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EVALUATION OF ANTIHYPERGLYCAEMIC EFFECTS OF ROOT EXTRACTS OF *Daniella oliveri* (CAESALPINIACEAE) AND *Sarcocephalus latifolius* (RUBIACEAE) IN EXPERIMENTAL DIABETIC RATS

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A THESIS SUBMITTED FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D) IN PHARMACOLOGICAL BIOCHEMISTRY, UNIVERSITY OF NIGERIA, NSUKKA

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FEBRUARY, 2007

CERTIFICATION

Iwueke, Adaku Vivien, a postgraduate student with Registration Number PG/Ph.D/02/33131 in the Department of Biochemistry has satisfactorily completed the requirements for research for the degree of Doctor of Philosophy (Ph.D) in Pharmacological Biochemistry.

The work embodied in this report is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

DR O.F.C. NWODO
(Supervisor)

PROF O. U. NJOKU
(Head of Department)

EXTERNAL EXAMINER 25.01.2009

DEDICATION

To my beloved husband, Chikezie Iwueke and children, Nnedimma, Chidera, Kezie, Onyekachi and the duo, Chinedum and Ngozichukwu

And

My parents, Kezie and Ngozi Eneze as they celebrate their 40 years in marriage.

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ABSTRACT

In the South Eastern part of Nigeria, the decoction of roots of *Daniella oliveri* and *Sarcocephalus latifolius* is used in the management of diabetes mellitus. The antihyperglycaemic potentials of acqueous and alcoholic root extracts of these plants were thus evaluated in normal and alloxan induced diabetic male Sprague-Dawley rats. The air dried roots were shredded and subsequently extracted in both water and ethanol to give *Sarcocephalus latifolius* aqueous extract (SLA), Sarcocephalus/Daniella aqueous extract (SDA) and *Sarcocephalus latifolius* ethanol extract (SLE). The phytochemical analysis of the extracts revealed the presence of flavonoids, saponins, glycosides and tannins. Tannins were absent in SLE. SLA and SDA (250 mg/kg) significantly ($P<0.05$) lowered fasting blood glucose levels of diabetic rats from 311±4.26 mg/dl and 261±3.02 mg/dl respectively to 73±2.23 mg/dl and 65±5.40 mg/dl within 6 hours. In normoglycaemic rats, the same dose did not produce significant ($P<0.05$) change (SLA: 55.50±7.76 to 55.00±8.16 and SDA: 59.00±3.70 to 60.20±5.56) in fasting blood glucose levels even after 12 hours of treatment. Both extracts increased the activities of hepatic hexokinase (HK), glucokinase (GK) and phosphofructokinase (PFK). They also induced increases in hepatic glycogen content of diabetic rats. SLE depressed the activities of GK and PFK, but increased the activity of HK. The results obtained indicated that relative to the control, SLA and SDA significantly reduced the activities of superoxide dismutase and catalase. The extracts also increased The concentration of reduced glutathione (GSH). SDA reduced the level of lipid peroxidation products assayed as malondialdehyde (MDA) in both liver and kidney homogenates of diabetic rats while SLA and SLE showed no effect on MDA relative to the diabetic (SLA, SLE and SDA). The extracts significantly decreased serum triglyceride (from 1.87±0.13 mMol/L to 0.87±0.18, 1.06±0.18 and 1.10±0.26 respectively) and total cholesterol (from 2.80±0.30 to 1.27±0.47, 1.54±0.22 and 1.95±0.21 mMol/L respectively) of diabetic treated rats. When compared to the diabetic control, SDA increased significantly ($P<0.05$) the level of HDL cholesterol by about 50%. Both acute and subacute toxicity testing in mice showed that the median lethal dose (LD_{50}) of each of the extract is greater than 5 g/kg. Moreover, the extracts showed no adverse effect on haematological parameters (WBC and RBC counts, Hb, Hct, MCV and MCH) as well as on serum liver enzyme markers (AST and ALT). However, the histopathological studies indicate mild degeneration of the hepatocytes. This investigation demonstrates that the plant extracts especially SLA and SDA have remarkable antihyperglycaemic effect with accompanying hypolipidaemic and antioxidant properties in diabetic rats. It further indicates clinical relevance in reducing complications of diabetes.

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LIST OF ABBREVIATIONS

AGE	Advanced glycation end product
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ADA	American diabetes association
AST	Aspartate aminotransferase
CDNB	1-Chloro-2,4-dinitrobenzene
DCCTRG	Diabetes control and complications trial research group
DKA	Diabetic ketoacidosis
Dm	Diabetes mellitus
DTT	Dithiotriethol
IDDM	Insulin-Dependent Diabetes Mellitus
FBGL	Fasting blood glucose level
GDM	Glutamic acid decarboxylase
GK	Glucokinase
GSH-P	Glutathione peroxidase
GSSG-Rx	Glutathione reductase
GST	Glutathione s-transferase
HK	Hexokinase
HDL	High-density lipoprotein
HLA	Human leucocyte antigen
HNE	Hydroxynonenal
IAA	Insulin autoantibodies
ICA	Islet cell autoantibodies
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IU/L	International unit per L
IRS	Insulin resistance syndrome
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NEFA	Non-esterified fatty acid

NIDDM	Non-insulin Dependent Diabetes Mellitus
OGTT	Oral glucose tolerance test
GSSG	Oxidized glutathione
PFK	Phosphofructokinase
PKC	Protein kinase C
PUFA	Polyunsaturated fatty acid
GSH	Reduced glutathione
SGOT	Serum glutamate-oxaloacetate transaminase
SGPT	Serum glutamate-pyruvate transaminase
ST2	Streptozotocin
TBA	Thiobarbituric acid
TCA	Trichloroactetic acid
UKPDSG	UK prospective diabetes study group
VLDL	Very low-density lipoprotein

CHAPTER ONE

INTRODUCTION

The mammalian system is composed of individual interacting dynamic units that are always maintained in equilibrium. This equilibrium and the co-ordinated intricate metabolic processes result in a well ordered system. However, any disruption in any aspect of the homeostasis gives rise to a pathological state or even death (Saeed and Al-Dabbali, 2003). A typical example of such is the pathological condition known as diabetes mellitus (Dm). Diabetes mellitus is a very common chronic endocrine/metabolic disorder characterised by improperly regulated metabolism of carbohydrates, proteins and lipids (Tuitoek *et al.*, 1996) by insulin (Tiwari and Rao, 2002). Cutting across every age, sex and socio-economic class, diabetes mellitus (Dm) has been associated with an increased risk for developing premature atherosclerosis (Scoppola *et al.*, 2001; Bierman, 1992).

The incidence of hyperglycaemia in Dm has been found to enhance the generation of reactive oxygen species (ROS) (Ha and Kim, 1999; Wolff *et al.*, 1991); in addition it affects antioxidant reactions catalysed by ROS scavenging enzymes (Uchimura *et al.*, 1999).

The disease is fast attaining an alarming prevalence rate in the developed countries, and is threatening the mere existence and economic survival of the populations in the developing countries (Osadebe *et al.*, 2004). The World Health Organisation has estimated that more than 180 million people worldwide have diabetes and that this figure is likely to double by 2030, if an urgent action is not taken (WHO, 2006; Zimmet, 1999). In 2005, an estimated 1.1 million people died from diabetes (WHO, 2006). Oral hypoglycaemia agents and/or insulin therapy afford relatively effective glycaemic control , but they are not very ideal because of their numerous side effects (Pari and Maheswari, 1999; Rang and Dale, 1991).

In recent years, research interests have shifted to the search for alternative and natural antihyperglycaemic agents, especially from plant sources (Krishna *et al.*, 2004; Pepato *et al.*, 2003).

A combination of the roots of *Sarcocephalus latifolius* (Rubiaceae) and *Daniella oliveri* (Caesalpiniaceae) is used in Igbo-Nigeria folk medicine as a herbal remedy for hyperglycaemia (Ezekwesili, 2004; personal communication). Although, the antihyperglycaemic potential of the leaves of *S. latifolius* has been demonstrated (Gidado *et al.*, 2005), there is no report in literature on the blood sugar lowering activity of the root of the plant in isolation or in combination with *D. oliveri* in both normal and diabetic rats.

1.1 DIABETES MELLITUS

Diabetes mellitus (Dm) can be defined as a group of metabolic disorders with different underlying aetiologies, each characterised by chronic hyperglycaemia owing to overproduction and/or underutilisation of glucose (Ugochukwu and Cobourne, 2003). Chronic hyperglycaemia which is the chief symptom of diabetes mellitus has been found to enhance the generation of reactive oxygen species and hence cause long term tissue damage especially to the nerves and blood vessels leading to diabetic complications.

1.1.1 TYPES OF DIABETES MELLITUS

There are four major classes of diabetes mellitus based on their aetiology and clinical manifestations (Mayfield, 1998).

a) Type I Diabetes mellitus

This was formally known as insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes. It is characterised by the loss of the insulin-producing beta cells of the islets of Langerhans of the panereas leading to a deficiency of insulin. This type of diabetes can affect children or adults but was traditionally termed juvenile diabetes because it represents a majority of cases in children.

b) Type 2 Diabetes mellitus

Type 2 diabetes mellitus – previously known as adult-onset diabetes, maturity-onset diabetes, or non-insulin dependent diabetes mellitus (NIDDM) is due to a combination of defective insulin secretion and insulin resistance or reduced insulin sensitivity (defective responsiveness of tissues to insulin) which almost certainly involves

the insulin receptor in cell membranes. In the early stage, the predominant abnormality is reduced insulin sensitivity, characterised by elevated levels of insulin in the blood. At this stage hyperglycaemia can be reversed by a variety of measures and medication that improve insulin sensitivity or reduced glucose production by the liver. As the disease progresses, the impairment of insulin secretion worsens, and therapeutic replacement of insulin often becomes necessary (Wikipedia, 2007).

c) Gestational diabetes mellitus (GDM)

Gestation diabetes also involves a combination of inadequate insulin secretion and responsiveness, resembling type 2 diabetes mellitus in several respects. It develops during pregnancy and may improve or disappear after delivery. Even though it may be transient, gestational diabetes may damage the health of the foetus or mother, and about 20-50% of women with gestational diabetes develop type 2 diabetes later in life. Gestational diabetes mellitus occurs in about 2-5% of all pregnancies. It is temporary and fully treatable but, if untreated, may cause problems with the pregnancy. These problems may include macrosomia (high birth weight), foetal malfunction and congenital heart disease (American Diabetes Association, 2005).

d) Other types

WHO (1999) proposed a new taxonomy for a type of diabetes mellitus resulting from rare causes and which do not fit into type 1, type 2 or gestational diabetes. Maturity onset diabetes of the young (MODY) is a typical example of this type of diabetes. MODY refers to a collection of different genetically caused forms of diabetes inherited in an autosomal dominant fashion. These forms of diabetes are neither Type 1 nor Type 2, though they are often misdiagnosed by doctors not familiar with these kinds of genetic diabetes (American Diabetes Association, 2005).

Also in this group are:

- Diabetes induced by certain medications or chemicals
- Diabetes caused by other diseases of the pancreas
- Diabetes associated with other hormone conditions

1.1.2 AETIOLOGY OF DIABETES MELLITUS

A number of factors have been implicated in the aetiology of diabetes mellitus, but the consensus is that the aetiology is a multifactorial interaction of environmental and genetic factors.

- **Environmental factors:** The environmental factors implicated in type 1 Dm could be viral, diet and stress. Several viruses like the coxsackie B4, retroviruses, rubella and cytomegalovirus have been implicated as one of the triggers of type 1 diabetes mellitus in genetically susceptible mice but not in those with a different genetic profile (Knip and Akerblom, 1999). Viruses may initiate immune-mediated damage to beta cells by direct destruction, by the generation of cytokines that can damage the beta cells or by molecular mimicry. It has been demonstrated that the autoimmune reactions are triggered by a similarity between a coxsackie viral protein and islet cell autoantibodies (ICA), insulin autoantibodies (IAA) or glutamic acid decarboxylase autoantibodies (GAD) (a protein in the outer surface of the pancreas) (Gottsater *et al.*, 1995). Studies have also shown that 60% of newly diagnosed diabetics had antibodies to the coxsackie virus (Landin-Olsson *et al.*, 1999 and Littorin *et al.*, 1999).

Dietary factors may also be involved in type 1 diabetes mellitus; consumption of high levels of N-nitroso compounds by parents at the time of conception of a progeny and by the progeny at infancy has been shown to cause an increased risk of developing type 1 diabetes mellitus (Virtanen and Aro, 1994).

Early introduction to cow's milk and short duration of breastfeeding to infants has a predisposing effect to type 1 diabetes mellitus (Kostraba *et al.*, 1993).

Environmental factors implicated in the aetiology of type 2 diabetes mellitus include lifestyle, malnutrition *in utero*, age and ethnicity.

Lifestyle – Overeating, obesity and sedentary lifestyle are high risk factors for type 2 diabetes mellitus. Studies have shown that immigrant Indians and Africans in the United Kingdom and USA and Singapore have a high incidence of diabetes compared to indigenes (King *et al.*, 1998). There is also increasing prevalence of developing diabetes in urban populations which has increased from 5.2% in 1986 to 11.6% in 1996 (Amos *et al.*, 1997). Central obesity (fat concentrated around the waist in relation to abdominal organs) is known to predispose for insulin resistance, possibly due to its secretion of

adipokines which impair glucose tolerance (American Diabetes Association, 2005). It has also been demonstrated that improved economic standard of living resulting in higher caloric/fat intake and increased sedentary lifestyle play a key role in the development of type 2 diabetes mellitus (Bennion and Grundy, 1977; WHO, 1994; Wokoma, 2002).

- **Malnutrition *in utero*:** Retrospective analysis has shown an inverse relationship between weight at birth and type 2 diabetes mellitus in late adulthood. Maternal malnutrition has been associated with deficient foetal nutrition and hence placental deficiency (Marles and Farnsworth, 1994; Bell and Hockaday, 1992). Foetal malnutrition can also lead to the development of insulin resistance which is a step to overt diabetes (Philips, 1996).

- **Genetic Factors:** In addition to environmental factors, genetic factors also play a major role in the aetiology of both type 1 and type 2 diabetes mellitus. There is a genetic element in individual susceptibility to some of the triggers of diabetes mellitus earlier mentioned, which has been traced to particular HLA genotypes. However, the majority of those who are genetically predisposed do not develop type 1 diabetes mellitus. On the other hand, genetic predisposition for type 2 diabetes mellitus is stronger than type 1. People with first-degree relatives with type 2 have a much higher risk of developing type 2, increasing with the number of those relatives.

1.1.3 COMMON SYMPTOMS OF DIABETES MELLITUS

The classical triad of diabetes symptoms include:

- Polyuria (frequent urination)
- Polydipsia (increased thirst and consequent increased fluid intake).
- Polyphagia (increased appetite).

The symptoms may develop quite fast in type 1, particularly in children. These symptoms may be subtle or completely absent as well as developing much more slowly in type 2 diabetes mellitus (American Diabetes Association, 2005).

In type 1 diabetes there may also be weight loss (despite normal or increased eating) and irreducible fatigue.

When the blood glucose concentration is above the renal threshold, reabsorption of glucose in the proximal renal tubule is incomplete and part of the glucose remains in

the urine giving rise to glycosuria (Nelson and Cox, 2005). Prolonged high blood glucose level causes glucose absorption and hence changes in the shape of the eye lens, leading to vision changes. Blurred vision is therefore a common symptom of diabetes mellitus. Diabetic patients may also present with diabetic ketoacidosis (DKA), which is an extreme state of metabolic dysregulation eventually characterised by the smell of acetone on the patients breath. This was clearly illustrated in Fig. 1.1 and 1.2.

1.1.4 RISK FACTOR FOR DIABETES MELLITUS

It has been observed that hypertension and diabetes share predisposing risk factors or rather each is a risk factor for the other (Reaven, 1988; Laakso, 1999; Pyorala *et al.*, 1987). These factors which are collectively referred to as 'metabolic syndrome' include obesity, impaired glucose tolerance (IGT), dyslipidaemia (elevated serum cholesterol or triglyceride levels), insulin resistance and hyperinsulinaemia (Janus *et al.*, 2000; Garg and Grundy, 1990) as well as decreased HDL-cholesterol. Metabolic syndromes or insulin resistance syndromes (IRS) are those metabolic changes that are associated with insulin resistance. It is known that about 25% of non-diabetic adults manifest this syndrome (Reaven, 1995).

Insulin resistance or sensitivity is defined as the reduced ability of body tissues to respond to insulin. Although type 2 diabetes mellitus results from both insulin resistance and insufficient insulin secretion (Defronzo *et al.*, 1992), insulin resistance is probably the primary defect because those prone to develop type 2 diabetes mellitus exhibit insulin resistance before the development of glucose intolerance (Erikkson *et al.*, 1989; Gulli *et al.*, 1992; Weyer *et al.*, 1999; Weyer., 2000).

