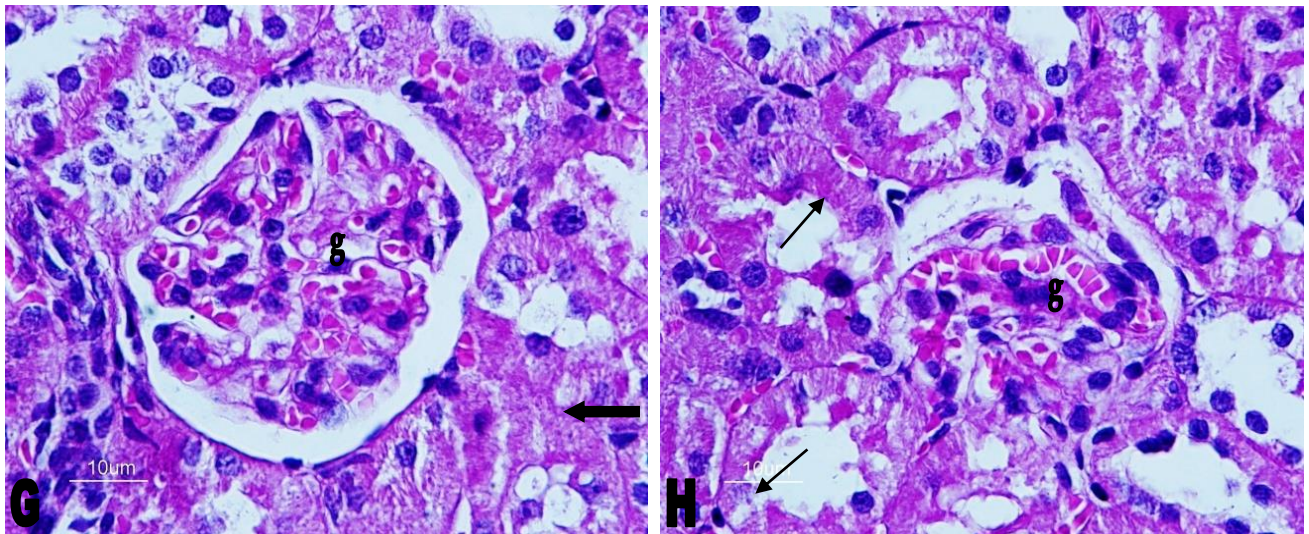
Figures 43 A and B. Renal cortex of control rats at day 21. Renal morphology is normal. DCT indicates distal tubule; PCT indicates proximal tubule; G indicates glomerulus. Periodic acid-Schiff; x600. (Scale bar = 10 µm).



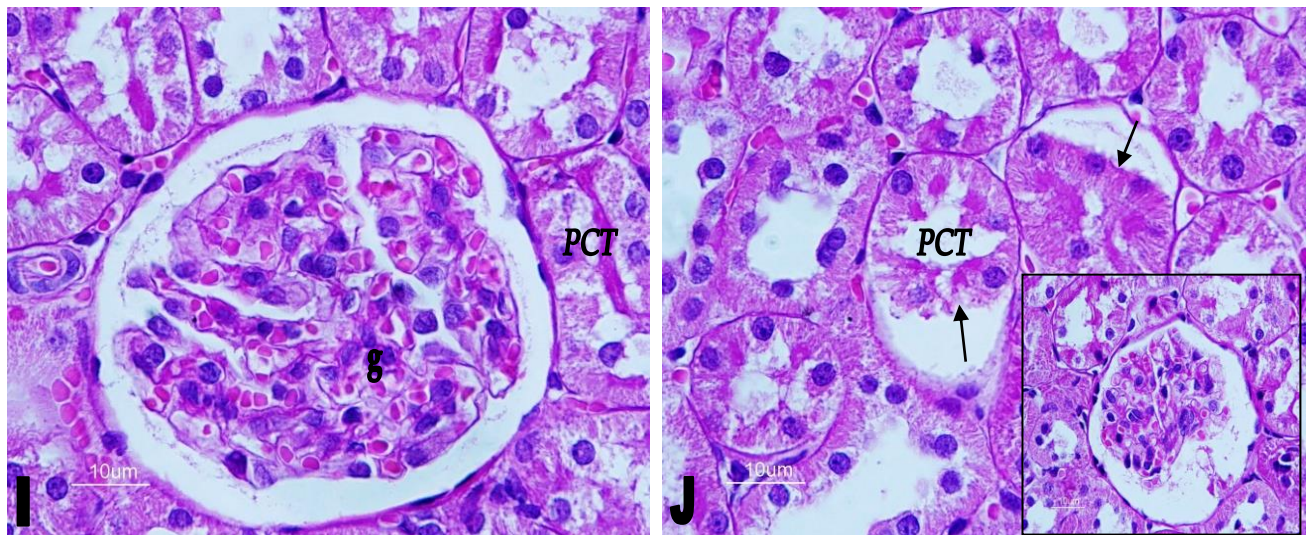
Figures 43 C and D. Renal cortex of untreated diabetic rats at day 21. Atrophic glomerulus (G), atrophic tubules (thick arrows) and loss of some brush border (thin arrows) are observable. C indicates capillary. Periodic acid-Schiff; x600. (Scale bar = 10 µm).



Figures 43 E and F. Renal cortex of neem-treated diabetic rats at day 21. Glomerular hypertrophy (Fig. 43 E) and distorted tubular cells (Fig. 43 F, arrows) are observable. A few glomeruli are also atrophic (Fig. 43 F); G indicates glomerulus. Periodic acid-Schiff; x600. (Scale bar = 10 μ m).



Figures 43 G and H. Renal cortex of metformin-treated diabetic rats at day 21. Distorted tubular cells (thick arrow), loss of brush border (thin arrows), and attenuation of basement membranes are observable. A few glomeruli are also atrophic (Fig. 43 H). g indicates glomerulus. Periodic acid-Schiff; x600. (Scale bar = 10 μ m).



Figures 43 I and J. Renal cortex of neem-treated non-diabetic rats at day 21. Certain tubular cells are detached from their basement membranes (arrows); while some glomeruli are partially atrophic (Fig. 43 J, inset). *PCT* indicates proximal tubule; *g* indicates glomerulus. Periodic acid-Schiff, x600. (Scale bar = 10 µm).

At 50 d of treatment, renal histological features of untreated diabetic rats were suggestive of diabetic nephropathy. Glomerular lesions observed at this point in time included thickened glomerular capillary basement membranes (GBM), mesangial sclerosis and nodular glomerulosclerosis (Kimmelstiel-Wilson disease) (Fig. 44 E). Strongly PAS-positive acellular nodules were seen in the glomeruli. Glomerular capillaries had been compressed by these nodules and glomerular tufts were thus largely obliterated. Capsular drops (adherence of glomeruli to the parietal epithelium of Bowman's capsules) were also observed (Fig. 44 E).

Furthermore, extensive Armanni-Ebstein lesions (cytoplasmic vacuolation) of proximal tubule (PCT) cells were a typical feature (Fig. 44 F). Tubular cells with these lesions showed clear, empty cytoplasm, with intact cell membrane. The non-vacuolated tubule cells appeared strongly PAS-positive. Thickening of the basement membranes of these cells had also occurred (as suggested by the accumulation of PAS-positive materials between tubular cells at certain loci (Fig. 44 E and 44 F).

Furthermore, the strong PAS-positive staining of renal tissue seen in figures 44 E and F (renal sections of untreated diabetic rats at 50 d) was lost following the treatment of the tissue with diastase prior to PAS staining (Fig. 44 G and H). Besides, figures 44 I and J show absence of sudanophilic materials (lipids) in the clear cytoplasmic vacuoles of the proximal tubule cells. However, certain non-vacuolated tubule cells contained sudanophilic granules, as shown in figures 44 I and J (white arrows).

In contrast to the foregoing, renal morphology of control, neem-treated diabetic and neem-treated non-diabetic rats was largely normal (Fig. 44 A, 44 B, 44 K and 44 L). The acute morphologic changes earlier observed in the latter two groups were absent at 50 d. On the other hand, metformin-treated diabetic rats showed morphologic features characterised by glomerular hypertrophy (Fig. 35 and 44 M), and distorted proximal tubules (Fig. 44 N). However, most of the renal glomerular and tubular lesions seen in the untreated diabetic rats at 50 d were absent in the metformin group (Fig. 44 M and N).

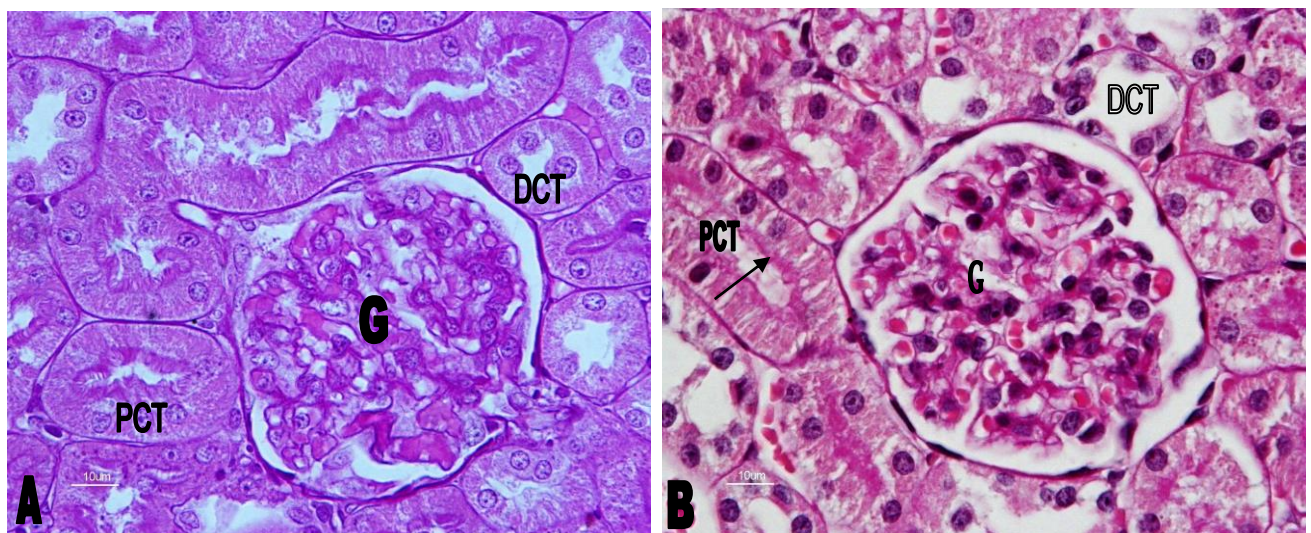
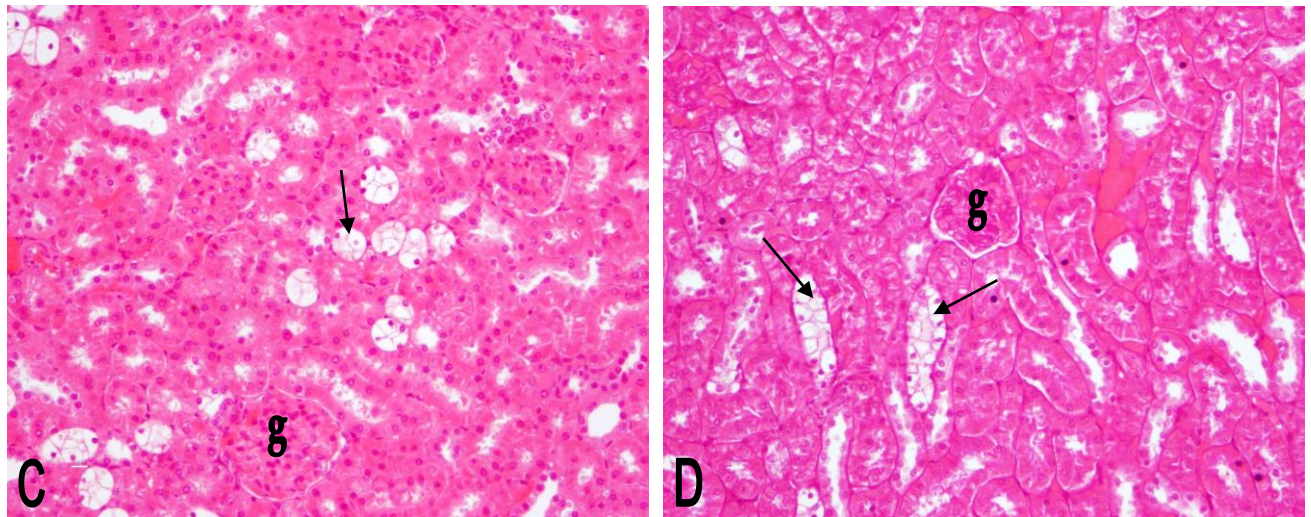
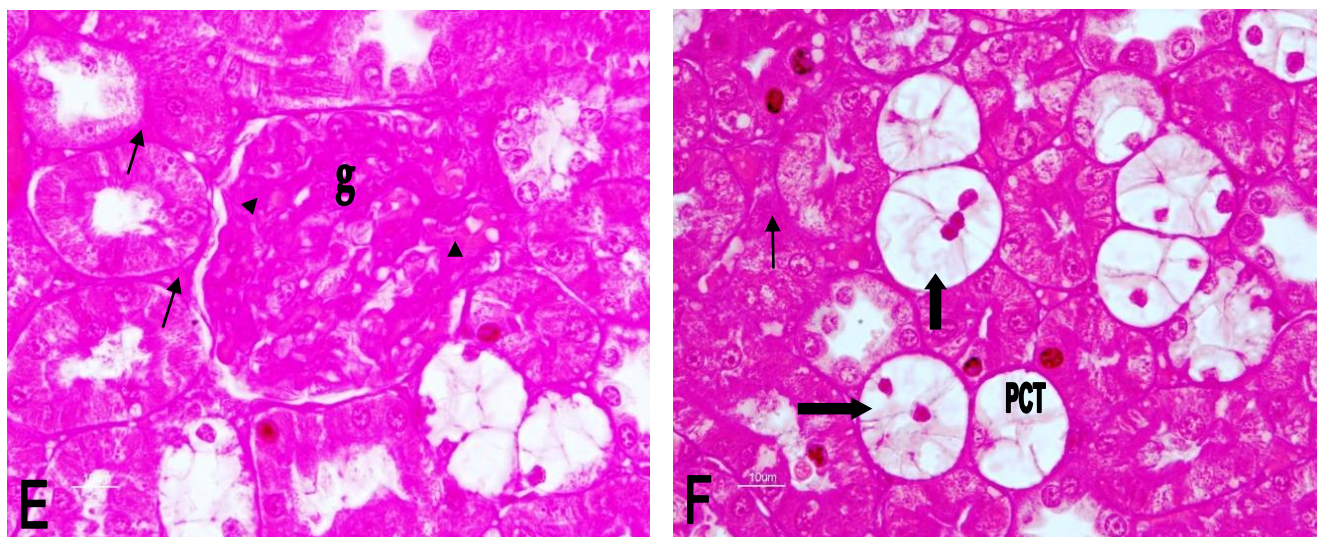


Figure 44 A. Renal cortex of control rats at day 50 of treatment. The glomeruli (G), proximal tubules (PCT) and distal tubules (DCT) are normal. Periodic acid-Schiff, x600. (Scale bar= 10 µm)

Figure 44 B. Renal cortex of neem-treated non-diabetic rats at day 50 of treatment. The glomerulus (G) is normal, while the proximal tubules (PCT) possess intact brush border (arrow). DCT indicates distal tubule. Periodic acid-Schiff, x600. (Scale bar = 10 μ m).

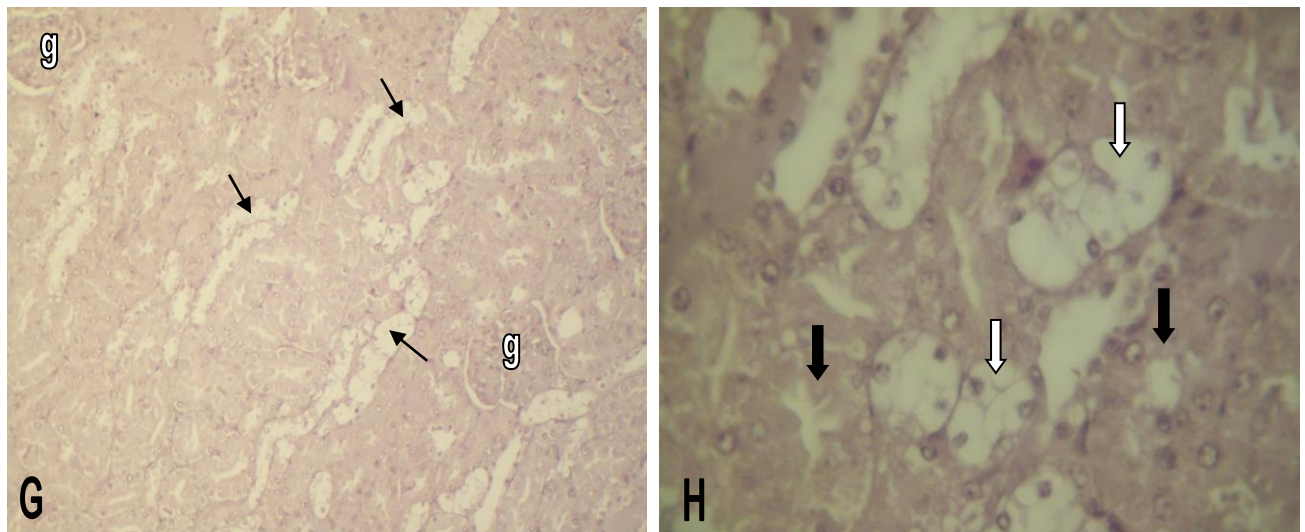


Figures 44 C and D. Renal cortex of untreated diabetic rats at day 50. Clear vacuoles are observable in renal tubules (arrows); figure C also shows increased eosinophilia of renal tissue. *g* indicates glomerulus. *C*, H&E, x200; *D*, Periodic acid-Schiff, x200. (Scale bar = 10 μ m).

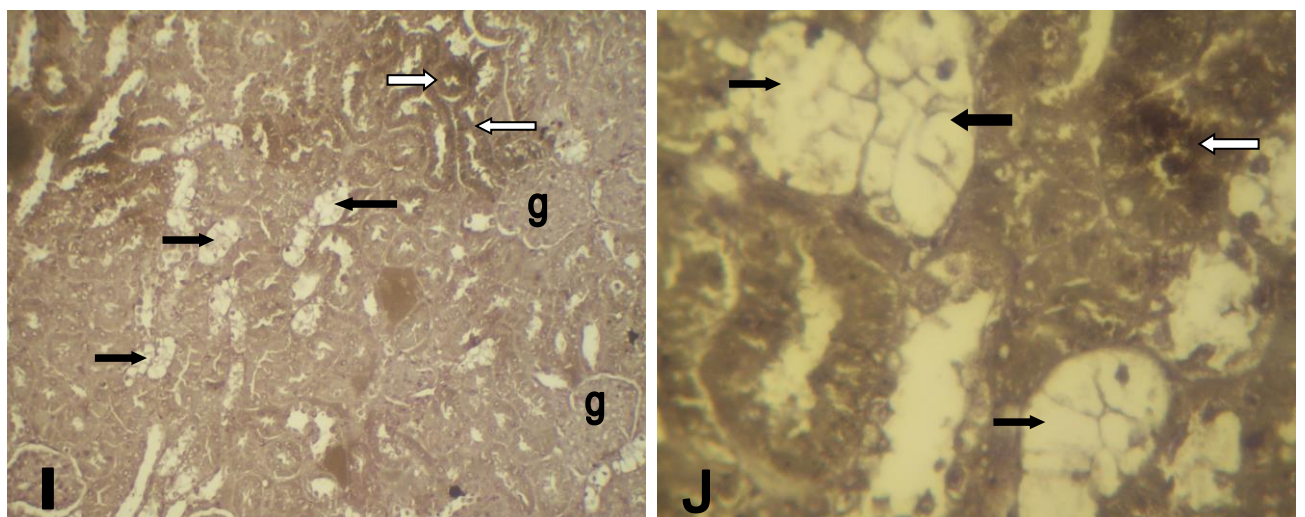


Figures 44 E and F. Renal cortex of untreated diabetic rats at day 50. Changes indicating diabetic nephropathy are observable. (**E**) Thickening of glomerular basement membranes has occurred (arrowheads); thickening of tubular basement membranes is also observable (thin arrows); *g* indicates glomerulus. (**F**) Armani-Ebstein cells (vacuolated PCT cells) are observable (thick arrows), while thickening of tubular basement membranes is evident (thin

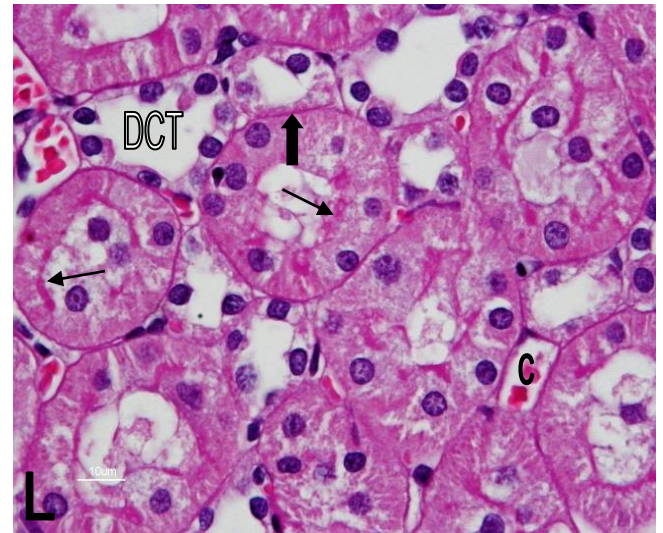
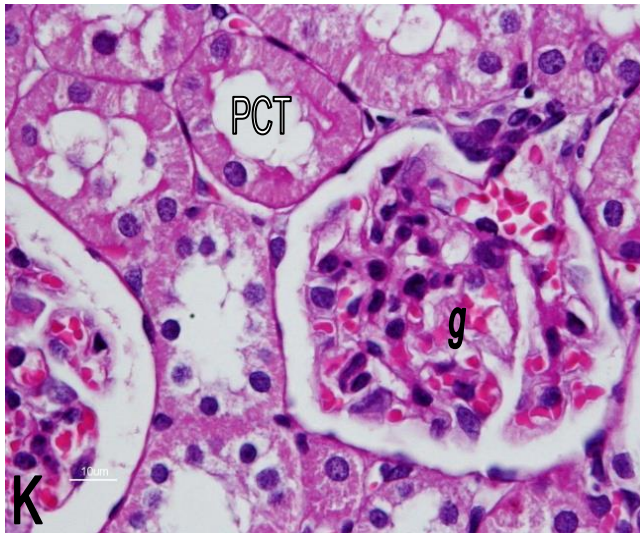
arrow). Strong PAS-positive staining of the entire renal tissue is also evident; (PCT= proximal tubule). Periodic acid-Schiff, x600. (Scale bar = 10 μ m).



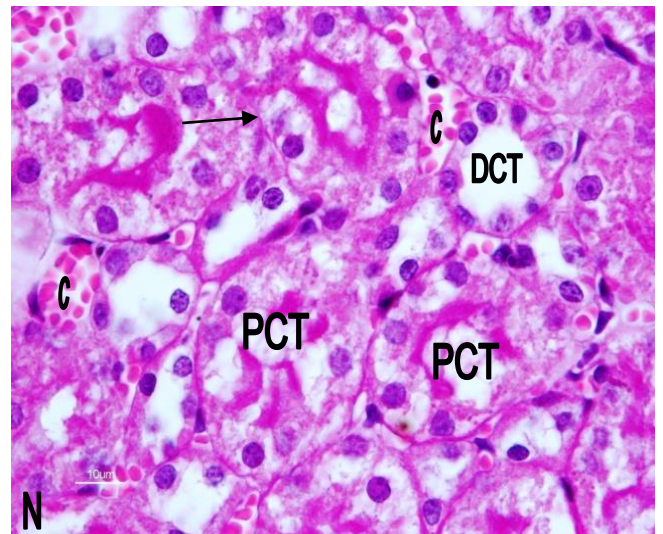
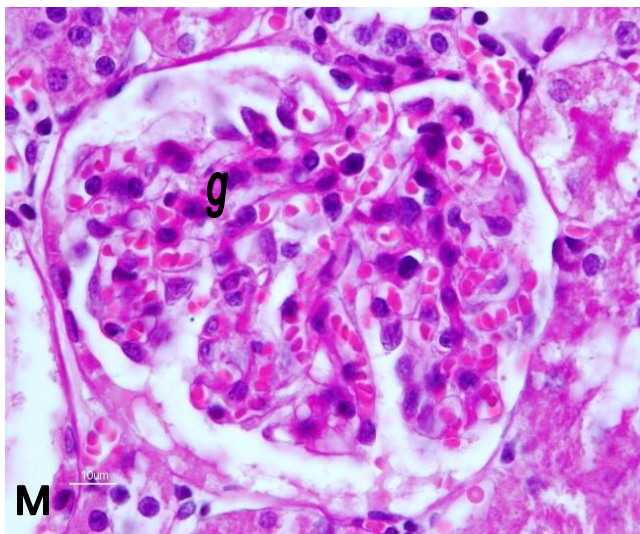
Figures 44 G and H. Renal cortex of untreated diabetic rats at 50 d. The sections were treated with salivary amylase and stained with PAS. Both sections show loss of PAS-positive material (glycogen and other periodic acid-reactive carbohydrates). In Fig 44 G, *g* indicates glomerulus; arrows indicate tubule cells with clear cytoplasmic vacuoles; x100. In Fig, 44 H, white arrows show proximal tubule cells with clear cytoplasmic vacuoles; dark arrows show PAS negative non-vacuolated tubule cells; x400. PAS with diastase control.



Figures 44 I and J. Renal cortex of untreated diabetic rats at 50 d. The sections show absence of sudanophilic material in the vacuoles of proximal tubule cells (dark arrows); white arrows show the presence of sudanophilic granules in some non-vacuolated proximal tubule cells. *g* indicates glomerulus. Sudan black B stain; *I*, x100; *J*, x400.



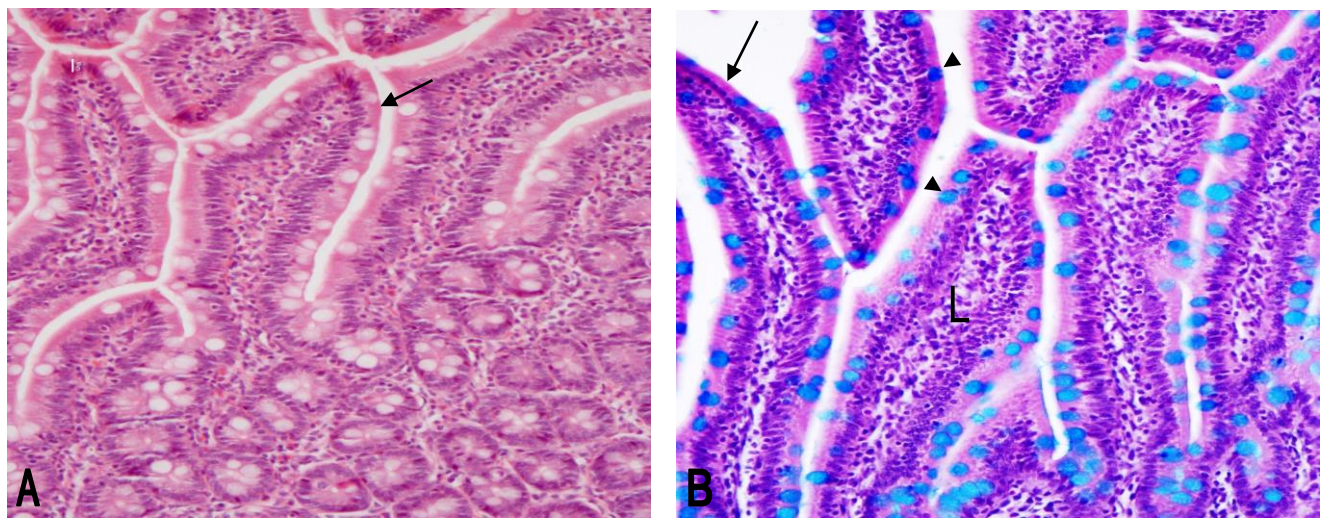
Figures 44 K and L. Renal cortex of neem-treated diabetic rats at day 50. Renal morphology is normal. The brush border (thin arrows) and basement membranes (thick arrow) are intact. PCT= proximal tubules; DCT= distal tubule; *g*= glomerulus; *c*= capillary. Periodic acid-Schiff, x600. (Scale bar = 10 μm).



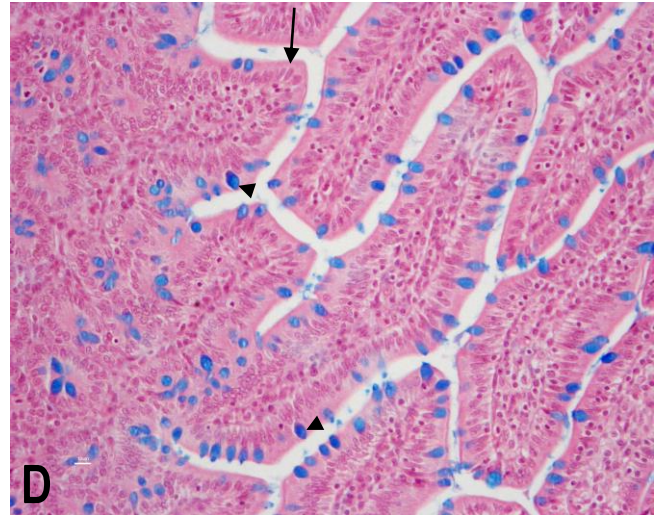
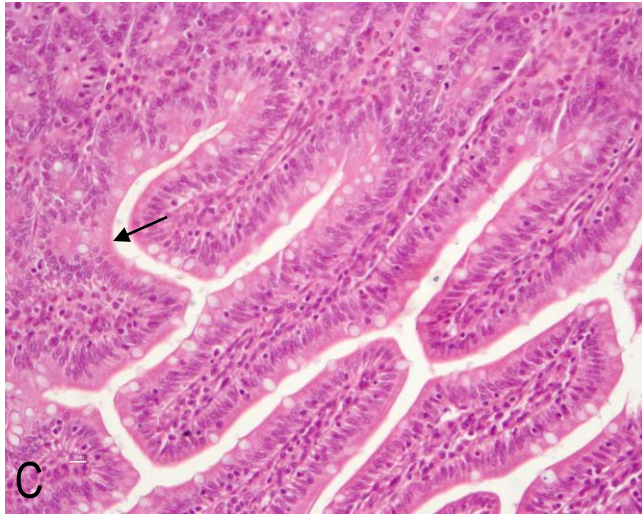
Figures 44 M and N. Renal cortex of metformin-treated diabetic rats at day 50. There is glomerular hypertrophy (as shown in Fig. 44 M) and attenuated basement membrane (thin arrow). Most proximal tubules (PCT) appear distorted. DCT= distal tubule; *g*= glomerulus; *c*= capillary. Periodic acid-Schiff, x600. (Scale bar = 10 μm).