There is considerable evidence in experimental animals that saturated fat in diet may lead to insulin resistance (Vessby, 1995) while ω-3 polyunsaturated fatty acid (PUFA) improves insulin sensitivity (Vessby, 1995; Storlien *et al.*, 1996). The mechanism is related to a change in membrane fluidity affecting processes around the insulin receptor and the recruitment of glucose transporters to the membrane.

Elevated concentration of non-esterified fatty acid (NEFA) is a common metabolic mechanism in insulin resistance (Reed *et al.*, 1999; Frayn *et al.*, 1996).

1.1.5 DIAGNOSIS OF DIABETES MELLITUS

The diagnoses of type 1 diabetes and many cases of type 2 diabetes mellitus are usually prompted by recent onset symptoms of excessive urination (polyuria) and excessive thirst (polydipsia), often accompanied by weight loss. These symptoms typically worsen over days to weeks and about 25% of people with type 1 diabetes mellitus have developed some degrees of diabetic ketoacidosis by the time the diabetes is recognised (WHO, 1999). The diagnosis of other types of diabetes is usually made in such other ways as:

i) Ordinary health screening: This is recommended for many people at various stages in life, and for those with any of several risk factors. For instance universal screening is recommended for adults at age 40 and above, obesed subjects, people with family history of diabetes, high risk ethnicity (Mestizo, native American, African American, Pacific Islanders).

ii) The presence of new signs and symptoms due to the diabetes, such as vision changes or unexplainable fatigue.

iii) Detection of hyperglycaemia while investigating a complication of other disease conditions.

Diabetes mellitus is characterised by recurrent or persistent hyperglycaemia, and is diagnosed by demonstrating any of the following (WHO, 1999):

- Fasting plasma glucose level at or above 126 mg/dl (7.0 mmol/l)
- Plasma glucose at or above 200 mg/dl or 11.1 mmol/l two hours after a 75 g oral glucose load (glucose tolerance test, OGTT)
- Random plasma glucose at or above 200 mg/dl or 11.2 mmol/l.

A positive result should be confirmed by another of the above-listed methods on a different day, unless there is no doubt as to the presence of significantly elevated glucose levels. By current definition, two fasting glucose measurements above 126 mg/dl or 7.0 mmol/l is considered diagnostic for diabetes mellitus.

Patients with fasting plasma glucose between 6.1 and 7.0 mmol/l (110 and 125 mg/dl) are considered to have "impaired fasting glucose" (IFG) and patients with plasma glucose at or above 140 mg/dl or 7.8 mmol/l after glucose tolerance test are considered to

have "impaired glucose tolerance" (IGT). 'Prediabetes' is either IFG or IGT; the latter in particular is a major risk factor for progression to full-blown diabetes mellitus.

While not used for diagnosis, an elevated glycosylated haemoglobin (HbA1c) of 6.0% or .higher (the 2003 revised US standard) is considered abnormal by most laboratories. HbA1c is primarily used as a treatment-tracking test reflecting average blood glucose levels over the preceding 90 days (approximately). People with diabetes who have HbA1c levels with the range of 6.5 - <7.0% have significantly lower incidence of complications from diabetes (Genuth, 2006)..

1.1.6 COMPLICATIONS OF DIABETES MELLITUS

The major implication of diabetes is that glucose accumulates in the blood instead of inside the cell where it could be metabolised for energy production in the system. This abnormality when uncontrolled gives rise to a lot of microvascular and macrovascular events. The compliments of diabetes are far less common and less severe in people who have well controlled blood sugar levels (DCCTRG, 1995; Nathan *et al.*, 2005). In fact, the better the control, the lower the risk of complications. Acute complications of diabetes mellitus (Dm) include the following:

- Diabetes ketoacidosis (DKA) which results from rapid mobilisation of fatty acid reserves, leads to ketone body formation and subsequent ionisation of the ketone bodies-producing acids. This is much more common in type 1 diabetes mellitus than in type 2 diabetes mellitus (United Kingdom Prospective Diabetes Study Group, 1995).
- Non ketotic hyperosmolar coma: This manifests more in patients with very high blood glucose levels (>300 mg/dl). Owing to excess glucose in the blood, the body tends to withdraw water osmotically from the cells into the blood in order to flush out the glucose. The kidneys will also be 'dumping' glucose into the urine, resulting in concomitant loss of water and hence increase in blood osmolarity causing dehydration.
- Hypoglycaemia (abnormally-low blood glucose): This may develop if the glucose intake does not cover the treatment. It may also lead to loss of consciousness, coma and/or seizures or brain damage.

- Amputation: Patients with poorly controlled diabetes and having wounds often heal slowly, even from, small cuts. This can result in infection and subsequent amputation.

Chronic complications include the following:

- Diabetic retinopathy: The result is the growth of friable and poor quality new blood vessels in the retina as well as macular oedema, which can lead to severe vision loss or blindness. Diabetes retinopathy is the most frequent cause of new cases of blindness among adults aged 20-70 years (Fong *et al.*, 2003). Studies have established that glycaemic blood pressure control can prevent and delay the progression of diabetic retinopathy in patients with diabetes (Diabetes Control and Complication Trial, 1993; UKPDS, 1998).
- Diabetic nephropathy: Damage to the kidney(s) is yet another chronic complication of diabetes mellitus (Dm). This can lead to chronic renal failure, and eventually requiring dialysis. Diabetes mellitus has become the most common cause of adult kidney failure worldwide in the developed world (American Diabetes Association, 2003). Recent studies have demonstrated that the onset and cause of diabetic nephropathy can be ameliorated to a very significant degree by several interventions, if insulated at a point very early in the course of the development of this complication (ADA, 2003).
- Diabetic neuropathy is a descriptive term indicating many types or syndromes of neural damage. It is the most common chronic complication of diabetes and is responsible for a large amount of morbidity. This progresses through initial biochemical abnormalities in the nerve, like accumulation of sorbitol, depletion of myo-inositol to impairment in nerve conduction and finally neuropathy.

1.1.7 PATHOGENESIS OF LONGTERM COMPLICATIONS OF DIABETES

Hyperglycaemia and its immediate biochemical sequence induce hyperglycaemia damage (Fig. 1.1) by ultimately acting via increased superoxide (reactive oxygen species) production by the mitochondrial electron transport chain. Under normal conditions

glucose is metabolised through the glycolytic pathway. An increase in intracellular glucose will lead to an increase in four pathways, namely:

1) Formation of advanced glycosylation end products (AGEs): Protein non-enzymatic glycosylation and formation of AGEs can crosslink with collagen and damage basement membranes (Bm) and also bind to receptors on many cells e.g. endothelial cells, macrophages and monocytes. These can lead to:-
   (a) Monocyte emigration, increased endothelial permeability, release of cytokines and increased synthesis of extracellular matrix (ECM).
   (b) Directly pathogenic to tissues e.g. nerves, kidney and retina.
2) The flux of glucose to sorbitol via the sorbitol (polyol) pathway: Glucose is metabolised by aldose reductase (AR) and sorbitol dehydrogenase (SDH) which is accompanied by increased oxidation of NADPH to $NADP^+$ and increased reduction of NAD^+ to NADH (Gabbay, 1975 and Oates, 2002). This pathway may impair endothelial function through three different mechanisms:
   (a) Accumulated sorbitol and fructose will lead to:
      (i) Increased intracellular osmolarity or osmotic stress with influx of water and osmotic cell injury.
      (ii) Decreased myoinositol leading to damaged schwann cells and retinal capillary pericytes resulting in neuropathy and retinopathy.
      (iii) Osmotic swelling of lens of eyes resulting in opacity of the lens - cateract.
   (b) Increase in cytosolic $NADH/NAD^+$ ratio resulting in a redox imbalance that resembles that which occurs in tissue hypoxia and therefore is termed hyperglycaemic pseudohypoxia (Williamson *et al.*, 1993).
   (c) The redox imbalance favours the accumulation of triose phosphates which increases the formation of methylglyoxal and AGEs and enhances oxidative stress which can be exacerbated by NADPH-deficiency-induced depletion of reduced glutathione.
3. Increased flux through the hexosamine pathway leading to an increase in fructose-6-phosphate (F-6-P). F-6-P is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT) and

subsequently to uridyldiphosphate-N-acetylglucosamine (UDPNAG). Incorporation of UDPNAG into serine and threonine residues of transcription factors leads to multiple effects on gene expression such as:

(a) Increased transcription of transforming growth factor B1 (TGFB1) which leads to increase vascular permeability and angiogenesis.

(b) Increased transcription of platelet activating factor inhibitor – 1 (PAI-1) leading to decrease fibrinolysis resulting in vascular occlusion.

4. Activation of protein kinase C (PKC) via denovo synthesis of diacylglycerol (DAG). PKC is synthesised denovo from DAG by a stepwise acylation of glycolytic intermediates, dihydroxyacetene phosphate (DHAP), glycerol-3-phosphate (G-3-P) and phosphatidic acid (PA). PKC activation has multiple effects on gene expression which include:

(a) Decreasing endothelial nitric oxide synthase (NOS) activity and or increasing endothelin-1 (ET-1) synthesis leading to blood flow abnormalities.

(b) Induction of vascular endothelial growth factor (VEGF) resulting in increased vascular permeability and angiogenesis.

(c) Increased fibroblast growth factor (FGF) leading to increased synthesis of collagen and fibronectin resulting in capillary occlusion.

(d) Increased expression of platelet activator inhibitor-1 (PAI-1) resulting in impaired fibrinolysis.

In addition, hyperglycaemia may lead to increase reactive oxygen species by activation of several NADPH oxidases, inactivation and reduced expression of the antioxidant enzymes catalase and superoxide dismutase (SOD) or uncoupling of endothelial nitric oxide synthase (NOS) resulting in oxidative stress.

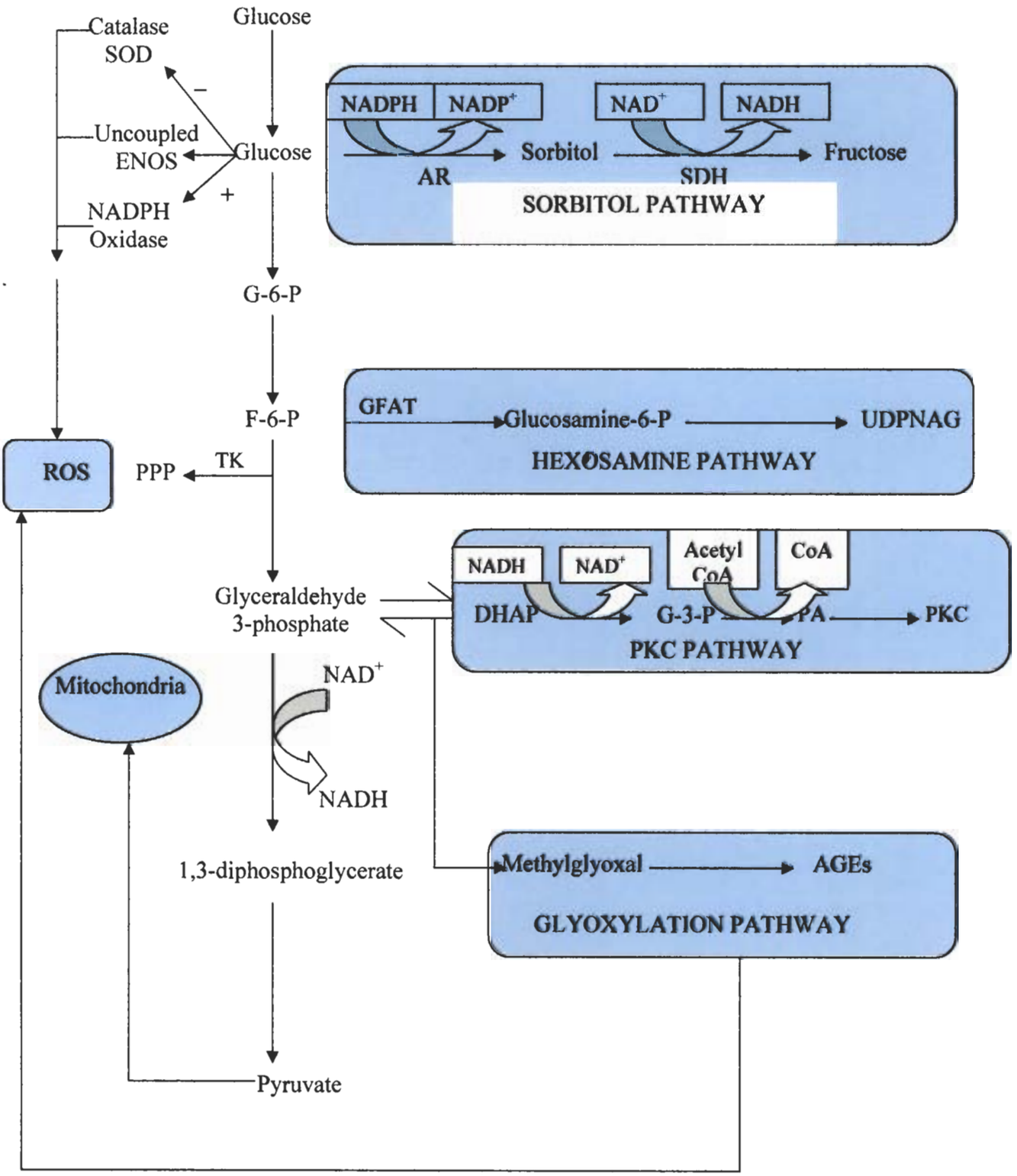


Fig. 1.1: Mechanisms by which hyperglycaemia and its immediate biochemical sequences or sequelae induce damage

Another hyperglycamia dependent metabolic abnormality that also play a role in the development of nephropathy is the polyols (Fig. 2). The tissues of the lens, peripheral nerves, kidneys and blood vessels do not require insulin for glucose transport and as such hyperglycaemia leads to an increase in intracellular glucose. These tissues contain two enzymes that constitute the polyol pathway – aldose reductase (AR) which uses NADPH to reduce glucose to sorbitol and sorbitol dehydrogenase (SDH) which uses NAD^+ to oxidase sorbitol to fructose. AR has a high Km for glucose. Flux through the polyol pathway is enhanced in hyperglycaemia. The resultant increase in tissue sorbitol contributes to the microvascular complications of diabetes.

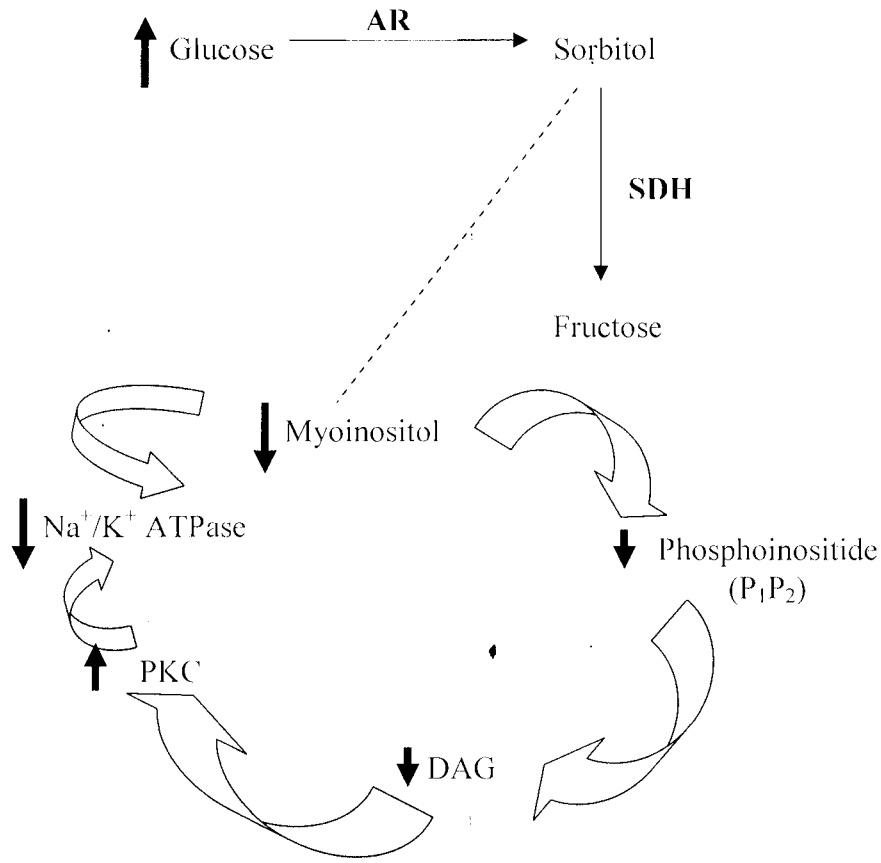


Fig. 1.2: The sorbitol pathway and effects on myoinositol in the pathogenesis of diabetic complications

1.1.8 TREATMENT/PREVENTION OF DIABETES MELLITUS

Diabetes mellitus is currently a chronic disease without a cure and medical emphasis must necessarily be on managing/avoiding possible short-term as well as lon-term diabetes related problems. Given the associated higher risks of cardiovascular disease, lifestyle modifications should be undertaken to control blood pressure and cholesterol by exercising, consuming appropriate diet and avoiding smoking (Adler *et al.*, 2000).