4.10.4 Histology of Jejunal Mucosa

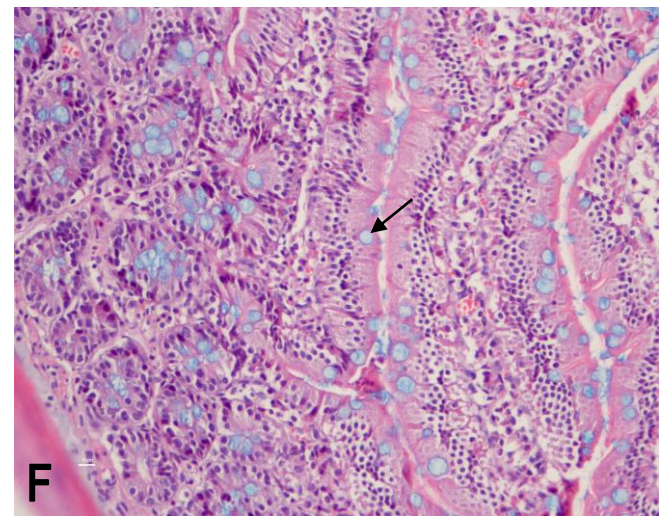
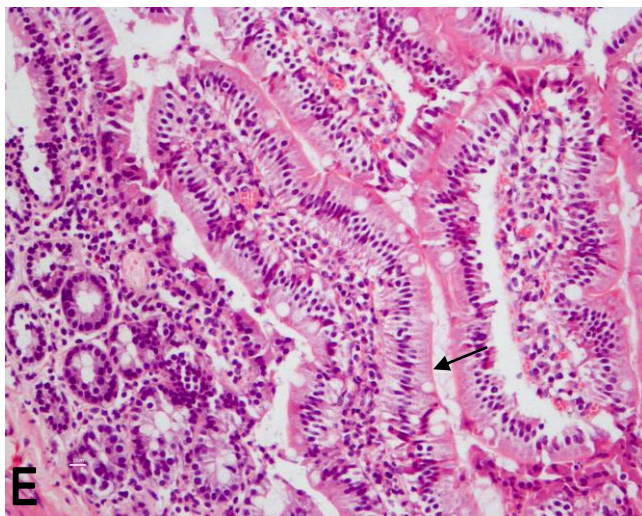
At 7 d of treatment, jejunal mucosa was intact in all the treatment groups. However, the secretory activity of goblet cells was markedly reduced in the untreated diabetic and neem-treated diabetic rats at this time. This finding was more pronounced in the latter group where mucin content of goblet cells was poor (Fig. 45 F). On the contrary, goblet cells were actively secretory in metformin-treated diabetic and neem-treated non-diabetic rats (Fig. 45 F).



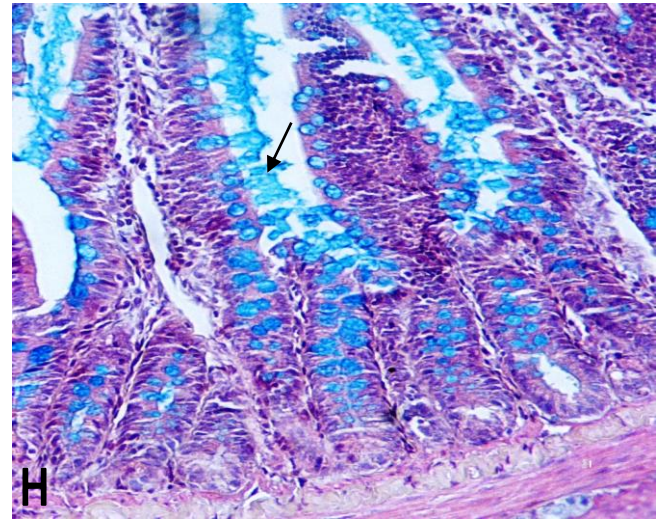
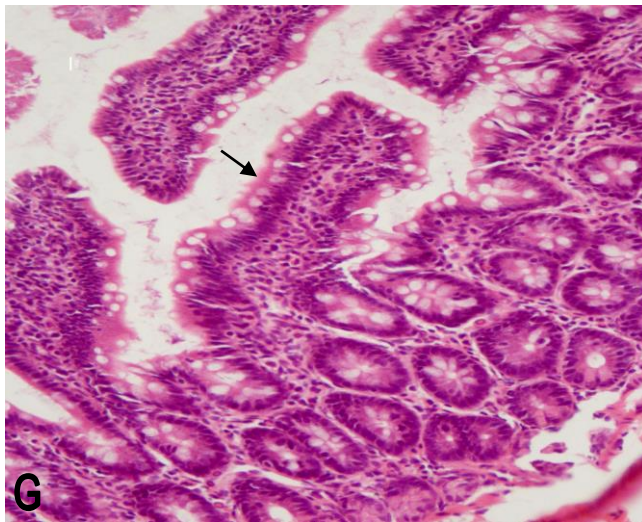
Figures 45 A and B. Jejunal sections of control rats at day 7. **(A)** Epithelial cells are intact (arrow); H&E, 200. Section **B** shows mucin-filled goblet cells (arrowhead) and intact lamina propria (L); epithelial cells are also intact (arrow); Alcian blue, x200.



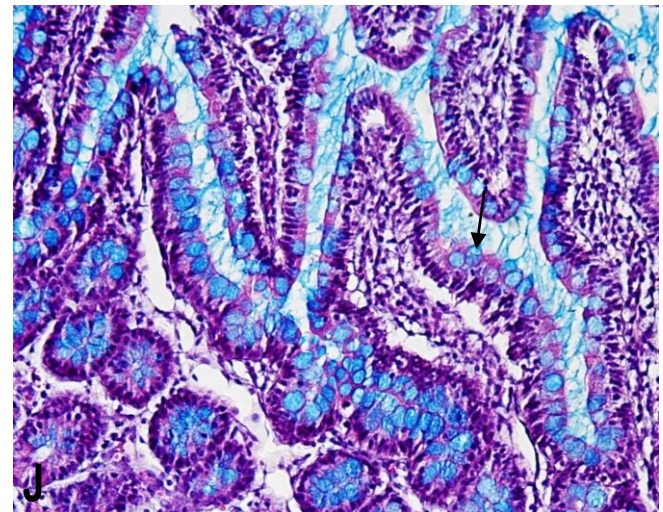
Figures 45 C and D. Jejunal sections of untreated diabetic rats at day 7. **(C)** The epithelium is intact (arrow); H&E, 200. **(D)** Goblet cells (arrow head) stained weakly for mucin when compared with control (Fig. 45 B); the epithelium is however intact. Alcian blue, x200. (Scale bar = 10 μ m).



Figures 45 E and F. Jejunal sections of neem-treated diabetic rats at day 7. **(E)** The mucosa is largely intact (arrow); H&E, x200. **(F)** Goblet cells stained weakly for mucin (arrow). Alcian blue, x200. (Scale bar = 10 μ m).



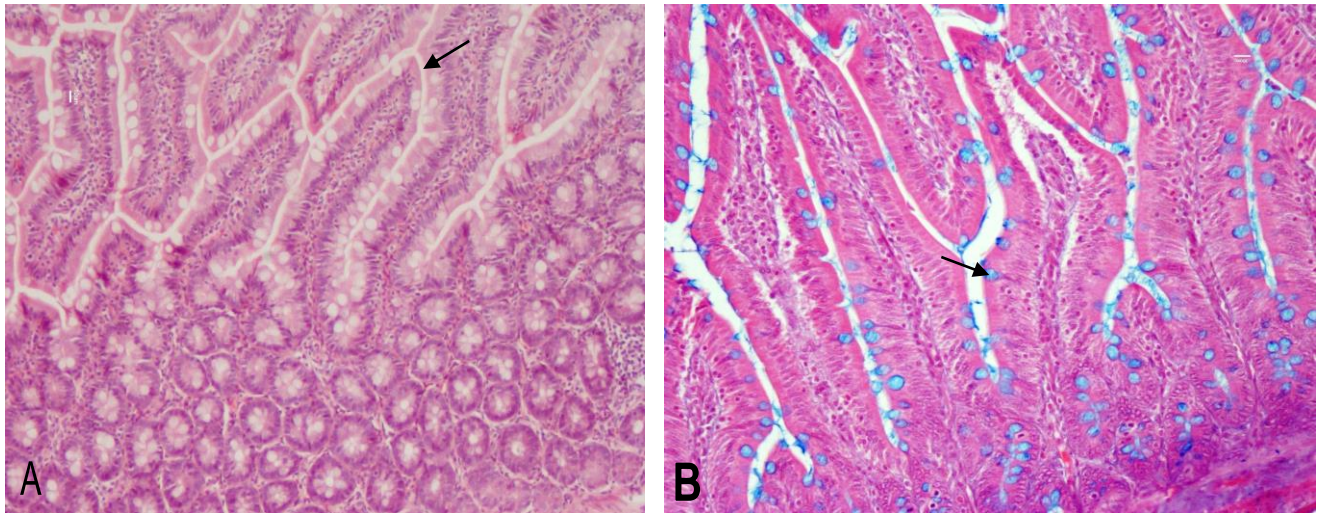
Figures 45 G and H. Jejunal sections of metformin-treated diabetic rats at day 7. **(G)** The epithelium is intact (arrow); H&E, x200. **(H)** Goblet cells are actively secretory (arrow); Alcian blue, x200. (Scale bar = 10 μ m).



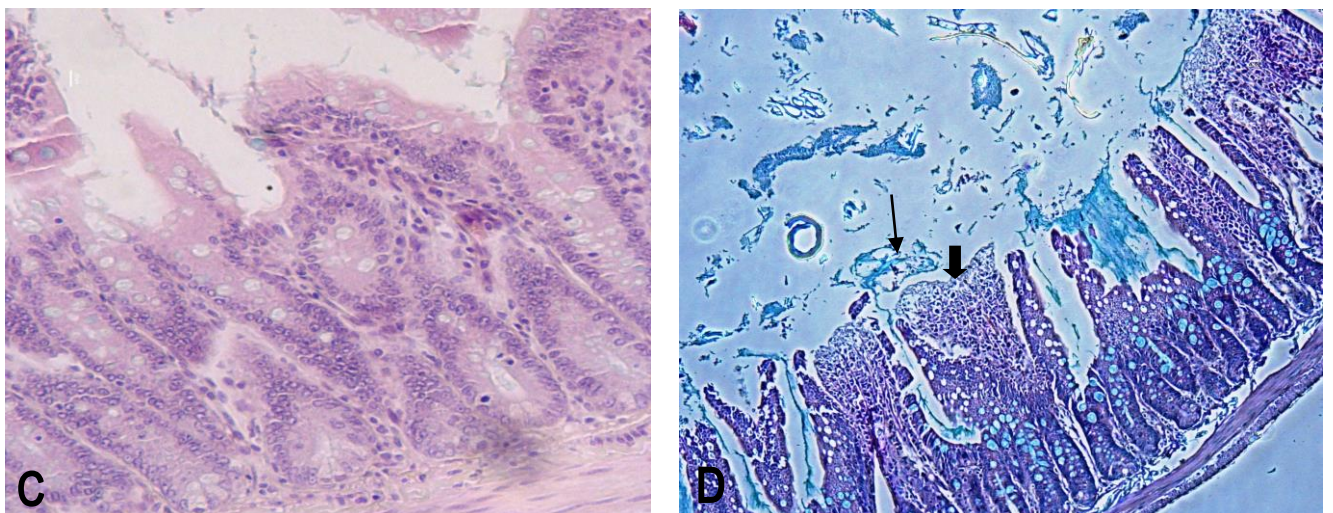
Figures 45 I and J. Jejunal sections of neem-treated non-diabetic rats at day 7. **(I)** The epithelium is intact (arrow); H&E, x200. **(J)** Goblet cells are actively secretory (arrow); Alcian blue, x200. (Scale bar = 10 μ m).

At 21 d, jejunal mucosa of animals in the untreated diabetic group was characterised by extensive superficial erosion of the epithelium, with blunting of the villi (Fig. 46 D). Most superficial goblet cells had become necrotic and were thus lost. However, most goblet cells in the crypts were preserved. Intestinal lumen contained debris of dead and desquamated tissues.

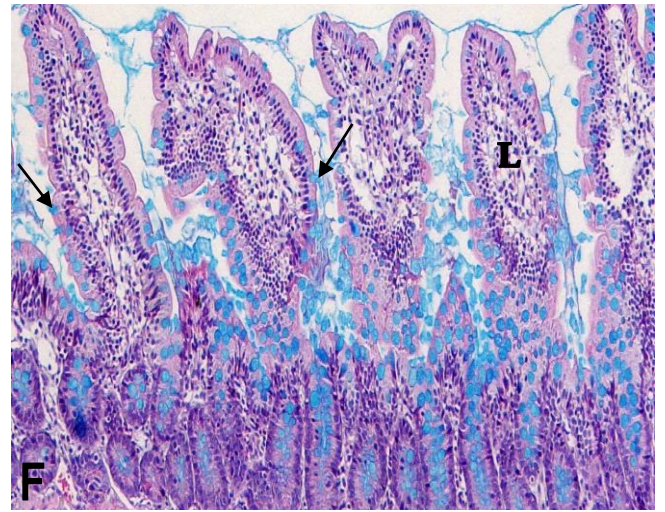
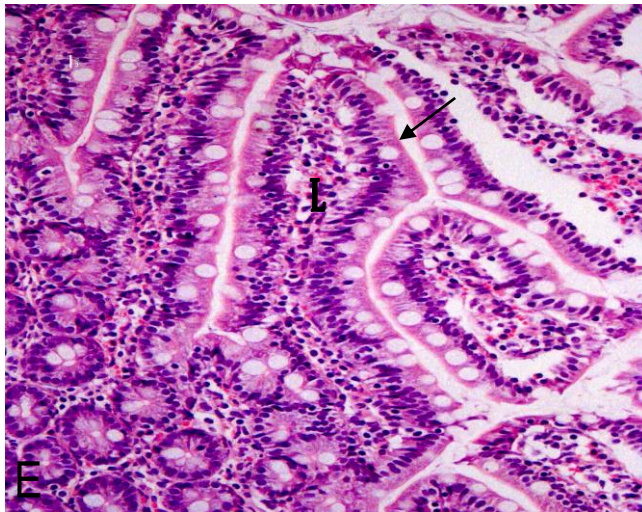
On the contrary, intestinal mucosa of neem-treated diabetic and neem-treated non-diabetic rats was similar to control; and there was higher secretory activity of goblet cells in the neem-treated diabetic rats at this time (Fig. 46 F). The mucosa of metformin-treated rats was also intact; and the goblet cells were highly secretory (Fig. 46 H).