Currently type 1 diabetes mellitus can be treated only with insulin, with careful monitoring of blood glucose levels. Apart from the common subcutaneous injection, it is also possible to deliver insulin by a pump (Wikipedia, 2007) which allows continous infusion of insulin 24 hours a day. It is also possible to deliver insulin with an inhaled powder. Type 1 diabetes mellitus treatment must be continued indefinitely. Type 2 diabetes mellitus is usually first treated by attempts to increase physical activity, decrease carbohydrate intake and lose some weight. These can restore insulin sensitivity and even achieve satisfactory glucose control, sometimes for years. However, the underlying tendency to insulin resistance is not lost and so adherence to diet and exercise must continue. The usual next step in type 2 diabetes mellitus treatment (if necessary) is treatment with oral administration of antidiabetic drugs which can still be used to improve insulin production (e.g. sulfonylureas), to regulate inappropriate release of glucose by the liver and attenuate insulin resistance to some extent (metformin:biguanides), and attenuate insulin resistance (e.g. thiazolidinediones).

Although there is no cure for diabetes mellitus, controlled studies have shown that intensive glycaemia control goes a long way to reduce the associated morbidity and risk for complications (Stratton *et al.*, 2000; DCCT, 1993; Ohkubo *et al.*, 1995).

Since the actual mechanism by which type 1 diabetes mellitus develops is not known, there are no preventive measures available for type 1 diabetes mellitus. Studies have attributed the protective effect of breast feeding on the development of type 1 diabetes mellitus (Kostraba *et al.*, 1999).

Lindström *et al.* (2006) and Knowler *et al.* (2002), have demonstrated that type 2 diabetes mellitus risk can be reduced in many cases by making changes in diets and increasing physical activity. This is also supported by American Diabetes Association

(2006) which recommends that good amount of fibre and whole grain may reduce the risk of type 2 diabetes mellitus. They maintained also that there is not enough consistent evidence that eating foods of low glycaemic index is helpful, but nutritious, low glycaemia-index foods are encouraged.

Recent reports showed delayed progression to diabetes in predisposed patients through the use of metformin (Knowler *et al.*, 2002), resiglitazone (Gerstein *et al.*, 2006) and Valsartan (Kjeldsen *et al.*, 2006).

Breastfeeding might also be correlated with the prevention of type 2 diabetes mellitus in mothers (Stuebe *et al.*, 2005).

1.1.9 PREVALENCE OF DIABETES

Diabetes is a highly prevalent chronic disease. Survey conducted between 1988 and 1994 estimated the prevalence of diagnosed and undiagnosed diabetes in people aged 20 and above at 15.6 million (Harris *et al.*, 1998). Of these people, about 90-95% have type 2 diabetes mellitus with a higher prevalence seen among Native Americans and Americans of African, Mexican and Japanese descent (Harris *et al.*, 1995; White *et al.*, 2003). It has been postulated that, with the growing obesity problem, diabetes will become an even more pervasive threat (Mokad *et al.*, 2001).

In Nigeria, diabetes mellitus is the most common endocrine disease with a prevalence of about 2.2% (Adebisi *et al.*, 2003; Alebiosu and Kadiru, 2003), and so constitutes a major health problem (Chukwak *et al.*, 2002; Fabiyi *et al.*, 2002). In 2006, according to the World Health Organisation (WHO, 2006), at least 180 million people worldwide suffer from diabetes. Its incidence is increasing rapidly, and it is estimated that by the year 2030, this number will double. The increase in incidence of diabetes in developing countries follows the trend of urbanisation and lifestyle changes, perhaps most importantly in 'Western-style diet', suggesting an environmental (dietary) effect.

1.1.10 ALTERNATIVE THERAPY

In addition to the orthodox hypoglycaemic therapies, there are also many plants and plant extracts which possess marked hypoglycaemic activity. From ancient times such materials have been used for the treatment of diabetes mellitus and still find

extensive use in traditional medicine worldwide. There have been several comprehensive reviews covering plants that possess hypoglycaemic principles (Oliver-Beaver and Zahnd, 1979, Ivorra *et al.*, 1989). Several reports have also shown a good number of plants with antidiabetic activity as well as their constituents, worldwide usage, and the chemical structures of their phytochemicals (Atta-Ur-Rahman and Zaman, 1989; Hamda *et al.*, 1989).

To mention but a few, the hypoglycaemic activities of *Gymena sylvestre* leaf (Shanmugasundaram *et al.*, 1990), *Trigonella foecum-graecium* seed (Sharma *et al.*, 1990), *Musa sapientum* (Pari and Maheswari, 1999), *Loranthus micranthus* (mistilloe) (Osadebe *et al.*, 2004) have been demonstrated in different animals models. Other studies by Ugochukwu and Babady (2003), Pushparaj *et al.* (2000), Grover *et al.* (2002) and Steven *et al.* (2004) described the antidiabetic properties of *Gongronema latifolium*, *Averrhoa bilinibi*, *Brassica juncea* seeds and *Vernonia amygdalina* leaf extracts respectively using animal models. There have also been successful clinical trials of some of these antidiabetic plants (Margollin *et al.*, 1998; Vuksan *et al.*, 2002).

It is generally agreed that medicinal plants and their products are relatively safer than synthetic drugs and offer a more wholistic approach to treatment. Perhaps the way nature made it, medicinal plant constituents mimic more closely the natural constitution of the animal (human) system (Evans, 2005).

About 80% of the world's population relies on herbal medicines, and governments of Third World countries, unable to sustain a complete coverage with Western-type drugs, have encouraged the rational development of traditional treatments (Evans, 2005). Presently, the World Health Organisation is taking an official interest in such developments in the bid to make health care available for all.

1.1.11 EXPERIMENTAL DIABETES

Diabetes mellitus is induced in experimental animals using chemicals which selectively destroy pancreatic β-cells. This is quite convenient and simple to use. The most common diabetogenic agents whose cytoxic mechanisms have been intensively elucidated are alloxan and streptozotocin (Szkudelski, 2001). Alloxan (2,4,5,6-tetraoxypyrimidine, 5,6-dioxyuracil) was first described by Brugnatelli in 1818 but

Wohler and Liebig first called it alloxan and described its synthesis (Lenzen and Panten. 1988). The diabetogenic properties of this substance were first reported by Dunn *et al.* (1943) in rabbits. Hence it has been utilised in inducing type 1 diabetes mellitus in animal model.

Alloxan exerts its diabetogenic action when administered parenterally (intravenous (i.v.), intraperitoneal (i.p.) or subcutaneously.). The dose required for diabetes induction depends on the animal species, route of administration and nutritional status. Human islets are considerably more resistant to alloxan than those of other animals (Eizirik *et al.*, 1994).

The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg/kg body weight (Gruppuso *et al.*, 1990; Boylom *et al.*, 1992). This dose must be doubled or tripled if it has to be given via other routes (Katsumata *et al.*, 1992; 1993). Fasted animals are more susceptible to alloxan (Katsumata *et al.*, 1992; Szkudelski *et al.*, 1998) while increased blood glucose provides partial protection (Bansal *et al.*, 1980 and Szkudelski *et al.*, 1998).

Using isolated islets and perfused rat pancreas respectively, Weaver *et al.* (1978) and Kliber *et al.* (1996) demonstrated that alloxan evokes a sudden rise in insulin secretion just after alloxan administration.

The alloxan induced insulin release which lasts shortly is then followed by complete suppression of the islet response to glucose (Kliber *et al.*, 1996). This action of alloxan in the pancreas is preceded by its rapid uptake by the β-cells (Boquist et al., 1983). There is finally formation of reactive oxygen species (ROS) that reduce alloxan to dialuric acid which forms a redox cycle with alloxan. The ROS formed actually target the DNA of pancreatic islets (Takasu *et al.*, 1991a). Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesised by Streptomyces achromogens and is used to induce both type 1 and type 2 diabetes mellitus. The frequently used single intravenous dose in adult rats to induce type 1 Dm (IDDM) is between the dose range of 40 to 60 mg/kg body weight (Ganda *et al.*, 1976; higher doses may be used. STZ is also efficacious when given i.p. and in multiple low doses. Type 2 can be induced in rats by an intravenous or intraperitoneal treatment with 100 mg STZ per kg body weight STZ on the day of birth (Portha *et al.*, 1974) or at 8 – 10 weeks of age and thereafter. The treated

rats manifest mild basal hyperglycaemia, an impaired glucose tolerance and a loss of β-cell sensitivity to glucose (Giroix *et al.*, 1983). West *et al.* (1996) reported that STZ at first abolished the β-cell response to glucose with a manifested hyperglycaemia and drop in blood insulin. Temporary return of responsiveness which then appears is followed by its permanent loss and the cells are damaged.

STZ is taken up by pancreatic β-cells via glucose transporter-GLUT 2. Recent studies have also proved that the main reason for the STZ induced β-cell death is alkylation of DNA (Delaney *et al.*, 1995; Elsner *et al.*, 2000). The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O^6 position of guanine. STZ was found to generate reactive oxygen species which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu *et al.*, 1991a; Bedoya *et al.*, 1996).

1.2 OXIDATIVE STRESS: ROLE IN DISEASE

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Johansen *et al.*, 2005).

Oxidative stress can also be referred to as the imbalance in favour of the generation of reactive oxygen species against the activity of the antioxidant defences (Aruoma, 1999). It has been observed by Vaya and Aviram (2001) that the pathogenesis of degenerative disease involves the shift in balance of the above mechanism. Some damages caused by oxidative stress include: oxidative lipid damage, oxidative damage to proteins and oxidative DNA damage (Bradley and Minn, 1992; Afanas'er, 1985) and even cell death.

Oxidative stress is associated with a lot of diseases such as cancer, atherosclerosis, diabetes, rheumatoids arthritis, Parkinson's disease, malaria and HIV infection/AIDS (Aruoma, 1993; Aruoma and Halliwell, 1998). The nature and extent of oxidative stress injury depends on the availability of weak bonds (such as double bonds) on the molecules with which the radicals will react. They are also affected by the availability of metal ions/catalysts such as iron and copper that will abstract electrons from reactive oxygen species. The presence of antioxidant defenses, the expression of

certain proteins (DNA repair enzymes) also affect them. The mechanism of oxidative stress induction through drugs, Fenton chemistry, trauma or enzymes activation. It can also be induced through the delocalisation of the singlet electron on the radicals (Friedberg *et al.*, 1995; Sancar, 1996).

1.2.1 OXIDATIVE LIPID DAMAGE (LIPID PEROXIDATION)

In the presence of the free radicals such as hydroxyl radicals, lipids undergo peroxidation. Lipid peroxidation involves the direct reaction of lipids with free radical intermediates and semistable peroxides. This auto-oxidation of lipids initiated by free radicals proceeds through a complex process involving rearrangement and destruction of double bonds in polyunsaturated fatty acids (PUFA) of membranes (Ceballos, 2000).

Lipid peroxidation contributes to the development of numerous diseases such as diabetes, cardiovascular diseases, and causes impairment of membrane functions (Orrenius *et al.*, 1989; Bast, 1993). The more unsaturated a lipid is, the more prone it is to peroxidation. End products of lipid peroxidation such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) can cause protein and DNA damage (Aruoma, 1999). Lipid peroxidation involves three major steps: initiation, propagation and termination. The initiation of lipid peroxidation involves the abstraction of hydrogen atom a double bond in the lipid by free radical. The products of initiation phase could undergo molecular rearrangement to form conjugated dienes. The propagation phase involves the abstraction of another hydrogen atom from another unsaturated lipid molecule by the lipid-peroxyl radical formed by the reaction of triplet oxygen with the initiation product at the initiation phase. This reaction leads to the formation of lipid peroxides and lipid hydroperoxides. The termination phase is accompanied by degradation or decomposition. The double bond then cleaves, leading to the formation of aldehyde such as malondialdehyde, 4-hydroxynonental or hexanal (Esterbauer *et al.*, 1992; Esterbauer, 1995). Most lipid peroxidation reactions are initiated by the hydroxyl radical which initiates a chain reaction (at very low concentration) involving triplet oxygen that leads to lipid peroxidation.

$OH^{\cdot} + RH \longrightarrow R^{\cdot} + H_2O$ ..... (1.1)

$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$ .....(1.2)

$ROO^{\cdot} + RH \longrightarrow R^{\cdot} + ROOH$ .....(1.3)

The lipid hydroperoxide is unstable and in the presence of a metal catalyst such as iron forms a reactive alkoxy radical (Reaction 4):

$ROOH + Fe^{2+} \longrightarrow OH^{-} + RO^{\cdot} + Fe^{3+}$ .....(1.4)

The above reaction amplifies the chain reaction involved in lipid peroxidation. Lipid peroxidation reactions are terminated when the peroxy radicals cross-link or form conjugated products (Reactions 5 – 7):

$R^{\cdot} + R^{\cdot} \longrightarrow R - R$ .....(1.5)

$R^{\cdot} + ROO^{\cdot} \longrightarrow ROOR$ .....(1.6)

$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2$ .....(1.7)

1.2.2 OXIDATIVE STRESS IN THE LIVER

The liver is a multi-lobed organ in the abdomen saddled with the responsibility of metabolising toxic substances in the body. The metabolic by-products in the liver, expected to be non toxic, sometimes become more toxic than the initial substance. This contributes to the development of liver diseases (Ashak *et al.*, 1991). The major functions of the liver can be detrimentally altered by liver injury resulting from acute or chronic exposure to toxicants.

The by-products of metabolism of toxic substances in the liver include oxygen-containing molecules that destroy vital cell components through oxidation (Fernandez-Checa *et al.*, 1997). The liver contains considerable amount of polyunsaturated fatty acids that are prone to damage by free radicals, through oxidative stress (Guo *et al.*, 2003).

Liver injury and altered metabolism in liver might be caused by inflammation-promoting substances called cytokines. For instance a cytokine, TNF-α (tumour necrosis factor alpha) causes cell damage by promoting oxidative stress in the mitochondria. This may therefore play a role in the development of liver diseases resulting from the intake of toxic substances, particularly if antioxidant defences are inadequate. Liver cells depleted

of mitochondrial glutathione, for instance, become more susceptible to oxidative stress. This is seen in alterations of mitochondrial structure and function, and a significant decrease in energy production (Fernandez-Checa, 1997).

The protective effect of antioxidants on oxidative liver injury has been reported (Naziroglu *et al.*, 2004; Ozdil *et al.*, 2004; Fernandez-Checa, 1997; Colell *et al.*, 1997; Garcia-Ruiz *et al.*, 1994; Fernandez-Checa *et al.*, 1993).

1.2.3 OXIDATIVE STRESS IN THE KIDNEY

Renal excretion is a major route through which toxic substances are removed from the body. Increased flux of substances into the kidney is associated with various changes and oxidative stress in the kidney (Braunlich *et al.*, 1994).

Hyperglycaemia is known to cause oxidative stress in the kidney via the formation of free radicals (Koya *et al.*, 2003). Hyperglycaemia induced increases in glucose antioxidation, protein glycation and subsequent oxidative degradation of glycated protein lead to enhanced production of reactive oxygen species (Kakkar *et al.*, 1997).

Oxidative renal disorder has been widely associated with diabetic conditions (Koya *et al.*, 2003; Limaye *et al.*, 2003; Ohkubo *et al.*, 1995; Kakkar *et al.*, 1997). This has been linked to hyperglycaemia (Brownlee, 2001; Kakkar *et al.*, 1997; Ha and Kim,1999). Oxidative stress has been suggested extensively as a potential mechanism for diabetic nephropathy since it promotes the formation of advanced glycation end products (AGE), and protein kinase C (PKC)/mitogen-activated protein kinase (MAPK) activation (Brownlee, 2001; Ha and Kim, 1999).

Indeed, the involvement of oxidative stress in diabetes has been indicated in the presence of lipid peroxidation products and 8-hydroxy deoxyguanosine in the kidney of streptozotocin induced diabetic rats (Horie *et al.*, 1997; Ha *et al.*, 1994). Protein oxidation products have also been found (Limaye *et al.*, 2003).

In diabetic situation, antioxidants have been found to reduce the oxidative stress induced in the kidney (Anyaneyulu and Chopra, 2004; Kedziora-Kornatowska *et al.*, 2003; Baydas *et al.*, 2002).

1.3 NATURAL DEFENCE AGAINST OXIDATIVE STRESS AND ANTIOXIDANTS

The body defences against ROS-induced damage can be eliminated by the number of enzymatic and non enzymatic antioxidant mechanisms. Antioxidants can be defined as substances which when present in low concentration compared to those of oxidisable substrate, significantly delay or inhibit oxidation of that substrate. They are complex and diverse group of molecules that protect key biological sites from oxidative damage (Halliwell, 1990; Gutteridge, 1994).

The enzymatic antioxidant systems include superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-PR) and glutathione reductase (GSSG-RX). Examples of the non enzymatic antioxidants are vitamins A, C and E; glutathione (GSH), lipoic acid, carotenoids, trace elements like copper, zinc and selenium; coenzymes Q10 (CoQ10) and cofactors like folic acid, uric acid, albumin and vitamins B1, B2, B6 and B12 (Johansen *et al.*, 2005).

Antioxidants can act by any of the following ways:

- By removing oxygen or decreasing local oxygen concentration
- By removing catalytic metal ions
- By inhibiting key reactive oxygen species or
- By directly scavenging free radicals like hydroxyl, alkoxyl and peroxyl species
- Breaking the chain of an initiated sequence
- Quenching or scavenging singlet oxygen

Antioxidants that protect lipids against free radical damage may actually accelerate damage to other molecules such as DNA, carbohydrates and protein under certain conditions (Gutteridge, 1994; Aruoma, 1999).

Decreased levels of these essential antioxidants in the circulation have been associated with increased risk of degenerative diseases such as diabetes, cancer and ageing (Sies, 1999).

In effect, a possible way of preventing ROS-mediated cellular injury is to augment endogenous oxidative defence system through dietary intake of antioxidants like the vitamins. Vitamin C acts as a strong antioxidants in plasma and presents a synergistic

effect with vitamin E (Kaneto *et al.*, 1999). Studies have also revealed that vitamin E intake improved several aspects of diabetes pathology. Vitamins break chain length of lipid radicals and scavenge the peroxy radicals thus formed (Vaya and Aviram, 2001).

1.3.1 SUPEROXIDE DISMUTASE (SOD) [EC 1.15.1.1]

Superoxide dismutase formerly known and identified with such names as erythrocuprein, indophenol oxidase and tetrazolium oxidase catalyses the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen as shown below (Ukeda *et al.*, 1997).

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{.....(1.8)}$$

Superoxide dismutases are ubiquitous metalloproteins that play a major role in living cells and have been widely used as pharmacological tools in the study of pathophysiological mechanisms.

Superoxide dismutases (SODs) are present in all aerobic organisms and most subcellular compartments that generate activated oxygen. It is therefore assumed that SOD has a central role in the defence against oxidative stress (Beyer *et al.*, 1991; Bowler *et al.*, 1992; Scandalis, 1993). There are three isozymes of superoxide dismutase classified on the basis of the metal cofactor; the MN-SOD found in the mitochondria of eukaryotic cells; the Cu/Zn-SOD found in the cytosol and chloroplast and the Fe-SOD found in the chloroplast (Bowler *et al.*, 1992). The Mn-SOD and Fe-SOD are also found in prokaryotes and eukaryotic algae: It has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stress.

Maritini *et al.* (2003) reported that diabetes has multiple effects on the protein levels and activity of this enzyme, further augment oxidative stress by causing a suppressed defence response.

Hence, modulation of this enzyme in target organs prone to diabetic complication such as the kidney may prove beneficial in the prevention and management of kidney failure (Johansen *et al.*, 2005).

1.3.2 CATALASE [EC 1.11.1.6]

Catalase is a haem-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen. The enzyme is present in all aerobic eukaryotes. It is important in the removal of hydrogen peroxide generated in peroxosomes (microbodies) by oxidases involved in ω-oxidation of fatty acids, the glyoxylate cycle and purine metabolism. Catalase was one of the first enzymes to be isolated in a highly purified state. Multiple forms of catalase have been described and all forms are tetramers (Redinbaugh *et al.*, 1988; Scandalis, 1990). Examination of the structure of beef liver catalase showed four NADPH binding sites per catalase tetramer (Fita and Rossmann, 1985), but these sites were not in close association with the hydrogen peroxide centre. Instead, NADPH functions in animals catalase to protect against activation by hydrogen peroxide (Kirkman *et al.*, 1987).

Catalase is very sensitive to light and has a rapid turnover rate similar to that of D1 protein of photosystem II in plants (Hertwig *et al.*, 1992). This may be as a result of light absorption by the haem group or perhaps hydrogen peroxide inactivation. Nevertheless, stress conditions which reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity (Hertwig *et al.*, 1992; Feirabend *et al.*, 1992).

The reactions catalysed by catalase are follows:

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{...(Catalytic activity) .....(1.9)}$$

(Decomposition)

$$ROOH + AH_2 \longrightarrow H_2O + ROH + A \quad \text{...(Periodic activity) .....(1.10)}$$

(Oxidation)

1.3.3 THE GLUTATHIONE SYSTEM

Glutathione (GSH), present in plants, animals and some bacteria, often at high levels, can be thought of as a redox buffer. It is derived from glycine, glutamate and aspartat. The oxidized form of glutathione (GSSG), produced in the course of its redox activities, contains two glutathione molecules linked by a disulfide bond (Nelson and Cox, 2005).

Taylor *et al.* (1993) ascribes important cellular defence against ROS to the glutathione system, which reacts both directly with radicals in non-enzymatic reaction. It also serves as an electron donor in the reduction of peroxides where it protects cells against injury. Hence, glutathione acts as a direct scavenger as well as a cosubstrate for glutathione peroxidase. It is a major intracellular redox system (Johansen *et al.*, 2005). The importance of intracellular GSH distribution and redox status of cells viability is supported by the evidence that the depletion of GSH or inhibition of its synthesis extensively affects several functions (Yoshida *et al.*, 1990; Chiba *et al.*, 1996).

The activities of glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rx) have been reported to be higher in cellular compartments i.e. mitochondria and microsomes where excess free radicals are known to occur.

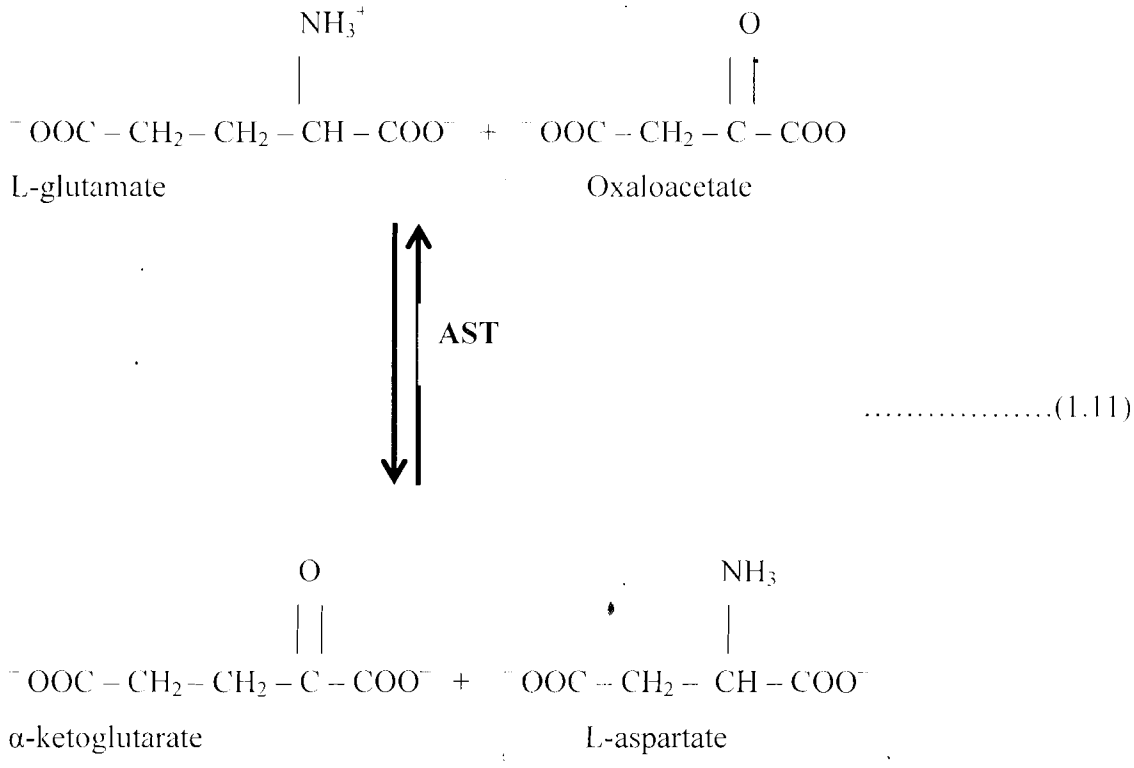
Glutathione S-transferase (GST, EC.2.5.1.18) is a family of multifunctional isozymes found in all eukaryotes (Rajurkar *et al.*, 2003). It catalyses both glutathione dependant conjugation and reduction (Ketter *et al.*, 1993).

GST detoxifies endobiotic and xenobiotic compounds by covalently linking glutathione to a hydrophobic substrate, forming less reactive and more polar glutathione S-conjugate (Neueferind *et al.*, 1997).

1.4 ENZYMES OF TISSUE DAMAGE

(a) Aspartate Amino Transferase (AST) [EC 2.6.1.1]

This enzyme which was formally known as serum glutamate oxalo-acetate transaminase (SGOT) is a cytosolic and mitochondrial enzyme that catalyses the transamination of L-aspartate and α-ketoglutarate, forming oxaloacetate and L-glutamate (Rxn 1.11) (Giannini *et al.*, 2005).

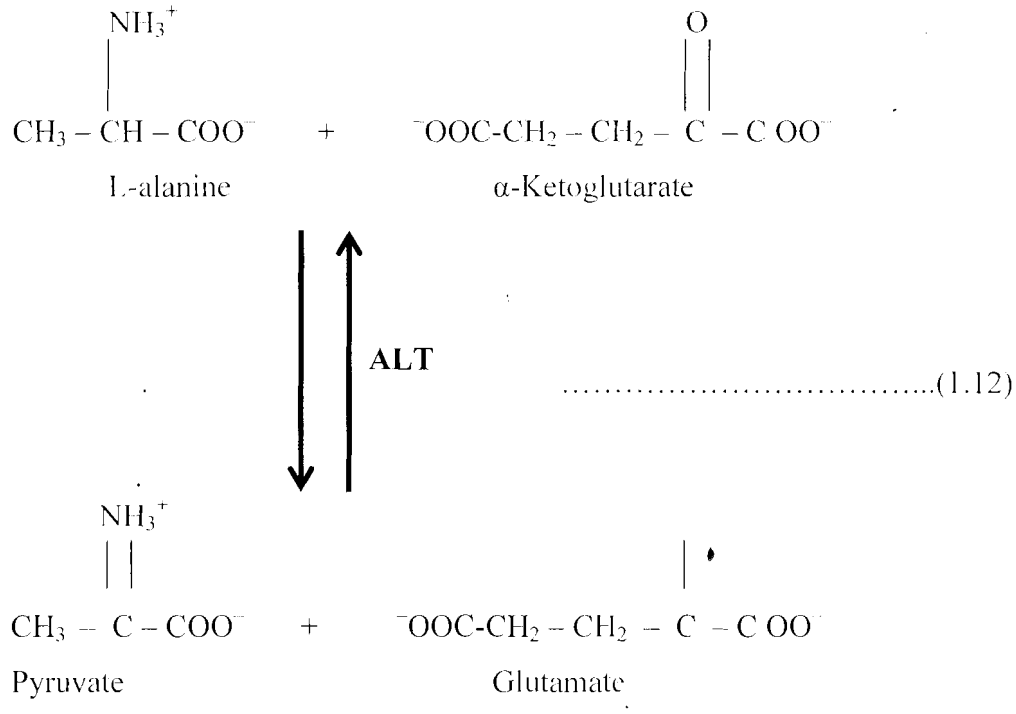


.....(1.11)

AST is activated by pyridoxamine 5-phosphate and inhibited by malate, adipate, succinate and glutamate. AST is present in many mammalian tissues such as the heart, muscle, liver, skeletal muscle, kidneys, intestines and plasma.

b) Alanine Aminotransferase (ALT) [EC 2.6.1.2]

This was previously known as serum glutamic pyruvic transaminase (SGPT). It is also correctly referred to as alanine aminotransferase (Kaufman and Greene, 1993; Stockham and Scott, 2002). Alanine aminotransferase (ALT) is a cytoplasmic enzyme found principally in the liver with only small amounts being present in other organs. ALT catalyses the transamination of α-ketoglutarate and L-alanine, forming glutamate and pyruvate in a reversible reaction as shown below (Rxn 1.12) (Bain, 2003).



.....(1.12)

These enzymes are important diagnostic tools in the diagnosis of heart and liver damage caused by heart attack, drug toxicity or infection. Liver degeneration caused either by chemical toxicity or infection is accompanied by leakage of these enzymes from injured hepatocytes into the blood. These enzymes also leak from the injured heart cells into the bloodstream after heart attack (Nelson and Cox, 2005). Determination of these activities of these enzymes is a relatively sensitive indicator of hepatic and or cardiac damage in certain animal species.

(c) Biochemical Roles of AST and ALT

Abnormal liver enzyme levels may signal liver damage or alteration in bile flow. The liver is a large complex origin that is well designed for its central role in carbohydrate, protein and fat metabolism. It is the site where waste products of metabolism are detoxified through processes such as amino acid deamination; which produces urea (Giannini *et al.*, 2005). In conjunction with the spleen the liver is involved in the destruction of spirit red blood cells and the reclamation of their constituents. It is

responsible for synthesising and secreting bile and synthesising lipoproteins and plasma proteins. including clotting factors (Burkitt *et al.*, 1993). It also maintains a stable blood glucose level by taking up and storing glucose as glycogen (glycogenesis), breaking this down to glucose when needed needed (glycogenolysis) and forming glucose from noncarbohydrate sources such as amino acids (gluconeogenesis).

Many of these biosynthetic functions use the products of digestion. With the exception of most lipids, absorbed food products pass directly from the gut to the liver through the hepatic portal vein. In order to accurately interprete biochemical abnormalities, it is necessary to understand how normal ranges are established and how to apply reference ranges. Aminotransferase levels vary according to age and sex (Dufer *et al.*, 2000). So care must be taken in choosing the reference limits. Levels of both AST and ALT may increase with strenuous exercise (Dufour, 1998). Other factors like hospital admission, physical activity and diet have been observed to induce these increase in AST and ALT levels in healthy subjects.

Alterations in liver enzyme levels encountered may vary according to the geographical location or the ethnicity of the patients (Whitehead *et al.*, 1999). The most common alterations in enzyme levels can be divided into either hepatocellular or cholestatic predominant. Injury to the liver, whether acute or chronic, eventually results in an increase in serum concentrations of aminotransferases.

1.5 *Daniella oliveri*

1.5.1 Botanical Outline of *Daniella oliveri*

Daniella oliveri (Caesalpinaceae) (Rolfe) Hutch and Dalz is a plant found in the Amazon region and other parts of South America and Africa (Langenhein, 1973; Gentry, 1993). The tree may reach a height of 100 feet and trunk diameter of 4 feet (Record and Mell, 1984). The plant is shown in Fig. 1.3.

Fig. 1.3: *Daniella oliveri* plant

1.5.2 Uses of *Daniella oliveri*

D. oliveri tree produces a liquid - oleoresin – which has been used as medicine by indigenous people for over 400 years (Gilbert, 2000). The oleoresin is produced in a tree trunk, stem and leaves and it consists of large but varying amount of volatile oil. The oleoresin is traditionally used as anti-inflammatory agent and in the treatment of a variety of genitor-urinary tract diseases and skin ailments (Raffauf, 1992; Duke and Vasquez, 1994).

Moreover, it is used as anti-rheumatic, antiseptic, antibacterial, diuretic and hypotensive agent, as well as an expectorant, laxative, purgative and vermifuge (Fleury, 1997). The leaves of *D. oliveri* are also claimed to be used in folk medicine as an anti-diabetic agent. Scientific studies have authenticated some of these medicinal uses of oleoresin such as its effectiveness as an anti-bacterial, anti-inflammatory and antioxidant agent (Verpoorate and Dahl, 1987; Basile *et al.*, 1988).

Daniella oliveri is known by the Igbos as 'Agba' and 'Ozia' by the Benis. Hausas call it 'maje'; Yorubas call it 'iya' while the Nupes and Fulanis know it as 'danchi' and 'kaharlahi' respectively (Olorode, 1984).

1.6 *Sarcocephalus latifolius* (Rubiaceae)

1.6.1 Botanical Outline of *Sarcocephalus latifolius*

Sarcocephalus latifolius formerly known as *Nauclea latifolia* is of the Rubiaceae family and is commonly called the pin cushion tree or 'Africana peach'. This plant is a straggling shrub or small tree native to tropical Africa and Asia as shown in Fig. 1.4. There are about thirty-five (35) species of Sarcocephalus widely distributed in the tropical savannah forest. It is known among as 'uvuru ilu' (Igbo), 'tafashiya' (Hausa) and 'egbesi' (Yoruba).