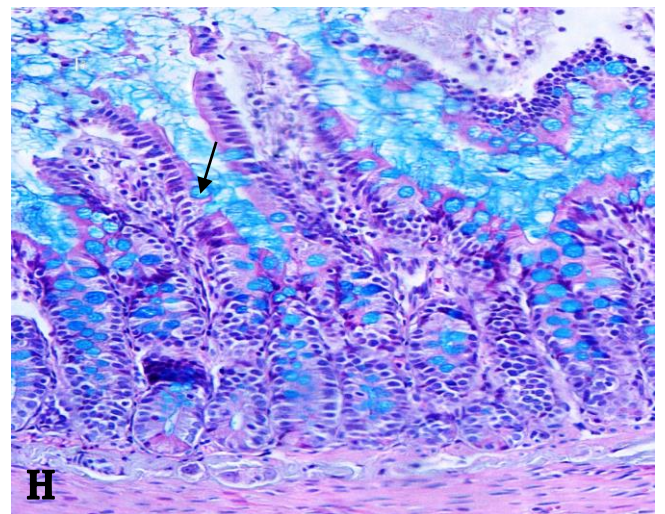
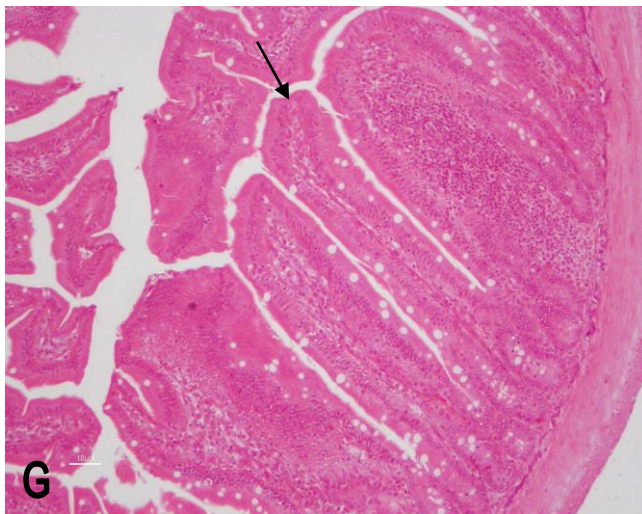
Figures 46 A and B. Jejunal sections of control rats at day 21 of treatment. **(A)** The epithelium is intact (arrow); H&E, x200. **(B)** Goblet cells are observable (arrow); Alcian blue, x200. (Scale bar = 10 μ m).



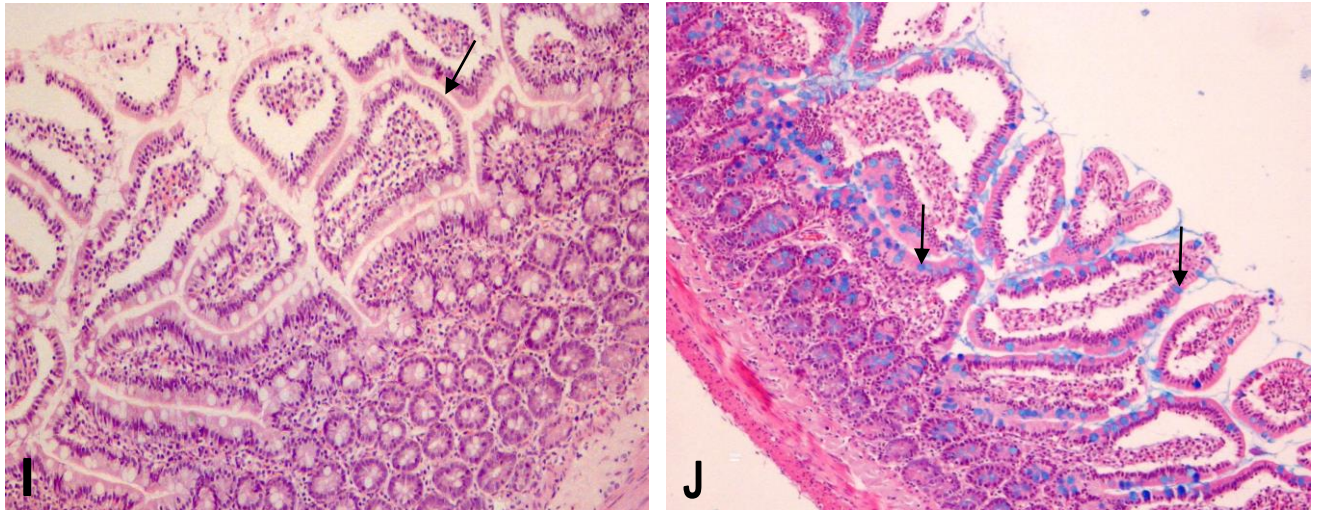
Figures 46 C and D. Jejunal sections of untreated diabetic rats at day 21. There was extensive superficial erosion of the epithelium, with blunting of the villi (Fig. 46 D; thick arrow). Most superficial goblet cells have become atrophic; while intestinal lumen contains tissue debris (Fig. D, thin arrow). **C**, H&E, x100; **D**, Alcian blue, x100.



Figures 46 E and F. Jejunal sections of neem-treated diabetic rats at day 21. **(E)** The mucosa is intact; arrow indicates epithelium, while L indicates lamina propria; H&E, x200. **(F)** Goblet cells are actively secretory (arrows) and the mucus coat is evident; L indicates lamina propria. Alcian blue, x200.

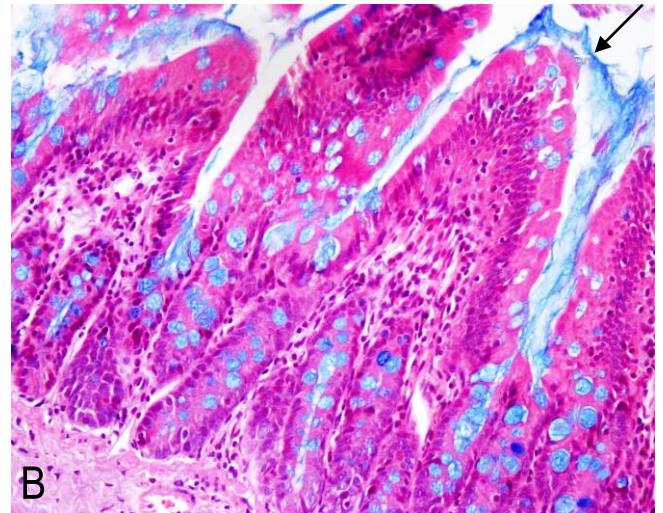
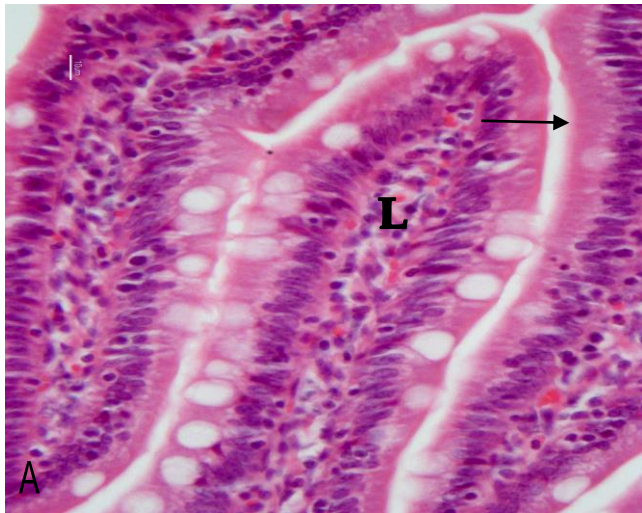


Figures 46 G and H. Jejunal sections of metformin-treated diabetic rats at day 21. **(G)** The epithelium is intact (arrow); H&E, x100. **(H)** Actively secretory goblet cells are observable (arrow); Alcian blue, x100. (Scale bar = 10 μ m).

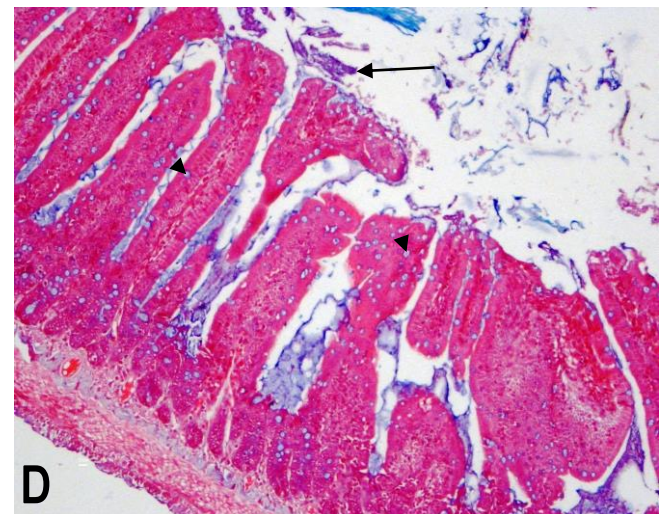
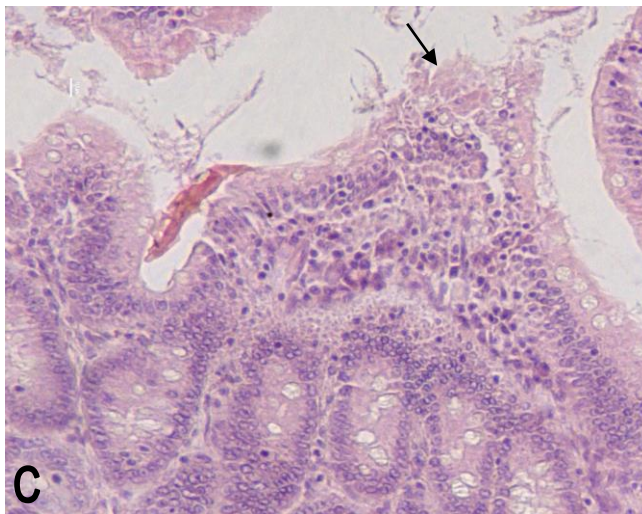


Figures 46 I and J. Jejunal sections of neem-treated non-diabetic rats at day 21. **(I)** The mucosa is intact (arrow); H&E, x200. **(J)** Goblet cells remain viable (arrows); Alcian blue, x100.

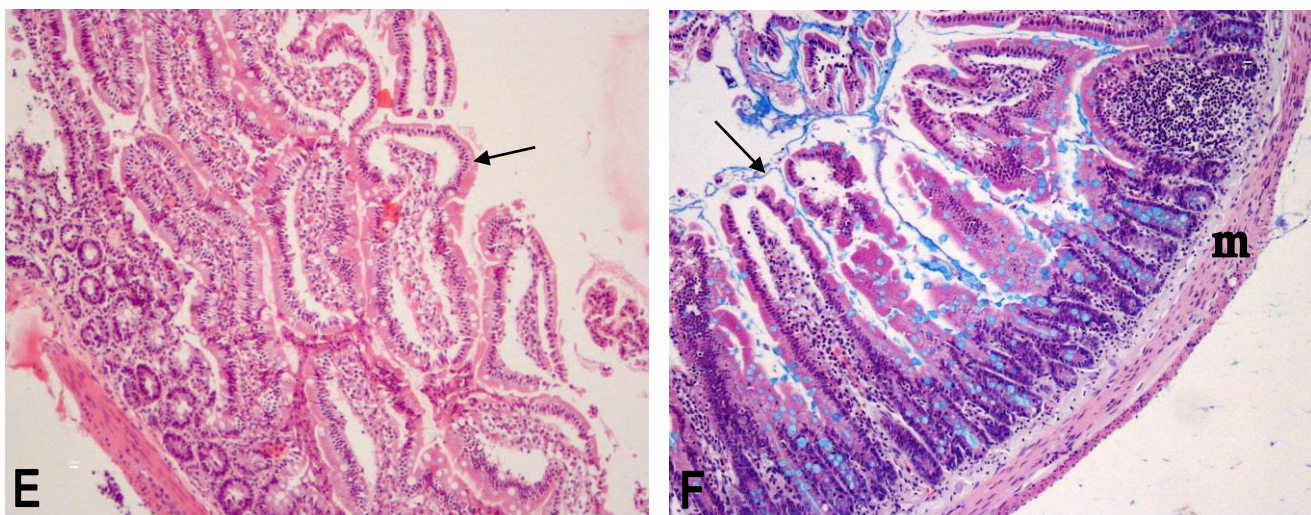
At 50 d, jejunal mucosa of the untreated diabetic rats was similar to that seen at 21 d (Fig. 46 D and 47 D). The mucosa was characterised by necrosis, low mucin content and poor secretory activity of goblet cells, as well as erosion of the surface epithelium. The epithelium was devoid of mucus lining; and desquamated cells and tissue debris were seen in the intestinal lumen. On the other hand, the mucosae of neem-treated diabetic and neem-treated non-diabetic rats were similar to control (Fig. 47 E, 47 F, 47 I and 47 J). The epithelium was intact and most goblet cells were actively secretory (Fig. 47 F and 47 J). The metformin group also had normal mucosa morphology except for the poor secretory function of the goblet cells (Fig. 47 G and 47 H). The surface epithelium, though intact, had a thin mucus lining (Fig 47 H).



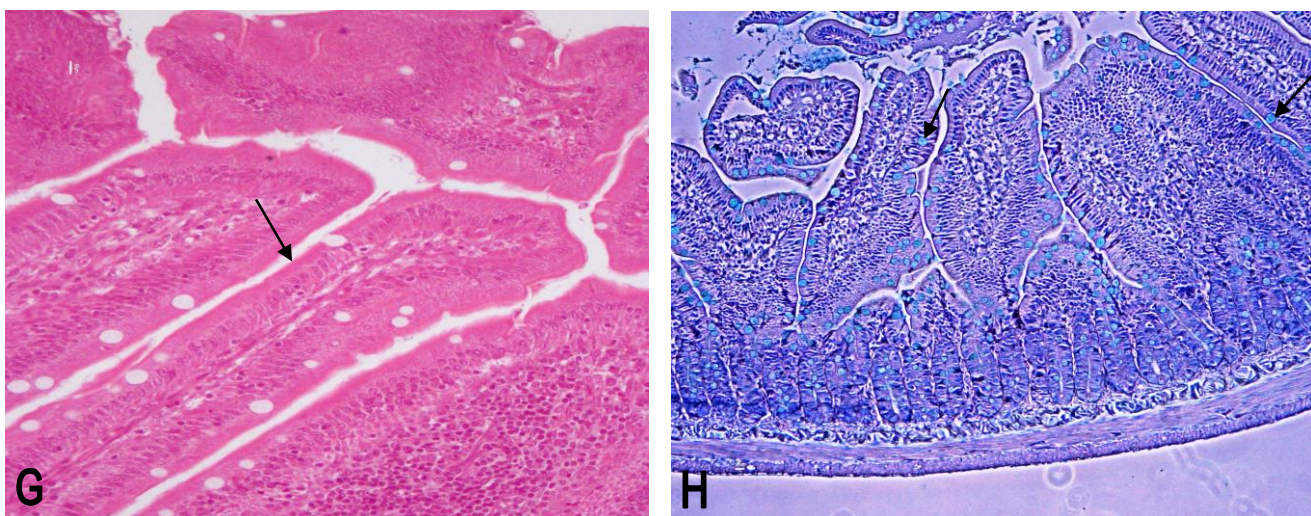
Figures 47 A and B. Jejunal sections of control rats at day 50. **(A)** The epithelium is intact (arrow); *L* indicates lamina propria; H&E, x400. **(B)** A coat of mucus (arrow) lines the mucosa. Alcian blue, x100. (Scale bar = 10 μ m).