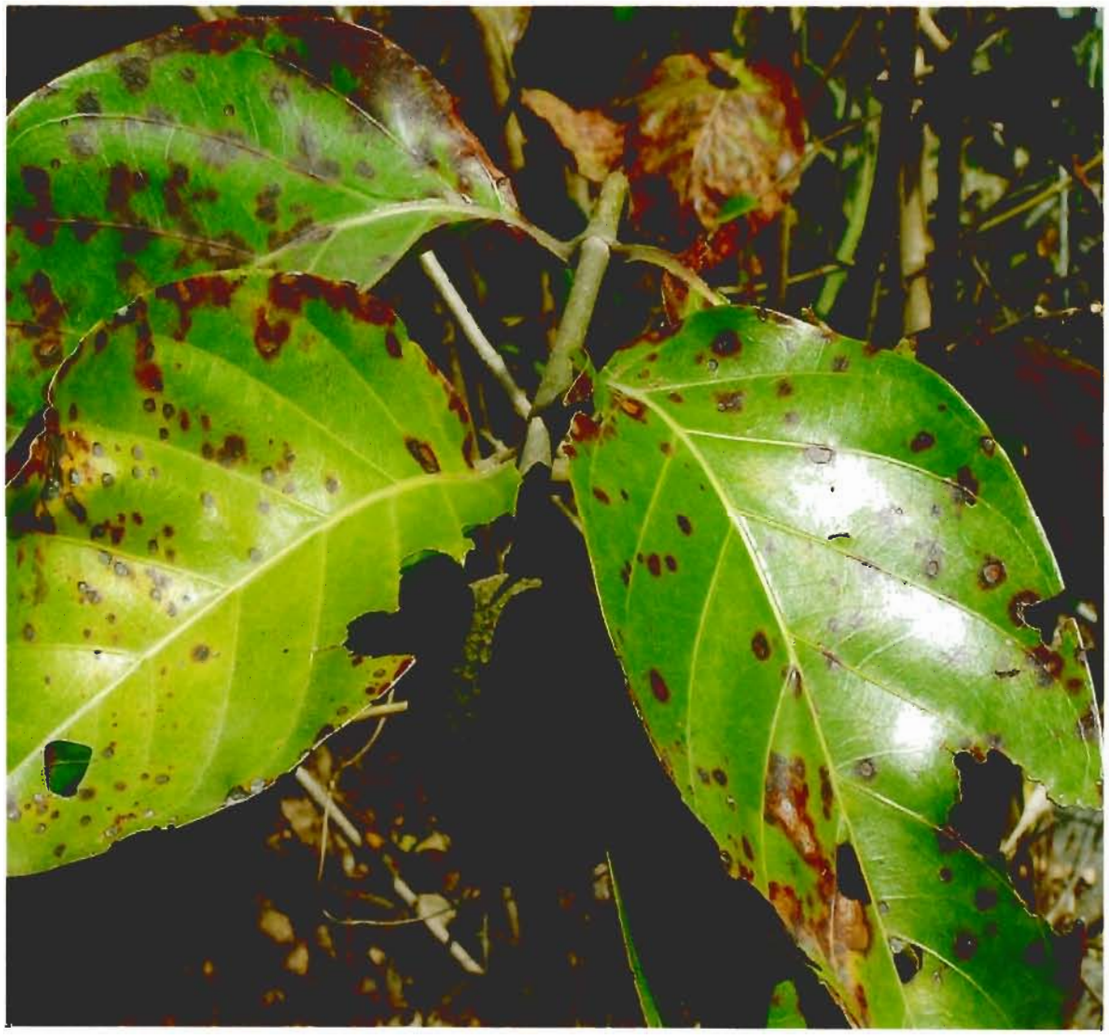


Fig. 1.4: *Sarcocephalus latifolius* plant

1.6.2 Uses of *Sarcocephalus latifolius*

Parts of the plant are commonly prescribed traditionally as remedy for diabetes mellitus. The plant is also used in the treatment of other ailments such as malaria (Kokwaro, 1976; Akubue and Mittal, 1982, Boye, 1990), gastrointestinal tract disorders (Madubunyi, 1995) and sleeping sickness (Kerharo, 1974) prolonged menstrual flow (Elujoba, 1995), hypertension (Akabue and Mittal, 1982). The stem of the plant is also used as chewing stick (Asubiojo *et al.*, 1982). The anti-diabetic effect of the leaves of this plant has been demonstrated by Gidado *et al.* (2005).

The key constituents of the plants are indole-quinolizidine alkaloids and glucoalkaloids and saponins. Studies have shown that the roots have anti-bacterial activity against gram positive and negative bacteria as well as anti-fungal activity (Iwu, 1993). It is most effective against *Corynebacterium, diphtheriae*, *Streptobaccilus sp*, *Streptococcus sp*, *Neisseria sp*, *Pseudomonas aeruginosa* and *Salmonella sp* (Deeni and Hassan, 1991).

1.6.3 PRESENT INVESTIGATION

Although a decoction of the roots of *Daniella oliveri* and *Sarcocephalus latifolius* in combination is used in the South Eastern Nigeria as a herbal remedy for hyperglycaemia, there is no empirical data or scientific reports to support the antidiabetic effect of the roots of these plants.

This investigation is aimed at verifying the blood sugar lowering effect and possible antihyperglycaemic potentials of these roots in combination as well as the roots of *Sarcocephalus latifolius*.

1.7 RESEARCH OBJECTIVES

The followings are the objectives of the study:

- To determine the hypoglycaemic and antihyperglycaemic effect of the root extracts of:-

(i) *Sarcocephalus latifolius* alone

(ii) *Sarcocephalus latifolius* in combination with *Daniella oliveri* in alloxan induced diabetic rats.

- To investigate the effect of these extracts on the activities of key enzymes of glucose metabolism and hepatic glycogen concentration in normal and diabetic rats.
- To determine the effects.of these extracts on oxidative stress in the liver and the kidney of normal control and alloxan induced diabetic rats.
- To determine the effect of these extracts on the serum lipid profile and haematological parameters of normal control and diabetic rats
- To establish the safety profile of the extracts by assessing (i) the activities of liver marker enzymes (ii) haematological parameters (iii) conducting histopathological examination on both normal and diabetic animals

CHAPTER TWO
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLANT MATERIALS

Fresh roots of *Daniella oliveri* and *Sarcocephalus latifolius* were collected in November, 2006 in Nsukka, Enugu State, Nigeria. The plants were identified and authenticated by A. Ozioko of the International Centre for Ethnomedicine and Drug Development. Nsukka, Nigeria, where the voucher specimens were deposited.

2.1.2 ANIMALS

Adult male Sprague-Dawley rats of between 10 and 16 weeks with average weights of 160 – 300 g and adult albino mice (15 – 35 g) of either sex were obtained from the laboratory Animal Facilities of the Faculty of Biological Sciences and Veterinary Medicine, University of Nigeria, Nsukka. The animals were acclimatised for about 7 days under standard environmental conditions, with a 12 hour light/12 hour dark cycle maintained on a standard feed (growers mash) and water *ad libitum*, before being employed for any determination.

2.1.3 CHEMICALS

The chemicals used for this study included analytical grades of ethanol, ethylene diamine tetracetate (EDTA), hydrochloric acid, sulphuric acid, glacial acetic acid (BDH Chemicals Ltd, Poole England), Sodium hydroxide, Trichloroacetic acid (TCA) and disodium hydrogen phosphate (May and Baker, England), glutathione, adenosine 5-diphosphate (ADP), adrenaline, 2-thiobarbituric acid (TBA), DL-dithiothreitol (DTT), 5, 5-dithiobis (2-nitrobenzoic acid), adenosine 5-triphosphate and 5-sulfosalicyclic acid and alloxan monohydrate (Sigma-Aldrich, USA), glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), nicotinamide adenine dinucleotide phosphoric acid (NADP), 1-chloro-2, 4-dinitrobenzene and adenosine 5-monophosphoric (5-AMP) (Fluka ~ Biochemika). All the other reagents and chemicals were also of analytical grade. The standard antidiabetic drug used was glibenclamide (Daonil$^{(R)}$).

2.1.4 EQUIPMENT

The following equipment were used: MSE High Speed Centrifuge (Labofuge–1, Netherland), Spectronic 20 (Bausch and Lamp), Analytical Balance (Ohaus Adventurer, China), Stopwatch, Water bath, One Touch Ultra Glucometer (Lifescan, USA), Homogeniser (Janke and Kunkel, Germany).

2.2 METHODS

2.2.1 EXTRACTION PROCEDURES

2.2.1.1 PREPARATION OF THE AQUEOUS EXTRACT

(i) The fresh roots of *Daniella oliveri* and *Sarcocephalus latifolius* were separately cleaned, chopped into pieces and shade-dried to a constant weight. About 500 g of the chopped roots of *Sarcocephalus latifolius* was extracted in 4 volumes (w/v) of distilled water by decoction. The extract was filtered and the filtrate was dried at room temperature to obtain a solid residue which was designated: SLA – *Sarcocephalus latifolius* in water.

(ii) Then 250 g each of the *Daniella oliveri* and *Sarcocephalus latifolius* roots were collectively subjected to the same treatment as above. The extract was designated SDA - *Sarcocephalus/Daniella* aqueous.

2.2.1.2 PREPARATION OF ETHANOL EXTRACT

A weight of 500g of *Sarcocephalus latifolius* roots was macerated in 5 volumes (w/v) of 95% ethanol and left to stand for 24 hours with intermittent shaking. This was filtered and the filtrate evaporated to dryness at room temperature. This extract was called SLE – *Sarcocephalus latifolius* in ethanol.

2.3 PRELIMINARY PHYTOCHEMICAL ANALYSIS

The three extracts SLA, SDA and SLE were individually subjected to phytochemical analysis based on procedures outlined by Harborne (1973) and Trease and Evans (1989).

2.3.1 Test for Carbohydrate

Molisch Test

A weight of 0.1 g of the extract was boiled with 2 ml of distilled water and filtered. To the filtrate, few drops of naphthol solution in ethanol (Molisch's reagent) were added. Concentrated sulphuric acid was then gently poured down the side of the test tube to form a lower layer. A purple interfacial ring indicates the presence of carbohydrate.

2.3.2 Test for Alkaloids

A volume of 20 ml of 3% sulphuric acid in 50 % ethanol was added to 2 g of the extract and heated on a boiling water bath for 10 minutes, cooled and filtered. The filtrate (2 ml) was tested with a few drops of Meyer's reagent (potassium mercuric iodide solution), Dragendorff's reagent (bismuth potassium iodide solution), Wagner's reagent (iodine in potassium iodide solution), and picric acid solution).

The remaining filtrate was placed in 100 ml separating funnel and made alkaline with dilute ammonia solution. The aqueous alkaline solution was separated and extracted with two 5 ml portions of dilute sulphuric acid. The extract was tested with a few drops of Mayer's, Wagner's, Dragendorff's reagent: reddish brown precipitate with few drops of Wagner's reagent; yellowish precipitate with few drops of picric acid and brick red precipitate with few drops of Dragendorff's reagent indicate the presence of alkaloids.

2.3.3 Test for Reducing Sugar

A given volume of 5 ml of a mixture of equal parts of Fehling's solutions I and II were added to 5 ml of aqueous extract and then heated on a water bath for 5 minutes. A brick red precipitate shows the presence of reducing sugar.

2.3.4 Test for Glycosides

A volume of 5 ml of dilute sulphuric acid was added to 0.1 g of the extract in a test tube and boiled for 15 minutes on a water bath, then cooled and neutralised with 20% potassium hydroxide solution. A given volume of 10 ml of a mixture of

equal parts of Fehling's solutions I and II was added and boiled for 5 minutes. A more dense brick red precipitate indicates the presence of glycoside.

2.3.5 Test for Saponins

A volume of 20 ml of distilled water was added to 0.25 g of the extract and boiled on a hot water bath for 2 minutes. The mixture was filtered while hot and allowed to cool and the filtrate was used for the following tests:

(a) Frothing Test

A given volume of 5 ml of the filtrate was diluted with 15 ml of distilled water and shaken vigorously. A stable froth (foam) upon standing indicates the presence of saponins.

(b) Emulsion Test

To the frothing solution was added 2 drops of olive oil and the contents shaken vigorously. The formation of emulsion indicates the presence of saponins.

2.3.6 Test for Tannins

One gramme (1 g) of the powdered material was boiled with 20 ml of water, filtered and used for the following tests:

(a) Ferric Chloride Test

To 3 ml of the filtrate, few drops of ferric chloride solution were added. A greenish black precipitate indicates the presence of tannins.

(b) Lead Acetate Test

To a little of the filtrate was added lead acetate solution. A reddish colour indicates the presence of tannins.

2.3.7 Test for Flavonoids

(a) Ethyl Acetate Test

A volume of 10 ml of ethyl acetate was added to 0.2 g of the extract and heated on a water bath for 3 minutes. The mixture was cooled, filtered and the filtrate was used for the following tests.

equal parts of Fehling's solutions I and II was added and boiled for 5 minutes. A more dense brick red precipitate indicates the presence of glycoside.

2.3.5 Test for Saponins

A volume of 20 ml of distilled water was added to 0.25 g of the extract and boiled on a hot water bath for 2 minutes. The mixture was filtered while hot and allowed to cool and the filtrate was used for the following tests:

(a) Frothing Test

A given volume of 5 ml of the filtrate was diluted with 15 ml of distilled water and shaken vigorously. A stable froth (foam) upon standing indicates the presence of saponins.

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2.3.6 Test for Tannins

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To 3 ml of the filtrate, few drops of ferric chloride solution were added. A greenish black precipitate indicates the presence of tannins.

(b) Lead Acetate Test

To a little of the filtrate was added lead acetate solution. A reddish colour indicates the presence of tannins.

2.3.7 Test for Flavonoids

(a) Ethyl Acetate Test

A volume of 10 ml of ethyl acetate was added to 0.2 g of the extract and heated on a water bath for 3 minutes. The mixture was cooled, filtered and the filtrate was used for the following tests.

(b) Ammonium Test

A volume of 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow colour in the ammoniacal layer indicates the presence of flavonoids.

(c) One Percent (1%) Aluminium Chloride Solution Test

Another 4 ml portion of the filtrate was shaken with 1 ml of 1% aluminium chloride solution. The layers were allowed to separate. A yellow colour in the aluminium chloride layer indicates the presence of flavonoids.

2.3.8 Test for Resins

A given weight of 0.2 g of the extract was extracted with 15 ml of 96% ethanol. The alcoholic extract was then poured into 20 ml of distilled water in a beaker. A precipitate occurring indicates the presence of resins.

(a) Precipitation Test

The weight of 0.2 g of the extract was extracted with 15 ml of 96% ethanol. The alcoholic extract was then poured into 20 ml of distilled water in a beaker. A precipitate occurring indicates the presence of resins.

(iii) **Colour Test**

The extract (0.2 g) was extracted with chloroform and the extract was concentrated to dryness. The residue was redissolved in 3 ml of acetone and another 3 ml concentrated hydrochloric acid was added. This mixture was heated in a water bath for 30 minutes. A pink colour which changes to magenta red indicates the presence of resins.

2.3.9 Test for Proteins

The extract (0.5 g) was extracted with 20 ml of distilled water and filtrate was used for the following tests:

(a) Picric Acid Test

To a little portion of the filtrate was added a few drops of picric acid. A yellow precipitate indicates the presence of proteins.

(b) Biuret Test

A volume of 3ml of Biuret reagent was added to 2ml of the filtrate and then two drops of potassium hydroxide were added. A purple or pink colour shows the presence of presence.

2.3.10 Test for Fats and Oil

The extract (0.1 g) of the extract was pressed between filter paper and the paper was observed. A control was also prepared by placing 2 drops of olive oil on filter paper. Translucency of the filter paper indicates the presence of fats and oil.

2.3.11 Test for Steroids and Terpenoids

Ethanol (9 ml) was added to 1.0 g of the extract and refluxed for a few minutes and filtered. The filtrate was concentrated to 2.5 ml on a boiling water bath, 5 ml of hot distilled water was added to the concentrated solution. The mixture was allowed to stand for 1 hour and the waxy matter was filtered off. The filtrate was extracted with 2.5 ml of chloroform using separating funnel. To 0.5 ml of the chloroform extract in a test tube was carefully added 1 ml of concentrated sulphuric acid to form a lower layer. A reddish brown interface shows the presence of steroids.

Another 0.5 ml of chloroform extract was evaporated to dryness on a water bath and heated with 3 ml of concentrated sulphuric acid for 10 minutes on a water bath. A grey colour indicates the presence of terpenoids.

2.3.12 Test for Acidic Compounds

The extract (0.1 g) was placed in a clear dry test tube and sufficient water added. This was warmed in a hot water bath and then cooled. A piece of water-wetted litmus paper was dipped into the filtrate and the colour change on the litmus paper was observed. Acidic compounds turn litmus paper red.