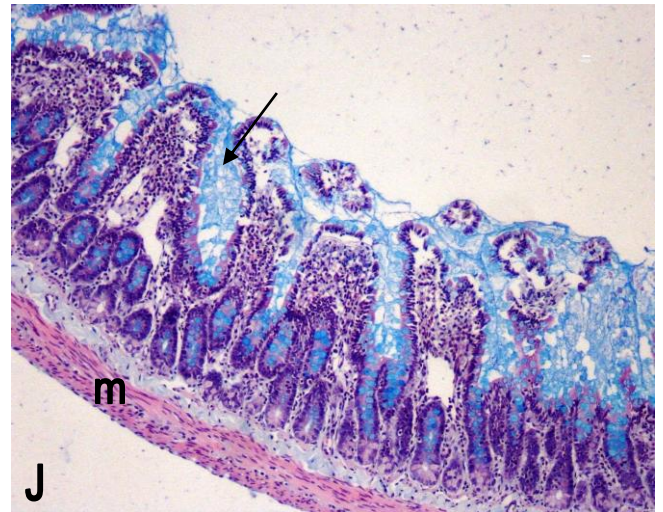
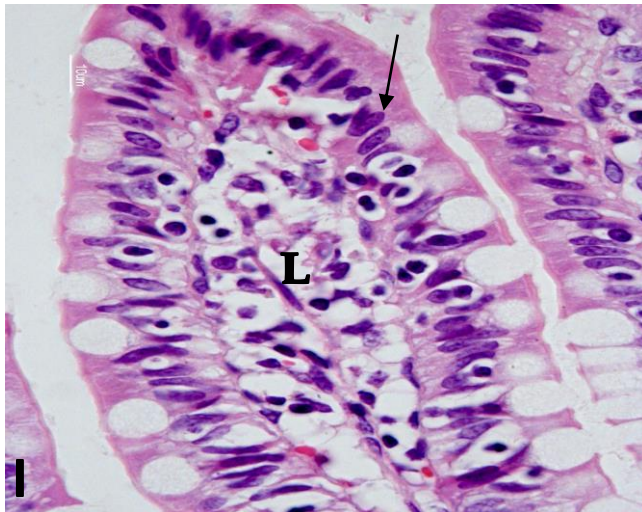
Figures 47 C and D. Jejunal sections of untreated diabetic rats at day 50. **(C)** Blunting of the villi is evident (arrow); H&E, x100. **(D)** Erosion of the mucosa is observable (arrow); and goblet cells show poor secretory function (arrowhead). Alcian blue, x100.



Figures 47 E and F. Jejunal sections of neem-treated diabetic rats at day 50. **(E)** Intestinal mucosa is largely normal (arrow); H&E, x100. **(F)** Goblet cells are active and a coat of mucus lines epithelial surface (arrow); *m* indicates muscularis externa. Alcian blue, x100.



Figures 47 G and H. Jejunal sections of metformin-treated diabetic rats at day 50. **(G)** Epithelium is intact (arrow); H&E, x200. **(H)** Note the poor secretory activity of goblet cells (arrows). Alcian blue, x100. (Scale bar = 10 μ m).



Figures 47 I and J. Jejunal sections of neem-treated non-diabetic rats at day 50. **(I)** Intestinal epitheliocytes are normal (arrow); *L* indicates lamina propria. H&E, x400. **(J)** The mucosa is intact, and goblet cells are actively secretory (arrow); *m* indicates muscularis externa. Alcian blue, x100. (Scale bar = 10 μ m).

Chapter Five

Discussion

5.1 Blood Glucose

In this study, treatment of STZ-induced hyperglycemic rats to a chronic regimen of the ethanolic leaf extract of *Azadirachta indica*, at a dose of 500 mg/kg bw/d, produced normoglycemia in 87.5% of the animals by the end of week 1 of treatment; and all animals had become normoglycemic by week 2 (Fig. 10). Comparison of the data from these animals to those of metformin-treated diabetic rats showed a relative advantage of *A. indica* (at 500 mg/kg bw/d) over metformin (at 350 mg/kg bw/d) in the maintenance of glucose homeostasis in STZ-induced hyperglycemic rats. Hypoglycemic activity of *A. indica* leaves had been reported in dogs (Murty *et al*, 1978); rats (Chattopadhyay, 1996, 1999; Ebong *et al*, 2008), and rabbits (Khosla *et al*, 2000). At day 0 of exposure, *A. indica* leaf extract produced its maximum effect on blood glucose 5 hours after the first dose of the extract (Fig. 9). A similar study by Murty *et al* (1978) showed that the hypoglycemic effect of *A. indica* leaves was most pronounced 1-2 hours post-dose. In this instance however, the drug was given intravenously to adrenaline-induced hyperglycemic dogs.

Proposed mechanisms of hypoglycemic effect of *A. indica* include inhibition of adrenalin-induced glycogenolysis (Chattopadhyay, 1996), and an insulintropic effect (Chattopadhyay, 1999). In the present study, a modulatory effect of *A. indica* on feed intake and body weight of diabetic (and non-diabetic) rats treated with the leaf extract was observed. By 50 d, gain in body weight was least in neem-treated rats (Fig. 11). Histological examination of intestinal mucosae of these animals showed intact epithelium (Fig. 45–47); and this suggests that the reduced feed intake and relatively low weight gain in these animals were not a result of impaired nutrient absorption. Rather, a modulatory effect of the extract on leptin production, secretion and/or action, or effects on body adiposity, might be involved.

A similar mechanism was reported for exendin-4, an antidiabetic substance from the salivary gland of the Gila monster lizard (Szayna *et al*, 2000). Future study on *A. indica* may thus test this hypothesis by quantifying the levels of leptin in the serum of neem-treated diabetic rats. Besides, 50-day sections of the pancreas of neem-treated hyperglycemia animals showed improved islet morphology (Fig. 38 F); and plasma insulin in these animals had increased significantly compared with untreated diabetic rats ($P<0.05$) (Fig. 18). Thus, morphologic improvement of the pancreatic islet, coupled with enhanced insulin secretion, might be an additional mechanism for the maintenance of normoglycemia in neem-treated diabetic animals. Chattopadhyay (1999) had previously reported the insulinitropic effect of neem in hyperglycemic rats; while Bhat *et al* (2009) reported similar effect in STZ-induced diabetic mice treated with chloroform leaf extract of *A. indica*.

A plethora of phytochemicals is obtainable from neem leaf (Siddiqui *et al*, 2004). Among these, quercetin, myricetin, kaempferol, rutin and their glycosides, have been implicated as responsible for the antidiabetic effect of neem leaves (Chattopadhyay, 1999). The work of Adewole *et al* (2007) showed the hypoglycemic activity of quercetin (25 mg/kg bw/d) in Wistar rats induced to hyperglycemia with 75 mg/kg bw of STZ. Similarly, Srinivasan *et al* (2005) reported the beneficial effect of rutin (200 mg/ kg bw) in male Swiss mice induced to diabetes with multiple low dose STZ (40 mg/kg bw) given intraperitoneally for five consecutive days. These findings suggest that the hypoglycemic effect of neem leaves may be mediated by its bioflavonoids. In contrast, Sanders *et al* (2001) and Dias *et al* (2005) reported lack of hypoglycemic activity of quercetin in STZ-induced hyperglycemic rats treated with 10 mg/kg bw/d and 150 μ mol/kg bw/d of quercetin, respectively. This negative finding may however be due to the use of relatively low doses of quercetin, when compared to the work of Adewole *et al* (2007), where the dosage of quercetin was much higher (25 mg/kg bw/d).

Furthermore, the negative reports of Sanders *et al* (2001) and Dias *et al* (2005) on the hypoglycemic effects of quercetin suggest that phytochemicals other than flavonoids may mediate or contribute to the hypoglycemic activity of neem leaves. This hypoglycemic activity may as well be owing to the synergistic effect of the phytochemicals (quercetin, rutin, etc) in the leaves of *A. indica*, as opposed to the use of isolated flavonoids.

5.2 Pancreas

Intraperitoneal injection of 70 mg/kg bw/d of STZ to fasted rats resulted in sustained hyperglycemia starting about 72 hours post-injection. Histopathological examination of the pancreatic islets at 21 d showed extensive necrosis that was most noticeable in the central parts of the islets in the untreated diabetic, diabetic + neem, and diabetic + metformin rats (Fig. 37). Less than 25% of islet β cells (in each of these groups) remained viable at this time (Fig. 34). Uptake of STZ into β cells is mediated by GLUT-2 transporter (Wang and Gleichmann, 1998). In these cells, STZ mediates its cytotoxicity via the DNA-alkylating activity of its *N*-methyl-*N*-nitrosourea moiety, especially at position O⁶ of guanine (Murata *et al*, 1999). The transfer of the methyl group from STZ to DNA results in DNA fragmentation and β -cell death (Yamamoto *et al*, 1981). Additional mechanisms involved in β -cell toxicity of STZ include over-activation of the poly (ADP-ribose) polymerase (in an attempt to repair damaged DNA), with the resultant depletion of cellular NAD⁺ and ATP (Uchigata *et al*, 1982); as well as glycosylation of proteins (Konrad and Kudlow, 2002).

As was also obtained in the present study (Fig. 27 and 28), increased pancreatic oxidative stress is associated with the progression of diabetes mellitus (Kakkar *et al*, 1998); and high ROS levels had been hypothesized as an additional mechanism of β -cell death in the pathogenesis of this disease (Robertson, 2004). This mechanism might thus contribute to the

death of β cells from STZ treatment in this study; and also to the failure of islet morphology to improve in the untreated diabetic rats in the latter part of the experiment.

At 50 d of exposure, morphometric analysis of the islets of *A. indica*-treated diabetic rats showed increased number of viable β -cell nuclei (Fig. 34). Histopathological assessment of the same tissue showed improved islet morphology (Fig. 38). This was in addition to the improved glycemia observed in these animals starting at week 1 (Fig. 10). In contrast, islet β -cell density in the untreated diabetic and metformin-treated diabetic rats remained relatively low at this time (50 d) (Fig. 34), and islet histomorphology did not improve (Fig. 38). These suggest that *A. indica* extract ameliorates islet lesions, possibly by enhancing β -cell proliferation. The latter may be owing to the upregulation of glucose-6-phosphate dehydrogenase (G6PDH) in β cells exposed to neem-leaf extract. Recently, Bhat *et al* (2009) reported elevated G6PDH activity and improved pancreatic islet morphology in STZ-induced mice treated with chloroform leaf extract of *A. indica* for 21 d. Besides, the findings of Diaz-Flores *et al* (2006) showed an inverse relationship between blood glucose and pancreatic G6PDH in STZ-induced diabetic Sprague-Dawley (SD) rats 15 d after the induction of hyperglycemia. G6PDH is the first key enzyme in the pentose phosphate pathway (Stryer, 1988); and its activity ultimately results in the synthesis of ribose-5-phosphate (R-5-P) (Kuo and Tang, 1999). The latter is converted to 5-phosphoribosyl-1-pyrophosphate that acts as the donor of the ribose phosphate unit in nucleotide biosynthesis (Kuo and Tang, 1999). Thus, G6PDH plays an important role in DNA synthesis and cell proliferation (Kuo and Tang, 1999). The work of Kuo and Tang (1999) showed that proliferation of NIH 3T3 cells was associated with overexpression of G6PDH in these cells. In the present study, reversal of hyperglycemia in neem-treated diabetic rats might result in the upregulation of pancreatic G6PDH, with increased production of R-5-P and the resultant increased DNA synthesis and

β -cell proliferation. This may explain the improved islet morphology seen in these animals at 50 d.

However, current opinions on the regenerative capacity of the β cells are rather conflicting. The long-held dogma that these cells are post-mitotic had been challenged by recent findings. In mice, the work of Dor *et al* (2004) showed that terminally-differentiated β cells retain a significant proliferative capacity *in vivo*. A similar finding had been reported by Nir *et al* (2007) in a murine model of diabetes. Furthermore, *in vivo* evidence that human β cells possess regenerative potential, even in the diabetic state, had been reported by Meier *et al* (2006b). However, recent *in vitro* studies of Parnaud *et al* (2008) on adult human β cells yielded contrary findings. This notwithstanding, their report on mice β cells provided additional evidence that these cells could, at least, divide in rodents.

Moreover, duplication of pre-existing adult β cells is not the only possible mechanism by which β -cell mass may be improved in diabetes. Extra-islet stem cells from the liver, spleen, bone marrow, and exocrine pancreas have been identified as potential insulin-producing cells (Meier *et al*, 2006a).

Though the mechanisms of improved islet morphology and enhanced cell mass (seen in diabetic rats treated with a chronic regimen of *A. indica* in this work) are unclear, it is noteworthy that such morphological improvement of the islet was absent in the untreated diabetic and metformin-treated diabetic rats within a similar time-frame. Indeed, *in vitro* treatment of primary β cells isolated from rats, as well as MIN6 cells, with biguanides (metformin and phenformin) had been reported to induce β -cell death (Hinke *et al*, 2007). In contrast, based on the present morphometric and morphological findings, it is suggested that the extract of *A. indica* is capable of enhancing β -cell proliferation *in vivo*. This study thus provides morphologic evidence supporting the tradomedical use of this drug as an

antidiabetic therapy (Abo *et al*, 2008), as well as its current use in certain antidiabetic formulations (Shekhar *et al*, 2002; Hsia *et al*, 2004). Morphological improvement of the pancreatic islets had been reported in related studies that employed the tetrapeptide pancreagene (Kvetnoi *et al*, 2007), the angiotensin-converting enzyme inhibitor ramipril (Ko *et al*, 2004), and the herbal drugs *dangnyosoko* (Kim *et al*, 2007), *hachimi-jio-gan* (Yamabe and Yokozawa, 2007), and *Aloe vera* (Noor *et al*, 2008). With respect to *A. indica* leaf extract and islet β -cell proliferation, future studies may consider BrdU or Ki67 staining of the islets of Langerhans of diabetic rats treated with a chronic regimen of this extract.

Oxidative stress is a confounding factor in diabetes mellitus, and it contributes to the pathogenesis (Robertson, 2004) and complications (Baynes, 1991) of this disease. Beta cells are especially vulnerable to oxidative insult in that they possess a relatively poor complement of antioxidants (Grankvist *et al*, 1981). In chronic hyperglycemia, oxidative stress is induced via several mechanisms (Wiernsperger, 2003; Wolff *et al*, 1989; Kennedy and Lyons, 1997; Kawamura *et al*, 1992). In streptozotocin diabetes, increases in pancreatic oxidative stress are associated with the progression of the disease (Kakkar *et al*, 1998), as was also obtained in this study (Fig. 27 and 28). STZ is a nitric oxide (NO) donor; and NO had been reported to mediate the destruction of pancreatic islet cells, probably via DNA damage (Kroncke *et al*, 1995). In addition to NO, STZ also generates ROS (from the action of this drug on the mitochondria and from increased xanthine oxidase activity) (Szkudelski, 2001). Thus, scavengers of NO (Kroncke *et al*, 1995) and ROS (Szkudelski, 2001) have been reported to possess beneficial effects against DNA damage and β -cell toxicity induced by these substances. In this work, lipid hydroperoxide and aqueous hydroperoxide levels of the pancreas of untreated diabetic rats had increased significantly above control at 50 d (Fig. 27 and 28). This indicates increased production of ROS in this organ. Besides, while lipid

peroxidation products were also significantly higher than control in the metformin-treated diabetic rats at this time, significantly low levels were obtained in the *A. indica*-treated diabetic (and non-diabetic) rats (Fig. 27 and 28). Given that the activities of SOD in all the treatment groups were not significantly different from control in the latter phase of exposure (Fig. 29), it is suggested that attenuation of oxidative stress in the pancreas of *A. indica*-treated diabetic rats was not a result of enhanced endogenous SOD (antioxidant) activity. Rather, this effect may partly be attributable to phytochemicals in the administered extract; and might be a factor contributing to the improved histological findings in the islets of *A. indica*-treated diabetic rats at 50 d. Active components of *A. indica* leaves had been reported to include quercetin, rutin, and their glycosides, etc (Chattopadhyay, 1999). Adewole *et al* (2007) reported that quercetin, at 25 mg/kg bw, given to adult Wistar rats for 30 days, resulted in improved morphology of the pancreatic islets. Similarly, Prince and Kamalakkannan (2006) reported the beneficial effects of rutin on islet morphology, oxidative status and glycemia in diabetic rats.

Moreover, in addition to the possible role of *A. indica* phytochemicals (antioxidants) in the amelioration of oxidative stress in the pancreas of experimental animals, the prompt reversal of hyperglycemia in *A. indica*-treated diabetic rats might be a contributory factor. As early as 7 d of treatment, normoglycemia had been restored in the larger percentage of these animals. Thus, hyperglycemia-induced, pancreatic ROS generation in this group could be minimized or abolished earlier. However, the normoglycemic status of the metformin-treated diabetic rats (which at 50 d still showed high pancreatic lipid peroxidation) suggests a possible role for *A. indica* phytochemicals in the attenuation of pancreatic oxidative stress in diabetic rats treated with this herb.

In conclusion, findings in this study confirm the hypoglycemic activity of *A. indica* leaves. Besides, the leaf extract of *A. indica* has the potential of attenuating islet lesions associated with established diabetes mellitus in Wistar rats. Furthermore, the low levels of lipid peroxidation products in the pancreas of diabetic rats treated with *A. indica* also suggest the beneficial effect of this herb in the amelioration of ROS-associated pancreatic islet lesions in diabetes mellitus.

5.3 Liver

In this study, hepatic glycogenosis (glycogenic hepatopathy) was observed in Wistar rats induced to hyperglycemia with 70 mg/kg bw of STZ. At euthanasia (50 d after attainment of hyperglycemia), liver weight had increased markedly in these rats (Fig. 13); and hepatocytes were glycogen-laden, as shown by the strong PAS-positive staining of their cytoplasm (Fig. 41 C and 41 E). Besides, plasma levels of certain liver enzymes (alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase) were raised in this group of animals (Fig. 26); and this indicates that diabetic hepatic glycogenosis is associated with hepatic injury, as was previously reported by Torbenson *et al* (2006) in human subjects.

In contrast, histological findings in the liver of neem-treated diabetic rats at 50 d (Fig. 41 G and 41 H) showed that, in addition to effective glycemic control, *A. indica* also prevents hepatic glycogen storage disease (glycogenic hepatopathy) that is associated with poorly-controlled diabetes mellitus. Glycogenic hepatopathy, described recently as under-recognised in diabetic patients with hepatomegaly, had been reported to be a common complication in type 1 diabetics whose blood glucose is poorly-controlled with insulin (Torbenson *et al*, 2006; Cuthbertson *et al*, 2007; Martocchia *et al*, 2008). Up to 80% of type 1 diabetes patients could be affected (Stone and Thiel, 1985). In such patients, marked accumulation of glycogen occurs in hepatocytes when insulin treatment leads to increased uptake of glucose by

hepatocytes, followed by rapid conversion of glucose to glycogen, and subsequently, trapping of the latter (Munns *et al*, 2000). Apart from diabetes, hepatic glycogenosis also occurs in Mauriac's syndrome (Mauriac, 1930), glycogen storage disease (Ozen, 2007), and following short-term high dose steroid therapy (Iancu *et al*, 1986).

In diabetic rats, hepatic glycogenosis may arise from upregulation of glycogen synthase. This enzyme had been reported to be upregulated in alloxan- and STZ-induced diabetes (Khandelwal *et al*, 1977; Bahnak and Gold, 1982). In the latter model, total glycogen synthase was reported to be significantly higher than control 40 days after induction of diabetes. Besides, the work of Ferrannini *et al* (1990) showed that longstanding insulin deficiency might actually enhance glycogen synthase activity.

Current approaches to the clinical management of glycogenic hepatopathy (GH) include administration of short- and intermediate-acting insulin (Duvnjak *et al*, 2004), dietary control and psychological support (Martocchia *et al*, 2008), as well as pancreas transplantation (Fridell *et al*, 2007). Our finding in this study suggests that neem prevents GH when used as an antidiabetic therapy. The marked glycogen accumulation observed in the hepatocytes of diabetic rats at 50 d was absent in those diabetic rats treated with neem leaf extract. This beneficial effect of neem perhaps arose from effective control of blood glucose by this herb.

However, though hepatocytes of neem-treated diabetic rats were devoid of glycogen, one characteristic histological finding in these rats (at 50 d) was the presence of aggregates of leucocytes in hepatic sinusoids (Fig. 41 H). This suggests mild lobular inflammation of the liver, and the development may be associated with hepatic oxidative stress present in these animals at 50 d (Fig. 30 and 31). Moreover, in this animals, plasma levels of ALT and ALP (but not ALP) were similar to control at 50 d ($P>0.05$; Fig. 26), and this largely suggests absence of hepatic lesions in this group.

The persistently high hepatic lipid peroxidation in diabetic rats treated with neem suggests high levels of reactive oxygen species (ROS) in hepatocytes, and perhaps in non-parenchymal hepatic cells, such as sinusoidal endothelial and Kupffer cells. The work of Vischer *et al* (1995) showed that high levels of ROS in vascular endothelial cells could stimulate exocytosis of Weibel-Palade bodies, and thus, expression of selectin platelet (P-selectin) on the surface of these endothelial cells. High ROS levels in sinusoidal endothelial cells could also enhance the transcription of selectin platelet (SELP) and other adhesion factors via the activation of the redox-sensitive NF κ B (Vischer *et al*, 1995). Expression of SELP on hepatic sinusoidal endothelial cell surface could mediate the tethering and adherence of leucocytes to vessel surface, leading to their aggregation (Ortmann and Brinkmann, 1997; Fernekorn *et al*, 2007). This phenomenon could explain the aggregation of leucocytes in hepatic sinusoids of neem-treated diabetic rats. A similar finding had been reported by Chosay *et al* (1997) in endotoxin-induced liver injury.

As shown in figures 30 and 31, significant increases in hepatic levels of lipid hydroperoxide and H₂O₂ occurred in the untreated diabetic and neem-treated diabetic rats throughout the 50-day treatment period. In the untreated diabetic rats, the persistent hepatic oxidative stress could be a result of chronic hyperglycemia, as explained elsewhere (Wolff *et al*, 1989; Yoshida *et al*, 1995; Blakytyn and Harding, 1992; Nishikawa *et al*, 2000b). However, the high hepatic lipid peroxide and H₂O₂ levels obtained in the neem-treated diabetic and neem-treated non-diabetic rats, largely in the absence of hyperglycemia, may not be readily explicable. A probable source of high ROS in these animals could be hepatic metabolism of the extract (neem) by the cytochrome P450 families of enzymes. The P450 families and subfamilies generally play active roles in the metabolism of drugs and xenobiotics (Ortiz de Montellano, 2005). Their induction and activity have been associated with enhanced ROS

production (Dostalek *et al*, 2008); and this suggests that metabolism of certain phytochemicals in neem could induce some of these enzymes. Such induction may result in increased generation of ROS. Previous studies had reported such an association between hepatic metabolism of drugs and xenobiotics by cytochrome P450, and ROS generation. For example, the toxic effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin was reported to arise from ROS generation via induction of the P450 enzymes (Shertzer *et al*, 1998). Ethanol could also induce certain families of P450 to produce free radicals (Albano *et al*, 1991). The work of Dasgupta *et al* (2004) showed that 80% hydroalcoholic extract of neem leaves, administered to mice at a dose of 200 mg and 500 mg/kg bw/d, could induce the phase II enzymes, which are involved in the metabolism of xenobiotics. Thus, generation of reactive oxygen species, which may arise from such cellular activities, could account for the high hepatic lipid peroxidation obtained in the present model.

However, in non-diabetic rats treated with neem, increases in hepatic lipid peroxidation products were limited to the early period of exposure (7 d and 21 d; Fig. 30 and 31). Lipid peroxidation was significantly lower than control at 50 d, in contrast to the neem-treated diabetic rats, where hepatic lipid peroxidation remained significantly high throughout the treatment period. This implies that neem-associated ROS generation (by whatever mechanisms) was limited to the early phase of neem exposure. Thus, the significant reduction in hepatic lipid peroxide levels in neem-treated non-diabetic rats (at 50 d) suggests that certain mechanisms were induced in the liver to suppress ROS generation (or scavenge these molecules) with continued exposure to the extract; and that such mechanisms were either inhibited or overwhelmed in the neem-treated diabetic rats. One cellular mechanism that could be involved in the suppression of ROS generation (or accumulation) in the neem-treated non-diabetic rats was upregulation of the antioxidant system. However, findings in

these animals (at 50 d) showed that SOD and GSH levels were significantly lower than control. This suggests that the reduced ROS levels in these animals might arise from reduced generation of ROS, rather than enhanced scavenging activity of antioxidants. Down-regulation of the cytochrome P450 enzymes in the latter phase of neem exposure might thus be responsible, but this is subject to further studies.

Furthermore, the significantly high hepatic lipid peroxide levels in neem-treated diabetic rats (at 50 d) could arise from ROS generation by leucocytes seen in higher number in the liver at this time. Leucocytes generate free radicals as part of their defensive mechanisms (Heyneman *et al*, 1990). However, they have been reported to produce significantly high amounts of ROS in experimental hepatitis (Bonder *et al*, 2004) and pancreatitis (Akpinar *et al*, 1995). In the present study, *A. indica* treatment could not prevent the generation of such high levels of hepatic ROS in these animals.

Furthermore, exacerbation of hepatic oxidative stress in neem-treated diabetic rats might also be associated with the time of exposure to the extract. Exposure to antioxidants after oxidative stress had been induced could actually promote the oxidative damage rather than ameliorate it (Halliwell, 2000). This had been demonstrated with ascorbate in paraquat-induced lung damage (Kang *et al*, 1998).

Most previous studies of the antioxidant activity of neem reported positive findings based on the high flavonoids content of this plant; and phytochemicals such as quercetin and rutin have been mostly implicated (Brahmachari, 2004). However, reports from *in vivo* studies of the antioxidant effect of quercetin in STZ diabetic rats had been conflicting. Suppression of hepatic oxidative stress was reported by Dias *et al* (2005), while the report of Sanders *et al* (2001) was contrary. In the present study, the consistently high hepatic lipid hydroperoxide levels and the presence of aggregates of leucocytes in the liver of diabetic rats treated with a

chronic regimen of *A. indica* suggest that this herb may not possess antioxidant activity in the liver of STZ-induced diabetic rats. However, in these neem-treated diabetic animals, plasma levels of liver enzymes (except alkaline phosphatase) were not significantly different from control at 50 d (Fig. 26). This largely suggests that chronic exposure to the leaf extract of neem is not associated with liver injury in this model; and that the raised hepatic oxidative stress in these animals may be a consequence of streptozotocin treatment and/or hepatic metabolism of *A. indica* leaf extract. Furthermore, the observed mild hepatic lobular inflammation in this group may be secondary to this increased oxidative stress, as explained above.

Moreover, hepatocellular ballooning, characterised by the presence of multivesicular intracytoplasmic vacuoles, were seen in all zones of hepatic acini in diabetic rats treated with metformin at 50 d of treatment (Fig. 41 I and 41 J). Besides, plasma ALT and AST levels were also elevated in these animals at 50 d (Fig. 26); and these findings, coupled with hepatocyte ballooning and vacuolation, suggest hepatic lesions in the metformin-treated diabetic rats at 50 d. In contrast, intracellular vacuoles and raised liver enzymes were not observed in neem-treated rats, nor in the metformin-treated diabetic rats at 7 d and 21 d.

Furthermore, the genesis and content of the vacuoles seen in the hepatocytes of metformin-treated rats are unclear. In diabetic patients, gross liver enlargement and ballooning of hepatocytes are generally interpreted as steatosis or steatohepatitis, arising from impaired hepatic lipid metabolism. The development of a steatotic liver in diabetes is associated with decreased plasma insulin, and consequently, increased lipolysis (Tolman *et al*, 2007). Such metabolic perturbations could imply increased delivery of exogenous fatty acids to hepatocytes; increased endogenous hepatic production of fatty acids; decreased hepatic disposal of fatty acids; or impaired export of VLDL from the liver. These can thus lead to

hepatic accumulation of lipids, especially in type 2 diabetic patients (Fong and Lindor, 2000). In the present study, staining of the ballooned hepatocytes with Sudan black B did not confirm the presence of lipid in these vacuoles (Fig. 41 K and L); and this indicates the absence of hepatic steatosis in this group.

Vacuolation and ballooning of hepatocytes had also been associated with anoxia that may occur at euthanasia in laboratory animals (Kimura and Abe, 1994). Trowell (1946) observed hepatic vacuolation from 5-15 minutes after death of animals from anoxia or asphyxia; while Sykes *et al* (1976) reported marked hepatocyte ballooning as early as 30 seconds post-euthanasia with nitrogen gas. Besides, the work of Li *et al* (2003) showed that hepatocyte vacuolation occurred in rats immediately after cessation of respiration, and that vacuolation progressed with increasing exposure to anoxic conditions. In the present study, animals were sacrificed under pentobarbital anaesthesia in a well-ventilated environment. Thus, ballooning of hepatocytes in this instance could not be a result of anoxia. Moreover, hepatocytes vacuoles were absent in other treatment groups euthanized in the same manner.

Furthermore, the study of Moore and Stegeman (1996) in pilot whales showed the link between autolytic floccular disintegration of hepatocyte cytoplasm and the interval between death and tissue sampling. The vacuoles observed in this study would not have arisen from autolytic changes as all samples were promptly excised from anaesthetized, live animals, and were fixed or frozen immediately on excision. Thus, in the present study, the content of hepatocyte vacuoles in the metformin-treated diabetic rats at 50 d is unclear.

In conclusion, findings from this study showed the beneficial effect of *A. indica* in the prevention of glycogenic hepatopathy in diabetic rats. However, the high hepatic lipid peroxidation and low liver GSH in neem-treated diabetic rats (at 50 d) suggest the association of hepatic oxidative stress with chronic *A. indica* treatment. However, in these animals,

plasma alanine aminotransferase and aspartate aminotransferase were not significantly different from control (Fig. 26), and this largely suggests absence of liver injury in these neem-treated diabetic rats at euthanasia.