The median lethal dose (LD_{50}) of the three different extracts SLA, SDA and SLE were determined in albino mice using the method of Lorke (1983). The studies were conducted in two stages for each of the extracts. In first stage, 3 groups of 3 mice each were treated, per os (p.o.) with 10, 100 and 1000 mg/kg of SLA, SDA and SLE respectively and observed for number of deaths and behavioural changes. Based on the percentage survival rates, 3 groups of mice were treated with 1500, 2900 and 5000 mg/kg of each of the extracts in the second stage and the animals observed for the next 72 hours.

The LD_{50} was calculated as the geometric mean of the highest non-lethal and the lowest lethal doses.

2.4.1.1 Sub-acute Toxicity Test

Six groups of four (4) healthy rats each received different doses of the extracts and distilled water as follows:

Group A received 5 ml/kg body weight of distilled water

Group B received 250 mg/kg body weight of SLA

Group C received 250 mg/kg body weight of SLE

Group D received 150 mg/kg body weight of SLA

Group E received 250 mg/kg body weight of SDA

Group F received 500 mg/kg body weight of SDA

The animals were treated with the above doses orally every day for 30 days using the above doses. At the end of the experiment, blood samples were collected retro-orbitally from the inner canthus of the eye using capillary tubes (micro haematocrit capillaries, Marienfeld). Blood (3 mls) was collected in fresh vials containing EDTA as anti-coagulant agent; while the blood samples for serum analyses were collected in fresh vials. The animals were euthanized using chloroform, and their livers as well as kidneys were removed immediately, washed differently in ice cold 1.15% KCl and fixed in 10% formalin for histopathological examination. The blood samples were used for the

2.4 EXPERIMENTAL/STUDY DESIGN

2.4.1 TOXICOLOGICAL STUDIES

2.4.1.1 Acute Toxicity and Lethality (LD_{50}) Test

The median lethal dose (LD_{50}) of the three different extracts SLA, SDA and SLE were determined in albino mice using the method of Lorke (1983). The studies were conducted in two stages for each of the extracts. In first stage, 3 groups of 3 mice each were treated, per os (p.o.) with 10, 100 and 1000 mg/kg of SLA, SDA and SLE respectively and observed for number of deaths and behavioural changes. Based on the percentage survival rates, 3 groups of mice were treated with 1500, 2900 and 5000 mg/kg of each of the extracts in the second stage and the animals observed for the next 72 hours.

The LD_{50} was calculated as the geometric mean of the highest non-lethal and the lowest lethal doses.

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assessment of white and red blood cells counts (WBS and RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), spartate aminotransferase (AST) and alanine aminotransferase (ALT).

2.4.2 ANTI-DIABETIC EVALUATION

2.4.2.1 DETERMINATION OF THE HYPOGLYCAEMIC ACTIVITY OF THE EXTRACTS

Healthy normal male rats were randomly divided into four (4) groups of four (4) rats and fasted for 16 hrs. Groups I and II received 250 mg/kg of SLA and SDA respectively, groups III received 3.0 mg/kg of glibenclamide while Group IV received 5mg/kg of distilled water; all the administrations were given orally. At the end of the fasting period taken as 0hr, , blood was collected from the tail vein and blood glucose levels determined (using the One Touch Ultra Glucometer) at time intervals (0, 1, 2, 3, 4, 5, 6, 8, 10 and 12 hours) after treatment.

2.4.2.2 INDUCTION OF EXPERIMENTAL DIABETES IN THE RATS

Rats fasted for 16 hours, but with free access to water were made diabetics by an intravenous injection of alloxan monohydrate (Sigma-Aldrich, U.S.A) (65 mg/kg) given in the tail vein. The alloxan was dissolved in the sterile normal saline just prior to injection. Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution after 3 hrs. The rats were kept for the next 12 hrs on 5% glucose solution bottles in their cages to prevent hypoglycaemia (Dhandapani *et al*., 2003). After 72 hours of alloxan injection, surviving rats with fasting blood glucose levels > 200 mg/dl were included in the study.

2.4.2.3 DOSE-REPONSE STUDY

Fasted diabetic rats were grouped into four (4) in a group and received 10 different doses (ranging from 50 to 500 mg/kg) of the extract SLA. Fasting blood glucose levels of these rats were determined 6 hours after oral administration of the extract.

2.4.2.4 DETERMINATION OF THE EFFECT OF THE EXTRACTS ON THE FASTING BLOOD GLUCOSE LEVELS (FBGL) OF ALLOXAN-INDUCED DIABETIC RATS

The alloxan-induced diabetic rats (blood sugar> 200 mg/dl) were divided into five groups; the fifth group was the normal control group, as shown below (Table 2.1).

Table 2.1: Fasting Blood Glucose Monitoring Design

Group	Number of Rats	Treatment
Diabetic Control (DC)	4	Distilled Water
Diabetic (DSLA)	4	SLA – 250 mg/kg
Diabetic (DSLE)	4	SLE – 250 mg/kg
Diabetic (DSDA)	4	SDA – 250 mg/kg
Diabetic-Glibenclamide	4	Glib – 5 mg/kg
Normal Control (NC)	4	Distilled Water

Blood samples were collected from the rats through the tail and blood glucose concentration were determined at 0 (initial), 1, 2, 3, 4, 5 and 6 hours after treatment using the one touch ultra glucometer.

2.4.2.5 DETERMINATION OF THE EFFECT OF REPEATED DOSES OF THE EXTRACT ON THE FASTING BLOOD GLUCOSE LEVEL

Diabetic rats were again grouped as in table 2.1 above. Treatment was administered orally twice daily using an intubator at a dosage of 250 mg/kg body weight of the three extracts (as previously determined in the dose-response study) for 3 weeks. Fasting blood glucose concentrations were determined every week. After 3weeks, blood samples were again withdrawn from the inner canthus of the eye as was previously described. The rats were euthanised using chloroform and their livers and kidneys were surgically removed, immediately washed with ice cold KCI (1.15%) and refrigerated until analysed. The blood samples and tissues were used for the biochemical analysis.

2.4.3 SAMPLE PREPARATION

2.4.3.1 PLASMA/SERUM PREPARATION

Both the blood samples collected in EDTA bottles and the clotted blood samples were centrifuged at 3,500 revolutions per minutes (rpm) for 5 minutes. The plasma and serum were decanted after centrifugation and stored in the freezer (below $4^{O}C$).

2.4.3.2 PREPARATION OF HOMOGENATES

2.4.3.2.1 Reagents

(a) Washing buffer (1.15% Potassium chloride, KCl)

KCl (11.5 g) was dissolved in distilled water and made up to one litre with distilled water. The pH was adjusted to 7.4 using HCl.

(b) Homogenising buffers

(1) 50 mM Tris/HCl, 1.15% KCl pH 7.4

(2) 100 mM KCl, 10 mM DTT, 50 mM Tris/HCl, pH 7.4 and 1 mM EDTA

(3) 50 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 30 mM DTT

(4) 50 mM Tris/HCl, pH 8.5, 5 mM $MgSO_4.7H_20$, 1 mM EDTA and 10 mM DTT

(1) Exactly 7.88 g of Tris/HCl and 11.5 g of KCl were dissolved and made up to one (1) litre with distilled water. The pH of the solution was adjusted to 7.4 using HCl.

(2) Exactly 7.88 g of Tris/HCl was dissolved in distilled water and made up to 500 ml with distilled water. The pH was adjusted to pH 7.4 with drops of HCl. This solution was added to a solution of 0.75 g of KCl + 1.54 g of DTT + 0.37 g of EDTA dissolved in 500 ml of distilled water.

(3) Exactly 7.88 g of Tris/HCl, 0.19 g of $MgCl_2$, 0.37 g of EDTA and 4.62 g of DTT were dissolved in distilled water and water up to 1 litre with distilled water. The pH of the solution was adjusted to pH 7.5 with drops of HCl.

(4) Exactly 7.88 g of Tris/HCl, 1.23 g of $MgSO_4.7H_2O$, 0.37 g of EDTA and 1.54 g of DTT were dissolved in distilled water and made up to one (1) litre with distilled water. The pH was adjusted to 8.5.

2.4.3.2.2 Procedure

The organs (liver and kidney) excised from the animals were washed separately in 1.15% KCl, dried on filter paper and weighed. Weighed amounts (0.5 g) of the different organs were separately homogenised in the stipulated volumes of the suitable homogenising buffer and centrifuged at $\leq 2^{O}C$ or as indicated in the procedure. The supernatants were collected and kept in the freezer at $\leq 4^{O}C$. Each time the supernatant was outside the freezer, it was kept in ice bags.

2.5 BIOCHEMICAL ANALYSIS

2.5.1 PROTEIN DETERMINATION

2.5.1.1 Principle

The protein content of the post mitochondrial fractions was determined by the Biuret method as described by Gornall *et al.* (1949) using bovine serum albumin (BSA) as standard. Potassium iodide was added to the Biuret reagent to prevent the precipitation of Cu^{2+} as cuprous oxide. In alkaline pH, Cu^{2+} forms complexes with proteins. The Biuret reagent contains copper sulphate, potassium iodide and sodium potassium tartarate. The blue complex formed by the reaction of Cu^{2+} of the Biuret reagent with protein has maximum absorbance at 540 nm.

2.5.1.2 Reagents

(a) **2 M NaOH:** This was prepared by dissolving 8 g of NaOH pellets in distilled water and made up to 1000 ml.

(b) **0.9% Normal Saline:** This was prepared by dissolving 8.5 g of NaCl in distilled water and made up 1000 ml.

(c) **Biuret reagent:** This was prepared by dissolving 3 g of copper sulphate pentahydrate ($CuSO_4.5H_2O$), 9 g of Na-K tartarate and 5 g of potassium iodide in 0.2 M NaOH.

(d) **Stock Bovine Albumin (BSA):** BSA (0.1 g) was dissolved in 0.9% NaCl and made up to 500 ml with normal saline; Concentration of BSA in the solution was therefore 20 mg/dl.

2.5.1.3 Procedure

The post mitochondrial fractions were diluted with 0.9% saline or normal saline solution. A volume of 2 ml of diluted samples each was added to 3 ml of Biuret reagent. The mixture was incubated at room temperature for 30 minutes after which the absorbance was read at 540 nm against a saline solution blank.

The protein concentrations were later extrapolated from the standard BSA curve.

2.5.1.4 PREPARATION OF BOVINE SERUM ALBUMIN STANDARD CURVE

Serial dilutions of BSA stock solution were made with 0.9% saline. Biuret reagent (3 ml) was added to 2 ml of each diluent. The mixture was thoroughly shaken and allowed to stand for 30 minutes after which the absorbance was read at 540 nm. The graph of absorbance against BSA concentration was later plotted.

2.5.2 DETERMINATION OF SERUM TOTAL CHOLESTEROL

2.5.2.1 Principle

The serum total cholesterol concentration was estimated by the method of King and Whooton (1959). This method was based on the fact that enzymatic hydrolysis and subsequent oxidation generates hydrogen peroxide which reacts with 4-aminoantipyrine in the presence of peroxidase and phenol to produce quinoneimine.

The indicator quinoneimine absorbs light at 500 nm.

$$\text{Cholesterol-ester} + H_2O \xrightarrow[\text{estarase}]{\text{Cholesterol}} \text{Cholesterol} + \text{Fatty acid} \quad \ldots\ldots\ldots\ldots\ldots 2.1$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{Cholesterol}} \text{Cholestene-3-one} + H_2O_2 \quad \ldots\ldots\ldots\ldots\ldots 2.2$$

$$2H_2O_2 + \text{Phenol} + \text{4-aminoamyipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinonemine} + H_2O \quad \ldots\ldots 2.3$$

2.5.2.2 Reagents (Randox, U.K.)

The commercially prepared reagents include:

(a) 0.30 mmol/l 4-aminoantipyrine

(b) 6.0 mmol/l Phenol

(c) ≥ 0.5 µl/ml Peroxidase

(d) ≥ 0.15 µl/ml Cholesterol esterase

(e) ≥ 0.1 µl/ml Cholesterol oxidase

(f) 80 mmol/l Pipes Buffer (pH 6.8)

(g) 5.17 mmol/l Cholesterol Standard (200mg of pure dry cholesterol was dissolved in 100 ml of glacial acetic acid.

2.5.2.3 Procedure for Total Cholesterol Determination

Table 2.2: Assay Procedure for Total Cholesterol Determination

	Reagent Blank (µl)	**Standard (µl)**	**Sample (µl)**
Distilled Water	10	-	-
Standard	-	10	-
Sample	-	-	10
Reagent	1000	1000	1000

The contents of each test tube were mixed and incubated for 10 minutes at room temperature or 5 minutes at 37^{O}C. The absorbance of the sample was measured against the reagent blank within 60 minutes at 500 nm wavelength and 1 cm light path.

$$Concentration\ of\ Cholesterol\ in\ the\ Sample = \frac{A_{Sample}}{A_{S\tan dard}} \times Conc.\ of\ S\tan dard\ (5.17 mmol/l)$$

2.5.3 DETERMINATION OF SERUM TRIGLYCERIDE

2.5.3.1 Principle

The serum triglyceride concentration was determined using the colometric method of Tietz (1990). This method is based on the fact that triglycerides undergo

enzymatic hydrolysis to yield H_2O_2. This hydrogen peroxide produces a quinoneimine when reacted with 4-aminophenazone and the colour reagent 4-chlorophenol. The quinoneimine absorbs light at 500 nm.

$$\text{Triglycerides} + H_2O \xrightarrow[\text{Lipases}]{} \text{Glycerol} + \text{Fatty acids} \quad \ldots\ldots\ldots\ldots\ldots\ldots 2.4$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[\text{kinase}]{\text{Glycerol}} \text{Glycerol-3-phosphates} + \text{ADP} \quad \ldots\ldots\ldots 2.5$$

$$\text{Glycerol-3-phosphates} + O_2 \xrightarrow[\text{oxidase}]{\text{Glycerol-3-(P)}} \text{Dihydroxyacetone} + \text{Phosphate} + H_2O_2 \quad \ldots\ldots\ldots 2.6$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + HCl + 4H_2O \quad \ldots\ldots\ldots 2.7$$

2.5.3.2 Reagents (Randox, U.K.)

The content of the commercially available Randox Diagnostic kit includes:

(a) 40 mmol/l Pipes Buffer, pH 7.6

(b) 5.5 mmol/l 4-Chlorophenol

(c) 17.5 mmol/l Magnesium ions

(d) 0.5 mmol/l 4-Aminophenazone

(e) 1.0 mmol/l Adenosine triphosphate

(f) $\geq$ 150 µ/ml Lipases

(g) 0.4 µ/ml Glycerol kinase

(h) $\geq$ 1.5 µ/ml Glycerol-3-phosphate oxidase

(i) $\geq$ 0.5 µ/ml Peroxidase

(j) 2.229 mmol/l Triglyceride standard

2.5.3.3 Assay Procedure for Serum Triglyceride Determination

Table 2.3: Serum Triglyceride Determination

	Reagent Blank (µl)	**Standard (µl)**	**Sample (µl)**
Sample	-	-	10
Standard	-	10	-
Reagent	1000	1000	1000

The contents of each test tube were mixed and incubated for 10 minutes at room temperature and the absorbance of the samples (A_{Sample}) and standard ($A_{Standard}$) were measured against the reagent blank within 60 minutes at 500 nm wavelength and 1cm light path.

The concentration of triglyceride was calculated using the relationship:

$$Triglyceride\ Concentration = \frac{A_{Sample}}{A_{Standard}} \times 2.229\ (mmol/l)$$

$$Triglyceride\ Concentration = \frac{A_{Sample}}{A_{Standard}} \times 200\ (mg/dl)$$

2.5.4 DETERMINATION OF SERUM HIGH DENSITY LIPOPROTEIN-CHOLESTEROL (HDL-CHOLESTEROL)/LOW DENSITY LIPOPROTEIN CHOLESTEROL (LDL-CHOLESTEROL)

2.5.4.1 Principle

HDL-Cholesterol concentration in the serum sample was determined spectrophotometrically as was described by Friedewald *et al.* (1972) as modified by Lopes-Virella *et al.* (1977). Low density lipoprotein (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the high density lipoprotein (HDL) fraction, which remains in the supernatant, is determined spectrophotometrically at 500 nm.

2.5.4.2 Reagents

The reagents used for this determination were provided in the commercially available Randox Kit as follows:

a) 0.55 mmol/l phosphotungstic Acid
b) 23 mmol/l Magnesium Chloride
c) Randox Aqueous Cholesterol standard
d) HDL-Cholesterol Precipitant

2.5.4.3 Assay Procedure for HDL-Cholesterol Determination

The determination was carried out in two stages

i) Precipitation: The samples (200 µl) were pipetted into centrifuge tubes and 500 µl of the diluted precipitant added to each of the sample tubes. The contents were mixed and allowed to stand for 10 minutes at room temperature. The tubes were then centrifuged for 10 minutes at 4,000 rpm. The clear supernatant was decanted within two hours and used for the determination.

ii) The Assay

Table 2.4: Assay Procedure for HDL-Cholesterol Determination

	Reagent Blank	**Standard**	**Sample**
Distilled Water	100 µl	-	-
Supernatant	-	-	100 µl
Standard	-	100 µl	-
Reagent	1000 µl	1000 µl	1000 µl

The contents of each test tube were again mixed, incubated for 5minutes at $37^{0}C$ and the absorbance of sample (A_{Sample}) and standard ($A_{Standard}$) measured against the reagent blank within 60minutes.