5.4 Kidney

In this study, nephropathy was observed in STZ-induced diabetic rats by 50 d post-induction of hyperglycemia (Fig. 44 E and 44 F). Glomerular lesions observed include basement membrane (BM) thickening and mesangial matrix expansion. Thus, glomerulosclerosis (Kimmelstiel-Wilson disease) was evident. Also noted in these rats were nephromegaly (Fig. 14) and reduced glomerular diameters (Fig. 35). Moreover, analysis of biomarkers of renal function showed elevated plasma creatinine and urea at day 50 (Fig. 26).

Using Wistar rats induced to diabetes with 85 mg/kg bw/d of STZ, Cohen and Klein (1979) reported glomerulopathy (thickened glomerular BM) 6 weeks post-induction of hyperglycemia. Similarly, glomerulopathy had been reported in an STZ model of nephropathy 7 months post-STZ injection to Wistar rats (Agardh *et al*, 2002). Diabetic glomerulosclerosis is a feature of human diabetic nephropathy (Dijk and Berl, 2004), with the same glomerular lesions as those reported in animal studies (Nobrega *et al*, 2004). Hyperglycemia and poor glycemic control have been implicated in the pathogenesis of glomerular lesions of diabetes mellitus (Diabetes Control and Complications Trial Research Group, 1993). Besides, hyperglycemia-dependent pathways are activated in diabetic kidney; and these include increased generation of reactive oxygen species (ROS), renal polyol formation, non-enzymatic glycation of proteins and induction of pro-sclerotic cytokines such as TGF- β (Saito *et al*, 2006). Besides provoking non-enzymatic glycation of proteins and its sequelae, chronic hyperglycemia also induces intracellular ROS in mesangial and tubular epithelial cells, perhaps by inducing PKC- and MAPK-mediated pathways (Wilmer *et al*,

2001). High glucose-induced PKC activation is one mechanism responsible for the generation of free radicals through advanced glycation end products (AGEs), polyol pathways and enhanced cytokine production in diabetic nephropathy (Craven *et al*, 1990). Glucose-induced oxidative damage and the production of TGF- β are final common mediators of extracellular matrix expansion and renal lesions of diabetic nephropathy (Ziyadeh and Han, 1997). The findings of Yamagishi *et al* (2003) showed that AGEs inhibit *de novo* protein synthesis and induces TGF- β mRNA expression, with associated deposition of extracellular matrix in renal tissue. Thus, glomerulosclerosis seen in our model might arise from chronic hyperglycemia and consequent upregulation of extracellular matrix production in the glomeruli of untreated diabetic rats.

In contrast to the foregoing, renal histological findings (Fig. 44 K and L) and analysis of plasma urea and creatinine (Fig. 24, 25 and 26) in neem-treated diabetic animals suggest that the leaf extract of *A. indica* is beneficial against diabetic nephropathy. These beneficial effects of neem might arise from effective glycemic control (Fig. 10) and activity of the antioxidant flavonoids in neem leaves (Tables 4 and 5). Quercetin and its glycosides are flavonoids available in neem (Chattopadhyay, 1999). The work of Anjaneyulu and Chopra (2004) showed the beneficial effects of quercetin against diabetic nephropathy. It was concluded that quercetin attenuates diabetic renal lesions via an anti-oxidative mechanism. Quercetin had been reported to inhibit lipid peroxidation by blocking xanthine oxidase (Chen and Breen, 2000); chelating iron (Da Silva *et al*, 1998), and scavenging hydroxyl and superoxide radicals (Robak and Gryglewski, 1988). This antioxidant is also beneficial against AGE formation (Asgary *et al*, 1999). Thus, the antinephropathic activity of neem leaf extract, as observed in the present study, might be owing to the activity of its antioxidant flavonoids.

Moreover, the renal tubular lesions seen in this model of nephropathy include thickening of tubule cell BM, vacuolation of several cells of the proximal tubules (PT), and strong PAS-positive staining of non-vacuolar tubular cells. Thickening of BM of tubular cells (Fig. 44 F), seen in this work, is characteristic of diabetic nephropathy; and its pathogenesis could be explained as described above for glomerulosclerosis. Besides, PAS-positive staining of non-vacuolated cells of the proximal tubules suggests glycogen deposition in these cells, and the presence of glycogen (and perhaps other periodate-reactive carbohydrates) was confirmed following diastase digestion of the PAS-positive materials of the non-vacuolated tubule cells, and the subsequent PAS-negative staining outcome of the sections (Fig. 44 G and H). Ebstein in 1882 demonstrated glycogen accumulation and cytoplasmic vacuolation in renal tubular cells of patients who died from diabetic coma. In the present study, this development was seen in cells of the proximal tubules of untreated diabetic rats at 50 d. Glycogen accumulation in tubular cells of diabetic Sprague-Dawley rats had also been reported recently (Kang *et al*, 2005). Furthermore, the presence of clear cytoplasmic vacuoles (Armanni-Ebstein phenomenon; glycogen nephrosis) in certain tubule cells (Fig. 44 F) had been explained as suggesting loss of accumulated glycogen from these cells during histological preparation of tissue for light microscopy (Kock and Vestergaard, 1994; Kang *et al*, 2005). In the same vein, Kim *et al* (2008) studied the effect of 4-week treatment with *Phellodendri cortex* extract on renal histology in streptozotocin-induced diabetic male Sprague-Dawley rats. They reported glycogen accumulation in several renal proximal tubule cells, as demonstrated by PAS-positive staining of those cells; as well as the presence of Armanni-Ebstein cells, characterised by clear vacuolation of the cytoplasm of some of these cells, consequent upon the removal of accumulated cytoplasmic glycogen during the staining procedure (Kim *et al*, 2008).

Recent findings however suggest that vacuoles of Armanni-Ebstein cells did not arise from loss of glycogen from these cells. The work of Nielsen *et al* (2003) and Thomsen *et al* (2006) showed accumulation of triglyceride in proximal tubule cells of patients in diabetic coma. In the present study, staining of renal sections of untreated diabetic rats for lipid yielded negative outcome, and this confirmed the absence of lipid in the clear vacuoles of proximal tubule cells (Fig. 44 I and J). Meanwhile, sudanophilic granules were observed in certain non-vacuolated tubule cells in this group, and this suggests the presence of lipid within such cells (Fig. 44 I and J). Thus, the staining outcome for renal sections of untreated diabetic rats at 50 d shows that the vacuolated proximal tubule cells are devoid of lipids, while the non-vacuolated cells of renal tubules contain a mixture of lipid and glycogen (as suggested by the presence of sudanophilic and diastase-digestible PAS-positive materials in such cells).

In hyperglycemic rats treated with neem, nodular glomerulosclerosis and Armanni-Ebstein phenomenon (seen in the renal sections of untreated diabetic rats at 50 d) were absent (Fig. 44 K and L). This beneficial effect of neem against diabetic nephropathy might arise from effective glycemic control by the extract (Fig. 10). Previous workers had reported improvement of diabetic nephropathy following improved glycemic control with insulin treatment (Rasch, 1979), islet transplantation (Wehner *et al*, 1980), and pancreas transplantation (Orloff *et al*, 1988). Besides, the work of Macedo *et al* (2002) showed the beneficial effects of insulin-acarbose treatment in ameliorating mesangial enlargement in diabetic rats. Moreover, chronic vasopeptidase inhibition in Zucker diabetic fatty (ZDF) rats was reported to prevent nephropathy in this animal model (Schafer *et al*, 2003). Agardh *et al* (2002) also emphasized the role of glycation and oxidative stress in the pathogenesis of diabetic nephropathy in rats and reported the beneficial effects of the antioxidant probucol in the inhibition of such processes. The importance of antioxidant intervention in the prevention

or amelioration of nephropathy was also reported recently by Anjaneyulu and Chopra (2004) in diabetic rats treated with quercetin. Thus, the beneficial effect of neem against diabetic nephropathy in this model of the disease might arise partly from effective glycemic control and ROS-scavenging activity of the antioxidant flavonoids in neem, of which quercetin and rutin are important components (Chattopadhyay, 1999).

5.5 Jejunum (Small Intestine)

Diabetic rat that were treated with neither neem nor metformin showed intestinal lesions characterised by superficial erosion of mucosal epithelium, blunting of villi, necrosis of goblet cells and absence of mucous coat at 21 d and 50 d of treatment (Fig. 46 D and 47 D). Such lesions were absent in metformin- and neem-treated hyperglycemic and neem-treated non-diabetic rats. In human, diabetic mellitus may or may not be associated with histological changes in the small bowel (Bjelakovic *et al*, 2005). In diabetic patients presenting with diarrhoea, with or without steatorrhoea, biopsy from a number of such patients showed microangiopathy of the small bowel, with atrophic, thickened and reduced villi, and stromal infiltration of the mucosa (De Las Casas and Finley, 1999). Blood vessels were seen to display prominent mural thickening and stenosis secondary to accumulation of hyaline material. In the present study, though staining of intestinal vessels for markers of microangiopathy was not done, this pathology might account partly for necrosis of intestinal epithelial and goblet cells seen in this study. Hyperglycemia had been reported to trigger arteriosclerosis in vessels via AGE- and ROS-mediated mechanisms (Hunt *et al*, 1988). Thus, chronic hyperglycemia in the untreated diabetic animals might result in arteriosclerosis of vessels of the small bowel, with thickening of vessel wall, stenosis of their lumen, ischaemia, and the resultant mucosal injury. In addition, intestinal lesions seen in the untreated diabetic rats at 50 d might as well arise from STZ toxicity. Epitheliocytes of the intestine possess

GLUT-2 transporter (Nolte and Karam, 2007). Cells expressing this transporter, including islet β cells, hepatocytes, renal tubular cells and epitheliocytes of the intestine, are able to take up STZ (Lenzen, 2008). Intestinal uptake of STZ might result in cell necrosis via DNA methylation and increased oxidative stress (Lenzen, 2008). In gastroduodenal lesions (ulceration), oxidative damage of the mucosa by ROS (Bhattacharjee *et al*, 2002), and apoptotic cell death (Biswas *et al*, 2003), had been reported as major causative factors. Oxidative damage of mucosa epitheliocytes by ROS (especially hydroxyl radical) is characterised by increased lipid peroxidation and protein oxidation, with depletion of thiols (e.g. glutathione) (Bandyopadhyay *et al*, 2004). The work of Chattopadhyay *et al* (2004) on Sprague-Dawley rats induced to gastric ulceration with indomethacin, stress, and ethanol, showed the beneficial effect of neem leaf extract against mucosal ulceration. Similar findings were reported by Ofusori *et al* (2008) against ethanol-induced gastric lesions. One mechanism proposed for this effect was the prevention of oxidative damage of gastric mucosa by attenuation of lipid peroxidation by neem; the extract was also reported to prevent apoptotic cell death from DNA damage (Chattopadhyay *et al*, 2004). Thus, the beneficial effect of neem leaf extract noted in the present work may arise from attenuation of STZ- and hyperglycemia-induced oxidative stress in the epitheliocytes and goblet cells of intestinal mucosa. Garg *et al* (1993) also reported that the extract of neem leaves could inhibit depletion of mucous lining of intestinal mucosa. In the present model, neem leaf extract enhanced mucus secretion in diabetic and non-diabetic rats (Fig. 45 J, 46 F, 47 F and 47 J). Thus, these histologic findings suggest that neem can augment the secretory activity of goblet cells, thereby conferring protection on intestinal mucosa. This beneficial activity of neem leaves against STZ-induced (diabetic) intestinal lesions might arise from the antioxidant activity of its phytochemicals.

Besides, absence of intestinal lesions in neem-treated non-diabetic rats suggests that oral treatment of healthy rats with neem is not associated with deleterious intestinal effects. However, the exact mechanism of this beneficial importance of neem in the GIT is unclear but may involve augmentation of intestinal mucous secretion by neem leaf extract, as suggested by the active secretory activity of intestinal goblet cells in this group (Fig. 45 J and 47 J).

5.6 Diabetic Dyslipidemia

Diabetes mellitus is almost always associated with changes in plasma lipoproteins (Howard, 1987); and such changes are recognized as a major determinant of atherosclerotic vascular disease in man (Rossetti and Goldberg, 2002). The commonest lipid abnormality in diabetic patients is the finding of raised serum VLDL-triacylglycerol or raised total triacylglycerol (TG) levels arising from decrease in the inhibitory effects of insulin on VLDL release from the liver; and decreased activity of lipoprotein lipase (LPL) that results in decreased hydrolysis of TG in the circulation (Tomkin and Owens, 1991). Diabetic hypertriacylglycerolaemia is associated with both type 1 and type 2 diabetes (Bierman *et al*, 1966). In type 1 diabetes, extreme elevations in plasma VLDL are a common occurrence in diabetic ketoacidosis (Bagdade *et al*, 1967), when insulin concentrations are minimal. In the present study, VLDL and total TG levels were significantly higher than control ($P<0.05$) in diabetic animals at 50 d (Fig. 22 and 23). Similarly, insulin concentrations at this stage were low in the untreated diabetic rats (Fig. 18). Thus, elevation of plasma TG, reported in this STZ model of diabetes mellitus, might arise from chronic insulin deficiency. This had been reported previously in diabetic human (Ginsberg *et al*, 1977) and animals (Yousef *et al*, 2006). Ginsberg *et al* (1977) found increased synthesis and decreased clearance of very low density lipoprotein-triglyceride (VLDL-TG) in a group of insulin-deficient type 1 diabetics,

and Greenfield *et al* (1980) reported an increase in VLDL-TG production in insulin-dependent diabetics. Associated with such raised serum TG is lowering of HDL concentrations (Glasgow *et al*, 1981). It is accepted that a low HDL level is an independent risk factor for atherosclerosis in the diabetics (Reckless *et al*, 1978) and non-diabetics (Gordon *et al*, 1977). In the present study, HDL levels in the untreated diabetic rats were significantly lower than control (Fig. 20). Such low plasma HDL had been reported to arise from low LPL activity, which leads to reduced formation of HDL during impaired lipolysis of VLDL (Taskinen, 1987). Such a metabolic perturbation would lead to impairment in the reverse transport of cholesterol from tissue to the liver for excretion (Tomkin and Owens, 1991), leading to increased risk of atherogenesis (Henry, 2001). Similar findings of dyslipidemia (elevated levels of total cholesterol, total TG, LDL-c, and VLDL-c; and decreased levels of HDL-c) had been reported in adult Wistar rats induced to hyperglycaemia with multiple low-dose STZ (40 mg/kg bw/d) after four weeks of hyperglycaemia (Komolafe *et al*, 2009)

As shown in figures 19 and 21, raised total plasma cholesterol and LDL levels could occur in chronic hyperglycemia. Plasma LDL has been shown to increase in poorly-controlled type 1 diabetic subjects (Lopes-Virella *et al*, 1981) and its level had been reported to correlate with the degree of glycemic control (Calvert *et al*, 1978). However, in many individuals with type 1 diabetes, LDL levels are either normal (Durringham, 1980) or even lower than control (Rosenstock *et al*, 1985). In addition to the possible alteration in the levels of plasma LDL in the diabetic state, interaction of LDL with its receptors can also be inhibited by chemical modification of the lysine and arginine residues of Apo B (Weisgraber *et al*, 1978). Glycosylation of approximately 40% of lysine residues completely blocks receptor-mediated LDL catabolism *in vivo* (Kesaniemi *et al*, 1983). Glycosylated LDL also seems to have altered interaction with endothelial cells (Lorenzi *et al*, 1984). Thus, glycosylation of LDL in

diabetes mellitus may accelerate the deposition of this lipoprotein in vessel walls. LDL is also liable to oxidation under diabetic conditions. Besides, diabetic patients typically have a preponderance of smaller, denser, oxidized LDL particles that may increase atherogenicity (Rubin *et al*, 1999). In uncontrolled type 1 diabetes, LDL fractional clearance is perhaps decreased since insulin appears to potentiate the binding of LDL to its receptors (Howard, 1987); and this may partly account for the raised plasma LDL seen in the present study; and in some diabetic patients (Lopes-Virella *et al*, 1981).