1. Concentration of Result:

$$Concentration\ of\ HDL-Cholesterol\ in\ Supernatant = \frac{\Delta A_{Sample}}{\Delta A_{Standard}} \times Conc.\ of\ Standard$$

2. LDL-Cholesterol

$$LDL-Cholesterol = Total\ Cholesterol - \frac{Triglycerides}{2.2} - HDL-Cholesterol\ (mmol/l)$$

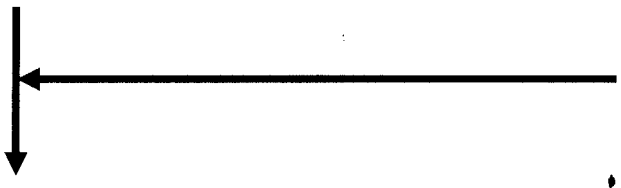
2.5.5 DERTERMINATION OF ALANINE AMINOTRANSFERASE (ALT) ACTIVITY

2.5.5.1 Principle

This was based on the method of Rietman and Frankel (1957). Alanine aminotransferase (ALT) activity was measured by monitoring the concentration of pyruvate hydrazone formed when pyruvate reacts with 2,4 – dinitrophernyl hydrazine.

Pyruvate is a reaction product of the transammase reaction catalysed by ALT.

α-Ketoglutarate + L-alanine



L-Glutamate + Pyruvate

2.5.5.2 Reagents

The reagents used for this determination were provided in the commercially available kit (Randox, U.K.):

a. 100 mmol/l Phosphate buffer (pH.7.4)
b. 200 mmol/l L - Alanine
c. 2.0 mol/l α – Ketoglutarate

} Solution 1

d. 2.0 mmol/l 2,4-Dinitropheylhydrazine } Solution 2

e. 20 mmol Stock standard pyruvate: This was prepared by diluting 220 mg of sodium pyruvate in 100ml of phosphate buffer above and refrigerated.

f. 4 mM Working pyruvate standard. This was prepared by diluting the stock standard 1: 5 with phosphate buffer and stored at -15^{O}C.

g. 0.4N Sodium hydroxide: this was prepared by dissolving 16 g of sodium hydroxide (NaOH) in water and made up to 1 litre.

2.5.5.3 Procedure

Table 2.5: Assay procedure for serum ALT Activity

Procedure	Reagent	Sample
Solution 1	0.5 ml	0.5 ml
Sample	-	0.1 ml
Distilled Water	0.1 ml	-

The contents of each tube were mixed and incubated for exactly 30 minutes at $37^{O}C$

Solution 2	0.5 ml	0.5 ml

Components were mixed and allowed to stand for 20 minutes at room temperature.

Sodium hydroxide	5.0 ml	5.0 ml

The contents of the tube were finally mixed and allowed to stand for 5 minutes. The absorbance was measured at 540 nm against the sample blank.

Calculation

The activity of GPT in the sera was determined using a calibration curve obtained from a standard calibration table.

Calibration curve

Table 2.6: Standard Calibration Table for Pyruvate

Absorbance	U/I	Absorbance	U/I
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77

0.200	34	0.450	83
0.225	39	0.475	83
0.250	43		

2.5.6 DETERMINATION OF ASPARTATE AMINOTRANSFERASE (AST) ACTIVITY

2.5.6.1 Principle

The principle according to the method of Reitman and Frankel (1957) is based on the fact that glutamate-oxaloacatate transaminase activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrphyenylhdrazine.

$$\text{Oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST (GOT)}} \text{L – glutamate} + \text{oxaloacetate} \quad \ldots\ldots\ldots\ldots 2.9$$

2.5.6.2 Reagents (Randox, U.K.)

a) 100mmol/l Phosphate buffer (pH 7.4)
b) 100mmol/ L – asparartate
c) 2 mmol/l α – Oxoglutarate

} Solution 1 (Buffer/Substrate Solution)

d) 2 mmol/l 2,4-dinitrophenylhydrazine } Solution 2

e) 0.4 N NaOH: This was prepared by dissolving 16g of sodium hydroxide (NaOH) in distilled water and made up to 1 litre.

2.5.6.3 Procedure

The solutions were pipetted into labelled tubes as follows:

Table 2.7: Assay Procedure for Serum AST Activity

	Sample Blank	**Sample**
Sample	-	0.1 ml
Solution 1	0.5 ml	0.5 ml

The contents of the tubes were mixed and incubated for exactly 30 minutes at $37^{O}C$

Solution 2	0.5 ml	0.5 ml

This was mixed and allowed to stand for 20 minutes at 20 to $25^{O}C$

Sodium hydroxide	5.0 ml	5.0 ml

This was finally mixed and the absorbance of the sample (A_{Sample}) read against the sample blank after 5 minutes.

Calculation

The respective activities of AST (GOT) in the samples were obtained from the Table below.

Table 2.8: Standard Calibration Table for Oxaloacetate

Absorbance	**U/l**	**Absorbance**	**U/l**
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.070	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

2.5.7 DETERMINATION OF PACKED CELL VOLUME (PCV/HAEMATOCRIT)

The centrifugation method was used for this determination. In this method, the microhaematocrit tubes were dipped into a sample of properly mixed anticoagulated blood. These tubes were centrifuged at 3,500 rpm for 10 minutes. After the centrifugation, the heights of the red cell column and the total blood column were measured.

The formular below was used to calculate the PCV:

$$PCV = \frac{Height\ of\ Red\ Cell\ Column}{Height\ of\ Total\ Blood\ Column}$$

2.5.8 DETERMINATION OF HAEMOGLOBIN (The Cyanomethaemoglobin Method)

In this method, the blood samples were diluted in a buffered solution of potassium ferricyamide and potassium cyanide to yield cyanomethaeglobin. The absorbance of the solution was read colorimetrically at a wavelength of 540 nm.

The final haemoglobin concentration was calculated as follows:

$$Hb = \frac{T \times C \times D}{A \times 1000} g/100ml$$

Where:

T = Test Absorbance at 540 nm

A = The Standard Absorbance at 540 nm

C = The Concentration of Cyanomethaemoglobin Standard (mg/100ml)

D = Dilution Factor

2.5.9 DETERMINATION OF RED BLOOD CELL COUNT (RBC)

Whole blood samples were diluted by washing 20 µl of blood taken into a 'shell back' or position displacement pipetted, into 4.0 ml of diluents to obtain a final dilution of 1 in 20. The diluted samples were then mixed, and the cells counted microscopically using an improved Neubauer ruled counting chamber. The cells were counted after the cells settled out of suspension.

The final red blood cell count was obtained as follows:

$$Red\ Cell\ Count = \frac{N \times DF \times 10^6}{A \times D} / litre$$

Where:

N = Number of cells

DF = Dilution Factor

A = Area of Counting Chamber

D = Depth of Counting Chamber

2.5.10 DETERMINATION OF WHITE BLOOD CELL COUNT

2.5.10.1 Principle

EDTA anticoagulated blood is used for this determination. The whole blood is diluted 1 in 20 in an acid reagent (precisely 2% acetic acid) tinged with gentian violet. This reagent, in addition to haemolysing the red cells, stained the white cell nuclei and hence making the white cells more readily visible to be counted. The cells are counted microscopically using an improved Neubauer ruled counting chamber (haemocytometer).

2.5.10.2 Procedure

Properly mixed anticoagulated blood sample (0.02 ml) was added to 0.38 ml of the diluting reagent and dispensed into a test tube.

One of the grids of the counting chamber was filled with the diluted blood sample using a Pasteur pipette, ensuring that the area is not overfilled. The filled chamber was left undisturbed for two minutes to allow time for the white cells to settle. The cells in the four large corner squares of the chamber were then counted microscopically using the × 10 objective.

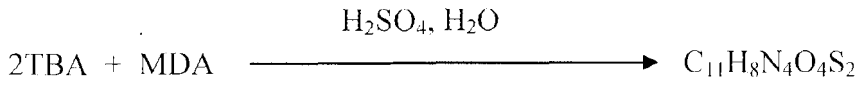
The final white cell count for the samples was calculated using the same basic formular:

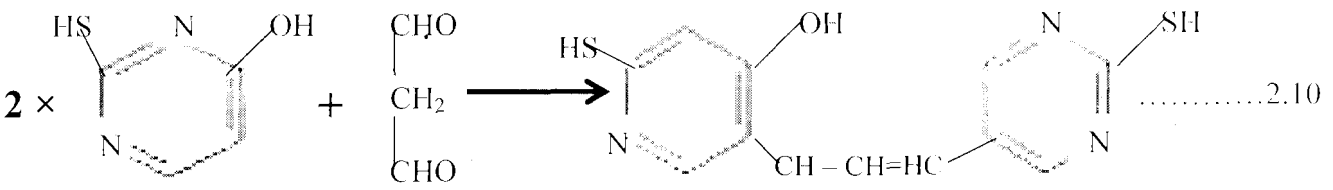
$$White\ Blood\ Cell\ Count = \frac{N \times DF \times 10^6}{A \times D} / litre$$

2.6.1 LIPID PEROXIDATION ASSAY

2.6.1.1 Principle

Lipid peroxidation in the supernatant fractions of the livers and kidneys was determined spectrophotometrically by assessing the level of thiobarbituric acid reactive substances (TBARS) as described by Varshney and Kale (1990). The results were expressed as malondialdehyde (MDA) formed relative to an extinction coefficient of 1.56 × 10^6 mol/cm. Small quantities of MDA are produced during lipid peroxidation. These react with thiobarbituric acid (TBA) to generate a pink coloured complex which in acid solution absorbs light at 532 nm and fluoresces at 532 nm. This complex is readily extractable into organic solvents such as butan-1-ol (Fig. 2.1).





TBA MDA Pink Coloured Complex

2.6.1.2 Reagents

a) **0.1 M HCl:** This was prepared by adding 3.65 ml of concentrated HCl to 996.35 ml of water.

b) **0.75% TBA:** TBA (0.75 g) was dissolved and made up to 100 ml with 0.1 M HCl. The solution was allowed to dissolve properly by shaking in a boiling water bath.

c) **20% Trichloroacetic acid (TCA):** Trichloroacetic acid (20 g) was dissolved in water and made up to 100 ml with distilled water. The solution was stored in the refrigerator.

d) 0.15 M Tris-HCl/KCl buffer:

(i) KCl (1.15 g) was dissolved and made up to 100 ml with distilled water.

(ii) Tris-HCl (2.36 g) was dissolved and made up to 100 ml with distilled water.

Solutions (i) and (ii) were then mixed together.

2.6.1.3 Procedure

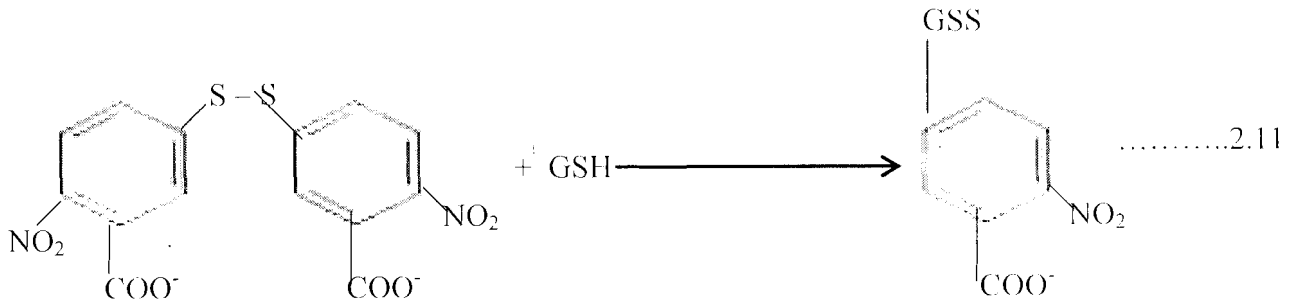
The liver and kidney post mitochondrial fractions were diluted 20 and 10 times respectively. Aliquot (0.4 ml) of each diluted sample was mixed with 1.6 ml of 0.15 M tris KCl buffer followed by the addition of 0.5 ml of 20% TCA. A quantity of 0.5 ml of 0.75% TBA was then added and the mixture placed in a water bath for 45 minutes at $80^{O}C$. At the expiration of the incubation time, the mixture was allowed to cool and centrifuged at 3000 rpm for 10 minutes. Butanol (20 ml) was finally added to extract the product. The absorbance of the clear supernatant was measured against a reference blank of distilled water at 532 nm. The concentration of MDA in each sample was calculated by dividing the absorbance with the molar extinction coefficient.

2.6.2 DETERMINATION OF REDUCED GLUTATHIONE (GSH)

2.6.2.1 Principle

This was based on the method of Jollow *et al.* (1974). Reduced glutathione (GSH) forms the bulk of non-protein sulfhydryl groups. This method is based on the formation of relatively stable yellow colouration when Ellman's reagent is added to a sulfhydryl compound as shown in Rxn 2.11.

The chromophoric product, 2-nitro-5-thiobenzoic acid reagent with reduced glutathione absorbs at 412 nm. The absorbance at 412 nm is therefore proportional to the glutathione content.



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Ellman Reagent

2-Nitro-5-thiobenzoic acid

2.6.2.2 Reagents

(a) **0.1 M Phosphate buffer, pH 7.4:** This was prepared by dissolving 7.163 g of Na_2HPO_4 and 1.36 g of KH_2PO_4 in distilled water and making up to 300 ml. The pH was adjusted to 7.4.

(b) **Reduced GSH:** GSH (40 mg) was dissolved in phosphate buffer above and made up to 100 ml with the buffer. This served as the stock solution for the GSH standard calibration curve.

(c) **Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid):** Ellman's reagent (40 mg) was dissolved in the phosphate buffer and made up to 100 ml with the same phosphate buffer.

(d) **4.0% Sulfo-salicyclic acid:** Sulfosalicyclic acid was dissolved in distilled water and made up to 100 ml with distilled water.

2.6.2.3 REDUCED GLUTATHIONE CALIBRATION CURVE

Serial dilutions of the stock GSH solution were made using the phosphate buffer for dilution (to a volume of 0.5 ml). Ellman's reagent (4.5 ml) was added to each solution. The absorbance of the coloured solution developed was read at 412 nm within 5 minutes of the colour generation. Optical density was plotted against glutathione concentration.

2.6.2.4 Procedure

One millilitre (1.0 ml) of each post mitochondrial fraction was deproteinized by adding an equal volume of 4% sulfo-salicyclic acid. The mixture was centrifuged at 7, 000 rpm for 15 minutes at $2^{O}C$. Supernatant (0.5 ml) was added to 4.5 ml of Ellman's reagent. A blank was prepared by adding 0.5 ml of 4% sulfo-salicyclic acid to 4.5 Ellman's reagent. The absorbance was read at 412 nm and the equivalent GSH concentration determined from the calibration curve.

2.6.3 DETERMINATION OF CATALASE ACTIVITY [EC 1.11.1.6]

2.6.3.1 Principle

The catalase activities of the supernatant fractions from the liver and kidney were determined according to the method of Sinha (1972). This method is based on the fact that dichromate in solution with acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide via the formation of perchromic acid which is an unstable intermediate. Chromic acetate produced is measured colorimetrically at 570 to 610 nm. The presence of dichromate in the mixture does not affect the colorimetric determination of chromic acetate, because dichromate does not absorb in the 570 to 610 nm region. The catalase preparation is allowed to split hydrogen peroxide for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate/acetic acid mixture; and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically.

2.6.3.2 Reagents

(a) **5% Potassium dichromate ($K_2Cr_2O_7$):** $K_2Cr_2O_7$ (5 g) was dissolved in distilled water and made up to 100 ml.

(b) **0.2 M H_2O_2:** This was prepared by adding 22.68 ml of 30% H_2O_2 (8.82 M) to distilled water and made up to 100 ml.

(c) **Dichromate/acetate acid:** This was prepared by mixing 5% solution of $K_2Cr_2O_7$ with glacial acetic acid in a 1:3 volume ratio.

(d) **0.1 M Phosphate buffer, pH 7.0:** Disodium hydrogen tetraoxophosphate (V) dodecahydrate ($Na_2HPO_4.12H_2O$) (3.58 g) and 1.19 g of Sodium dihydrogen tetraoxophosphate (v) dihydrate ($NaH_2PO_4.2H_2O$) was dissolved in distilled water and made up to 100 ml with distilled water. The pH was adjusted to 7.0.