Diabetic dyslipidaemia is managed clinically by means of pharmacological and non-pharmacological measures. The latter involves diet and exercise (American Diabetes Association, 2001). Pharmacological approach involves the use of the statins and fibric acid derivatives. Others include nicotinic acid, bile-acid binding resins, and certain antidiabetic drugs such as thiazolidinediones (Henry, 2001). Statins and resins lower LDL (American Diabetes Association, 2001). Elevated TG is reduced with fibric acid derivatives; while HDL may be raised with the use of statins, fibric acids and nicotinic acid (Henry, 2001).

Findings from the present study suggest that the leaf extract of *Azadirachta indica* is effective as an alternative or complimentary therapy for the prevention (and/or management) of diabetic dyslipidaemia. Dyslipidaemia observed in the untreated diabetic rats was absent in neem-treated diabetic animals. Similar findings had been reported in acute study of Chattopadhyay and Bandyopadhyay (2005) in streptozotocin-diabetic Wistar rats treated with 500 mg/kg bw/d of *A. indica* leaf extract for 7 d. Hyperglycemic rats were reported to develop dyslipidemia characteristic of diabetes mellitus within this short period. Such impairment of lipid metabolism was not observed in those hyperglycemic animals treated with neem leaf extract. In the present study, this beneficial effect of neem on diabetic dyslipidemia could arise from effective glycemic control resulting from enhanced insulin

secretion in diabetic rats treated with neem. Effective glycemic control and improved plasma insulin had been linked with the attenuation of diabetic dyslipidaemia (Dunn *et al*, 1985). Thus, increases in plasma insulin levels in neem-treated hyperglycemic rats at 50 d may be the key factor mediating amelioration of dyslipidemia in these animals, possibly from enhanced activity of insulin-dependent lipoprotein lipase (Howard, 1987).

In conclusion, the present findings suggest that neem-leaf therapy may be beneficial against morbidity and mortality arising from increased cerebrovascular, cardiovascular, and peripheral vascular lesions that often result from chronic dyslipidemia and accelerated atherosclerosis of diabetes mellitus.

Chapter Six

Conclusions

6.1 Conclusions

Findings from the present study show that:

- i. Ethanolic leaf extract of *Azadirachta indica* possesses hypoglycemic effect. This underscores the potential of this botanical therapy in the management of diabetic hyperglycemia.
- ii. Chronic treatment with ethanolic leaf extract of *Azadirachta indica* is associated with improvement of islet morphology, with elevated plasma insulin. Neem therapy may thus be beneficial in ameliorating diabetic islet lesions.
- iii. Chronic treatment with ethanolic leaf extract of *Azadirachta indica* prevents diabetic oxidative stress in the pancreas. This might contribute to the improvement of islet morphology in diabetic rats.
- iv. *Azadirachta indica* treatment is beneficial against diabetic glycogen storage disease (hepatic glycogenosis) and elevated liver enzymes in streptozotocin-induced diabetes.
- v. Furthermore, *Azadirachta indica* leaf extract is beneficial against diabetic nephropathy. Thus, the extract may reduce morbidity and mortality arising from this complication of diabetes mellitus.
- vi. Increased hepatic lipid peroxidation and poor hepatic antioxidant status are associated with chronic treatment with ethanolic leaf extract of *Azadirachta indica* in STZ-induced diabetic rats. These suggest association of hepatic oxidative stress with chronic neem therapy.

- vii. *Azadirachta indica* ameliorates diabetic dyslipidemia in Wistar rats. It may thus be beneficial in reducing the morbidity and mortality associated with increased cardiovascular events that are characteristic of diabetic dyslipidaemia.
- viii. *Azadirachta indica* possesses no adverse effects on intestinal mucosa in healthy rats. Besides, it prevents STZ-induced intestinal lesions in this animal model.
- ix. *Azadirachta indica* modulates body weight and feed intake in rats. It may thus be beneficial in the management of obesity and type 2 diabetes.

6.2 Future Research Directions

- i. Using skeletal muscle cell lines or adipocytes cell lines, the expression of GLUT-4 transporter protein or mRNA following exposure to neem leaf extract may be studied *in vitro*. Neem may produce its hypoglycemic effect in part by enhancing glucose uptake into these cells through up-regulation of this transport molecule.
- ii. The hypothesis that “Neem modulates the production, secretion and/or action of leptin” may be tested by studying this hormone in neem-treated diabetic rats.
- iii. The hypothesis that “Neem treatment enhances β -cell proliferation” could be tested by performing Ki67 or BrdU immunostaining in pancreatic islets of neem-treated diabetic rats. Histochemical and biochemical studies of islet glucose-6-phosphate dehydrogenase may also be done.

7.0 References

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8.0 Appendix

8.1. Reagents for the Colourimetric Estimation of Total Flavonoid Content of

Azadirachta indica Dry Leaves

HEPES buffer 40 mM, pH 7.4

HEPES (CalBiochem, CA, USA), 40 mM	0.0191 g
Imidazol (Sigma, MO, USA), 10 mM	0.0209 g
6-aminohexanoic acid (Sigma, MO, USA), 5 mM	0.01312 g
Distilled water	20 ml

NaNO₃, 5%

NaNO ₃ (Sigma, MO, USA)	5 g
Distilled water	100 ml

AlCl₃, 10%

AlCl ₃ (Sigma, MO, USA)	10 g
Distilled water	100 ml

NaOH, 1M

NaOH (Sigma, MO, USA)	4 g
Distilled water	100 ml

8.2. Preparation of Citrate Buffer

- Citrate buffer 0.1 M, pH 4.5

Citric acid (Sigma-Aldrich Chemie, Steinheim, Germany)	2.10 g
Sodium citrate, dihydrate (J.T Baker Chemicals,	

Deventer, Holland)	2.58 g
Bidistilled water	100 ml

8.3. Preparation of Reagents for Total Cholesterol Assay

- Cholesterol probe: This was dissolved in 220 µl of DMSO
- Cholesterol esterase: This was dissolved in 220 µl of cholesterol reaction buffer
- Enzyme mix: This was dissolved in 220 µl of cholesterol reaction buffer.

For each well (96-well plate), the reaction mix was prepared as follows:

- 44 µl of cholesterol reaction buffer
- 2 µl of cholesterol probe
- 2 µl of enzyme mix
- 2 µl of cholesterol esterase

8.4. Preparation of Reagents for Plasma Total Triglyceride Assay

Reconstituted triglyceride reagent

- Triglyceride reagent, 5x 10 ml
- Deionised water 10 ml
- Mix by repeated inversion

Reconstituted free glycerol reagent

- Free glycerol reagent, 10x 40 ml
- Deionised water 40 ml
- Mix by inversion

Triglyceride working reagent

- Reconstituted free glycerol 40 ml

Reconstituted triglyceride reagent	10 ml
Triglyceride reagent blank	
Reconstituted free glycerol	4 ml
Deionised water	1ml
Standard solution	
Glycerol standard solution (Sigma, MO, USA)	2.5 mg/dl

8.5. Preparation of Reagents for Plasma Insulin Assay

Enzyme conjugate solution

Enzyme conjugate, 11x	600 µl
Enzyme conjugate buffer	6 ml

Wash buffer

Wash buffer, 21x	35 ml
Distilled water	700 ml

8.6. Preparation of Working Solution for Plasma Alkaline Phosphatase Assay

Assay buffer	18 ml
Mg acetate	0.45 ml
pNPP	0.18 ml

8.7. Reagent Preparation for Plasma Aspartate Aminotransferase Assay

- Reconstitution of reagent mix: This was done by adding 27 ml of deionised water to the reagent mix powder, followed by mixing by repeated inversion. Before use, the temperature of the reconstituted reagent mix was raised to 37 °C.

8.8. Reagent Preparation for Alanine Aminotransferase Assay

- ALT assay buffer (10x): this was diluted by adding 36 ml of distilled water to 4 ml of the assay buffer concentrate
- ALT substrate: The vial content was dissolved with 30 ml of the diluted assay buffer.
- ALT cofactor: The content of the vial was dissolved with 1.5 ml of diluted assay buffer.

8.9. Reagent Preparation for Plasma Urea Assay

Working reagent:

- | | |
|-------------|-------|
| • Reagent A | 50 ml |
| • Reagent B | 50 ml |

8.10. Preparation of Reagents for Plasma Creatinine Assay

Working solution: This was prepared by mixing equal volumes of reagent A and reagent B (50 ml each).

Reconstituted standard solution

- | | |
|---|--------|
| Creatinine standard solution (50 mg/ml) | 8 µl |
| Distilled water | 192 µl |

8.11. Preparation of 0.15 M KCl-Tris Buffer, pH 7.4

- KCl (Sigma, MO, USA), 0.15 M 11.18 g
- Tris (tris(hydroxymethyl)aminomethane) (Sigma, MO, USA), 0.05 M 6.06 g
- Double-distilled water 1000 ml

8.12. Preparation of Reagents for Lipid Hydroperoxide Levels of the Pancreas and Liver

- Organic peroxide colour reagent:
 - Organic peroxide colour reagent 1 vial
 - Methanol (J.T Baker, Deventer, Holland), 90 %
 - (prepared with deionised water) 120 ml

(After reconstitution, the colour reagent contained 4 mM BHT and 125 μ M xylene orange)

- Working colour reagent:
 - Ferrous ammonium sulphate reagent 100 μ l
 - Organic peroxide colour reagent 10 ml
- *tert*-Butyl hydroperoxide (t-BuOOH) standard solution, 200 μ M: This was prepared by serial dilution of 70 % solution of t-BuOOH (7 M) with methanol.

8.13. Preparation of Reagents for Aqueous Hydroperoxide Levels of the Pancreas and Liver

- H₂O₂ standard solution (100 μ M): This was prepared by serial dilution of the 30% (9.8 M) H₂O₂ solution with deionised water to obtain a final concentration of 100 μ M
- Working reagent solution
 - Ferrous ammonium sulphate reagent 100 μ l
 - Aqueous peroxide colour reagent 10 ml

8.14. Preparation of Stock Solutions for GSH Assay

- DTNB stock solution, 1.5 mg/ml

DTNB	8 g
DMSO	5.33 ml

- NADPH stock solution, 40 mg/ml

NADPH	25 mg
Bidistilled water	0.625 ml

- 5% 5-Sulphosalicylic acid (5% SSA)

5-Sulphosalicylic acid	2.5 g
Bidistilled water	50 ml

- Glutathione (GSH) standard stock solution, 10 mM

GSH	0.3 g
Bidistilled water	0.1 ml

Working Solutions for GSH Assay

- Assay buffer, 12 ml

Assay buffer, 5x	2.4 ml
Distilled water	9.6 ml

- Enzyme solution, 6 U/ml

Glutathione reductase (400 U/ml)	3.8 ml
Assay buffer, 1x	246.2 μ l

- NADPH solution (0.16 mg/ml)

NADPH stock solution (40 mg/ml)	10 ml
Assay buffer, 1x	2.5 ml

- Working mixture

Assay buffer 1x	8 ml
Diluted enzyme solution (6 U/ml)	228 µl
DTNB stock solution (1.5 mg/ml)	228 µl
Vortex-mixed	

- Glutathione standard solution, 50 µM

GSH standard stock solution (10 mM)	1 µl
5 % SSA	199 µl

8.15. Preparation of Working Solutions for Superoxide Dismutase Assay

- WST working solution

WST solution	1 ml
Buffer solution	19 ml

- Enzyme working solution

Enzyme solution	15 ml
Dilution buffer	2.5 ml

8.16. Reagents for Protein Assay by the Bradford Method

- Bradford solution (BioRad, Hercules, CA, USA)
- Bovine serum albumin (BSA) (Sigma, MO, USA), 100 µg/ml
- Distilled water

8.17. Reagents for Paraffin Embedding

- Bouin's fluid

Picric acid (Carlo Erba, Milan, Italy)	750 ml
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37 % Formaldehyde (Carlo Erba, Milan, Italy)	250 ml
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Acetic acid, glacial (Carlo Erba, Milan, Italy)	50 ml
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Whatman No. 1 filter paper

- Absolute alcohol (Carlo Erba, Milan, Italy)
- Xylene (Carlo Erba, Milan, Italy)
- Paraffin wax, 56-58 °C (Bio-Optica, Milan, Italy)
- 4% Paraformaldehyde

37 % Formaldehyde (Carlo Erba, Milan, Italy)	4 ml
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Phosphate buffered solution (PBS)	96 ml
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8.18. Preparation of Reagents for Haematoxylin and Eosin Staining

- Carazzi's haematoxylin

Haematoxylin (Sigma, MO, USA)	0.2 g
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Potassium aluminium sulphate (Carlo Erba, Milan, Italy)	18 g
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Potassium iodate (Carlo Erba, Milan, Italy)	0.04 g
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Glycerol (Sigma, Mo, USA)	40 ml
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Distilled water	160 ml
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- Eosin, 0.5%

Eosin Y (Sigma, MO, USA)	0.5 g
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Bidistilled water	100 ml
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8.19. Reagents for Periodic Acid-Schiff Staining

- Periodic acid, 0.8 % (Applichem, Germany)
- Schiff reagent (Sigma, MO, USA)
- Carazzi's haematoxylin

- Eosin Y (Sigma, MO, USA), 0.5%
- Absolute ethanol (Carlo Erba, Milan, Italy)
- Xylene
- Human salivary amylase

8.20. Reagents for Sudan Black B Staining

- Sudan black B

Sudan black (Sigma, MO, USA)	1 g
70% Ethanol	100 ml
Whatman No 1 filter paper	

(Sudan black B was dissolved in 70% ethanol at 70 °C, with mixing)
- Glycerine medium

Phosphate buffered solution (PBS)	1 ml
Glycerol (Sigma, MO, USA)	1 ml

8.21. Preparation of Reagents for Masson's Trichrome Staining

8.21.1. Ponceau 2R Solution

- Ponceau 2R (Sigma, MO, USA) 0.5 g
- Acid fuchsin (Sigma, MO, USA) 0.5 g
- Glacial acetic acid (Carlo Erba, Milan, Italy) 1 ml
- Distilled water 99 ml

8.21.2. Light Green

- Light green SF(Fluka, Steinheim, Germany) 2 g
- Glacial acetic acid (Carlo Erba, Milan, Italy) 2 ml

- Distilled water 100 ml

8.21.3. Orange G solution (Merck, Germany)

8.22. Preparation of Reagent for Alcian Blue Staining

- Alcian blue, pH 2.5
 - Alcian blue 8GX (Sigma, MO, USA) 0.5 g
 - Glacial acetic acid (Carlo Erba, Milan, Italy), 3% 100 ml
 - Whatman No. 1 paper