2.6.3.3 PREPARATION OF STANDARD HYDROGEN PEROXIDE (H_2O_2) CURVE

To prepare this curve, different concentrations of H_2O_2 ranging from 10 to 100 micromoles were pipetted into test tubes. Dichromate/acetic acid (2 ml) was added to each tube. On adding the dichromate/acetic acid, an unstable blue precipitate of perchromic acid was instantaneously produced. Heating for 10 minutes in a boiling water

bath changed the colour of the solution to stable green due to the formation of chromic acetate. After cooling at room temperature, the volumes of the samples were made to 3 ml with distilled water and the optical density measured at 570 nm. The curve was gotten by plotting absorbance on the vertical axis against concentration of H_2O_2 on the horizontal axis.

2.6.3.4 Procedure

The supernatant fractions were properly diluted (50 times for the liver and 20 times for the kidney). H_2O_2 solution (4 ml of 0.2 M) was added to 5 ml of phosphate buffer. Properly diluted fractions (1.0 ml) were added to the H_2O_2/buffer mixture and the mixture was gently mixed at room temperature. One millilitre (1.0 ml) portion of the reaction mixture was withdrawn and blown into 2 ml dichromate/acetic acid reagent at one minute interval and the steady absorbance reading taken at 570 nm.

2.6.3.5 Calculation

The monomolecular velocity constant, K, for the decomposition of hydrogen peroxide by catalase was determined by using the equation for a first-order reaction:

$$K = \frac{1}{t} \log \frac{S_o}{S}$$

Where S_0 is the initial H_2O_2 concentration and S is the concentration of H_2O at a particular time interval given as t (minutes). The values of K are plotted against t, and the velocity constant of catalase K[o] at 0 minute determined by extrapolation (that is, the interval on the vertical axis). The catalase contents of the samples were expressed in terms of catalase feiahigkeit or Kat.F`.

$$Kat.f. = \frac{K[0]}{mg\ protein/ml}$$

2.6.4 DETERMINATION OF SUPEROXIDE DISMUTASE (SOD) ACTIVITY [EC 1.15.1.1]

2.6.4.1 Principle

This determination was carried out as was described by Fridovich (1989). The ability of superoxide dismutase to inhibit the autoxidation of adrenaline was the basis of the SOD assay. Superoxide generated by the xanthine oxidase reaction is shown to cause the oxidation of adrenaline to adrochrome. The yield of adrenochrome produced per superoxide introduced increase with increasing pH (Valerino and McCormack, 1971) and also with increasing concentration of adrenaline. These led to the proposal that autoxidation of adrenaline proceeds by at least two distinct pathways: one of which is a free radical chain reaction involving superoxide radical and hence could be inhibited by SOD.

2.6.4.2 Reagents

a) **0.05 M Phosphate buffer; pH 7.8:** This was prepared by dissolving 6.97 g of K_2HPO_4 and 1.36 of KH_2PO_4 in distilled water and making up to 1000 ml with distilled water. The pH was adjusted to 7.8.

b) **0.059% Adrenaline solution:** It consists of 0.01 g of adrenaline dissolved in 17 ml of distilled water.

2.6.4.3 Procedure

The post mitochondrial fractions were properly diluted (20 times for the liver and 10 times for the kidney). A volume of 0.2 ml of each diluted sample was added to 2.5 ml of 0.05 M phosphate buffer. The mixture was equilibrated in the spectrophotometer before adding adrenaline solution. The reaction started with the addition of 0.3ml of freshly prepared adrenaline solution to the mixture followed by quick mixing by inversion of the cuvette. The reference cuvette therefore contained 2.5 ml buffer, and 0.3 ml of adrenaline. The increase in absorbance was taken at 480 nm for 150 seconds at 30 seconds interval.

2.6.4.4 Calculation

$$Increase\ in\ Absorbance\ per\ Minute = \frac{A_3 - A_0}{2.5}$$

where A_0 = Absorbance after 30 seconds

A_3 = Absorbance after 150 seconds

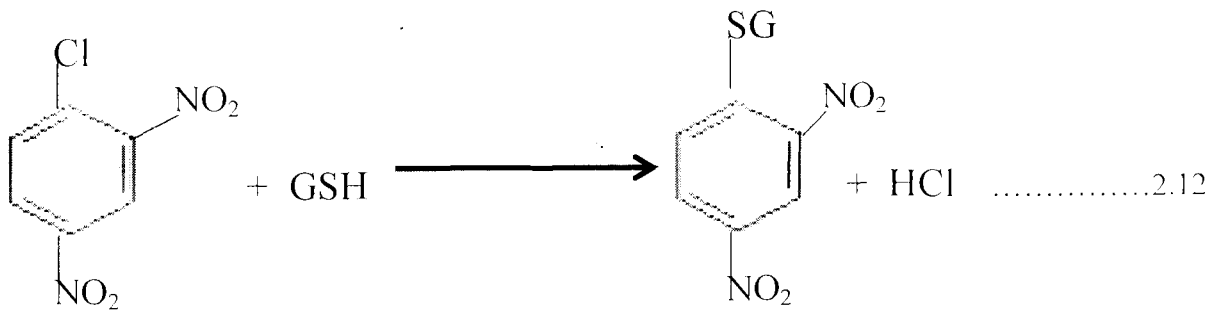
$$\%\ Inhibition = \frac{100 - (increase\ in\ absorbance\ for\ substrate)}{(increase\ \ in\ absorbance\ for\ blanck} \times 100$$

One of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.

2.6.5 DETERMINATION OF GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY [EC 2.5.1.18]

2.6.5.1 Principle

The GST activity of the tissue fractions was determined according to the method of Habig *et al.* (1974) and as reported and modified by Rajurkar *et al.* (2003). It involves the formation of a complex by the enzymatic conjugation of GSH with the aromatic substrate, 1-Chloro-2, 4-dinitribenzene. The complex formed absorbs at 340 nm.



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1-Chloro-2,4-dinitrobenzene

2.6.5.2 Reagents

(a). **20 mM 1-Chloro-2,4-dinitrobenzene (CDNB):** A weight of 3.37mg of 1-Chloro-2,4-dinitrobenzene (CDNB) was dissolved in 1 ml of absolute ethanol in simple ratio to required volume.

(b) **0.1 M Phosphate buffer, pH 6.5:** This was prepared by dissolving 4.96 g of K_2HPO_4 and 9.73 g of KH_2PO_4 in distilled water. The pH was adjusted to 6.5.

(c) **0.1 M Reduced Glutathione:** 30.37 mg of glutathione was dissolved in 1 ml of 0.1 M phosphate buffer, pH 6.5; in simple ratio to the required volume.

2.6.5.3 Procedure

1-Chloro-2,4-dinitrobenzene (0.15 ml) was added to 2.79 ml of the buffer. The mixture was incubated at $37^{O}C$ for 15 minutes. After incubation, the reaction was started by adding 0.03 ml of diluted tissue fraction (20 times for the liver, 10 times for the kidney). The absorbance was read at 30 second intervals for 3 minutes. The incubated reaction mixture without the tissue fraction was used as the blank in each case. The readings were taken immediately after incubation. The enzyme activity was calculated with an extinction coefficient of 9.6 mM/cm for CDNB and was expressed in µM/min at $25^{O}C$ and the specific enzyme activity was expressed in µM/min/mg protein.

2.6.5.4 Calculation

$$GST\ Activity = \frac{Absorbance/\min}{9.6} \times \frac{Total\ reaction\ Volume}{mg/0.03ml\ Protein}$$

= µM/min/mg Protein

2.6.6 DETERMINATION OF HEPATIC HEXOKINASE (EC 2.7.1.1) ACTIVITY

2.6.6.1 Principle

The hepatic hexokinase activity was determined according to the method of Lapeir and Rodnick (2001) as reported by Ugochukwu and Babady (2003). This method is based on the fact that the primary reaction of glycolysis.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6-phosphate} + \text{ADP}$$ is coupled to the first reaction of the pentose phosphate pathway:

$$\text{Glucose 6-phosphate} \xrightarrow{\text{G-6-PDhase}} \text{6-Phosphogluconolacetone}$$

NADP NADPH

The rate of glucose utilization (hexokinase activity) is then monitored at $37^{0}C$ by following the appearance of NADPH at 340 nm for 3 minutes at 30 seconds interval.

2.6.6.2 Reagents

(a) Homogenising buffer; pH 7.5 as 2.4.3.2.1 (3).

(b) Reaction mixture components.

(i) **50 mM Tris-HCl:** Tris-HCl (7.88 g) was dissolved in 1 litre of distilled water.

(ii) **0.8 mM EDTA:** Ethylene diamine tetraacetate (0.00297 g) was dissolved in 10 ml of distilled water.

(iii) **7.5 mm $MgCl_2$:** Magnesium chloride (0.1425 g) was dissolved in 200 ml of water.

(iv) **1.5 mM KCl:** Potassium chloride (0.0022 g) was dissolved in 20 ml of distilled water.

(v) **2.5 mM ATP:** Adenosine triphosphate (0.025 g) was dissolved in 200 ml of distilled water.

(vi) **0.7 IU.ml Glucose 6-phosphate dehydrogenase (G-6-PD):** G-6-PD (0.038 mg) was dissolved in 10 ml of the homogenising buffer.

(vii) **0.4 mM β-NADP, pH 7.4:** β-NADP (2.97 mg) was dissolved in 10 ml of distilled water.

(viii) **10 mM D-Glucose:** Glucose (0.1802 g) of glucose was dissolved in 100 ml of distilled water.

2.6.6.3 Procedure

Liver tissues (0.5 g) were homogenised with ten times their weight in volume of the homogenising buffer. The homogenates were centrifuged at 20,000 rpm for 15 minutes at $4^{O}C$ and the supernatants used for the enzyme assay. A volume of 0.1 ml each of the reaction mixture components was pipetted into a cuvette containing 0.04 ml of G-6-PD. Next, 20 µl of the diluted liver homogenate (1:20 in homogenising buffer) was added to the cuvette at $37^{O}C$. The reaction was initiated by adding 0.1 ml of 10 mM glucose. The enzyme activity (change in absorbance) was measured by following the appearance of NADPH at 340 nm for 3 minutes at 30 second interval.

2.6.6.4 Calculation

$$\frac{Change\ in\ Absorbance}{Extinction\ Coefficient\ (\varepsilon_0)} = \frac{Concentration}{Time}$$

Since NADPH $\varepsilon_0 = 620.0$

$$Specific\ Enzyme\ Activity = \frac{Concentration/Time}{mg\ Protein\ in\ 20\ \mu l}$$

2.6.7 DETERMINATION OF HEPATIC GLUCOKINASE (EC 2.7.1.3) ACTIVITY

2.6.7.1 Principle

The hepatic glucokinase activity was determined according to the method of Newgard *et al.* (1983) as modified and reported by Ugochukwu and Babady (2003). The

production of Glucose 6-phosphate (total glucose phosphorylating capacity) by glucokinase in the presence of ATP was linked to the reduction of NAD by glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*.

2.6.7.2 Reagents

(a) Homogenising buffer (2); pH 7.4 as in 2.4.3.3

(b) Reaction mixture components

(i) **100 mM Tris/HCl:** Tris/HCl (3.152 g) was dissolved in 100 ml of water and the pH adjusted with drops of HCl.

(ii) **7.5 mM $MgCl_2$:** $MgCl_2$ (0.1425 g) was dissolved in 200 ml of distilled water.

(iii) **5 mM ATP:** ATP (0.025 g) of ATP was dissolved in 100 ml of distilled water.

(iv) **1 mM NAD:** NAD (0.00332 g) was dissolved in 5 ml of distilled water.

(v) **5.5 I.U. Glucose 6-phosphate dehydrogenase (G-6-PD):** G-6-PD (0.0304 mg) was dissolved in 10 ml of homogenising buffer.

(vi) **10 mM D-Glucose:** Glucose (0.1802 g) was dissolved in 100 ml of distilled water.

2.6.7.3 Procedure

Liver tissues (0.5 g) were homogenised in nine volumes of the homogenising buffer. After centrifugation at 12,000 rpm for 20 minutes at $4^{O}C$, the supernatants were used to measure the enzyme activity.

The reaction mixture contained a final volume of 1 ml with 0.3 ml of G-6-PD, 10 µl of the diluted (1:10) liver homogenate and 0.1 ml of 10 mM D-glucose. The blank cuvette was devoid of glucose and G-6-PD. The production of NADH was monitored at 340 nm.

2.6.7.4 Calculation

$NADH_{340} = 6220$

$$Specific\ Enzyme\ Activity = \frac{\Delta A/\min}{\varepsilon_{()}} = \frac{Concentration\ (mmoles)/\min}{mg\ Protein\ 10\mu l}$$

2.6.8 DETERMINATION OF HEPATIC PHOSPHOFRUCTOKINASE (PFK) (EC 2.7.1.11) ACTIVITY

2.6.8.1 Principle

This assay was based on the fact that the rate determining reaction of glycolysis is catalysed by phosphofructokinase (PFK):

Fructose 6-phosphate $\xrightarrow{PFK}$ Fructose-1,6-bisphosphate was coupled to the reduction of NAD to NADH.

The enzyme activity was measured according to the method of Castano *et al.* (1979) modified by Ugochukwu and Babady (2003) at 0.25 mM fructose 6-phosphate and the activity was determined as Vmax.

2.6.8.2 Reagents

(a) Homogenising buffer; pH 8.5 as in 2.4.3.3 (4).

(b) Reaction mixture components.

(i) 70 mM Tris/HCl: This was prepared by dissolving 1.576 g of Tris in 200 ml of water.

(ii) 100 mM KCl: KCl (0.0745 g) was dissolved in 100 ml of distilled water.

(iii) 5 mM $MgCl_2$: $MgCl_2$ (0.0047 g) was dissolved in 10 ml of distilled water.

(iv) 1.5 mM ATP: ATP (0.0076 g) was dissolved in 10 ml of distilled water.

(v) 1 mM NAD:

(vi) 5 mM Pi: Disodium hydrogen phosphate (0.0094 g) was dissolved in 100ml of distilled water.

(vii) 0.1 mM AMP: Adenosine monophosphate (0.365 mg) was dissolved in 100 ml of distilled water.

(viii) 1 mM NH_4Cl: NH_4Cl (0.001 g) was dissolved in 20 ml of distilled water.

(ix) 5 mM Fructose 6-phosphate: F-6-P (0.024 g) was dissolved in 20 ml of distilled water

(ix) **1 Unit of Phosphoglucose isomerase:**

2.6.8.3 Procedure

Liver tissues (0.4 g) were homogenised in 10 volumes (w:v) of the homogenising buffer. The homogenates were centrifuged at 20,000 rpm for 15 minutes at $4^{O}C$.

Supernatant (50 µl) was used for the enzyme assay. The supernatant and all the fraction mixture components in a final volume of 1 ml were incubated at $37^{O}C$ and the change in absorbance (appearance of NADH) was followed at 340 nm for 3 minutes at 30 second intervals.

2.6.8.4 Calculation

$NADH_{340} = 6220$

$$Specific\ Enzyme\ Activity = \frac{Concentration\ (mmoles)/\min}{mg\ Protein\ in\ 50\ \mu l}$$

2.6.9 DETERMINATION OF HEPATIC GLYCOGEN CONTENT

2.6.9.1 Principle

Glycogen content was determined as described by Ong and Khoo (2000). This method is based on the fact that hot concentrated KOH digests the glycogen contained in the liver tissues. The glycogen is then determined colorimetrically as glucose using anthrone reagent.

2.6.9.2 Reagents

(a) **30% KOH:** KOH (30 g) was dissolved in 100 ml of distilled water and refrigerated.

(b) **Anthrone reagent:** This was prepared by dissolving 1 g of anthrone in 500 ml of concentrated H_2SO_4.

(c) **Stock Glucose solution (1 mg/ml)**.

2.6.9.3 Procedure

Liver tissues (0.5 g) were homogenised in 10 ml volumes (w:v) of ice-cold 30% KOH and boiled at 100^{O}C for 30 minutes.

The homogenates were precipitated with 5 volumes of 95% ethanol. The precipitate was washed with another 5 volumes of ethanol and then resolubilised in water.

One millilitre (1 ml) of the resolubilised samples were pipetted into separate test tubes and 5 ml of freshly prepared anthrone reagent was introduced into each tube. The contents were mixed properly, covered and immediately boiled for 20 minutes. After 20 minutes the colour developed, the tubes were cooled and the absorbance read at 625 nm.

2.6.9.4 PREPARATION OF THE GLUCOSE STANDARD CURVE

Serial dilutions of the stock glucose solution were made with distilled water. Five millilitres (5 ml) of freshly prepared anthrone reagent were added to 1 ml of each diluent. The mixture was thoroughly mixed, covered and boiled for 20 minutes after which the absorbance was read at 625 nm. The graph of absorbance against glucose concentration was later plotted (Appendix 14).

2.7 HISTOPATHOLOGIC EXAMINATION

(A.) Fixation and Washing:

Formalin (10%) was used as the fixative and for the purpose of preservation. A thin section of the tissue (about 1 to 2 cm in diameter) was trimmed with a sharp razor blade. The small pieces of the tissue were placed in the 10% formalin, the container was shaken gently several times to make sure that the fluid had reached all surfaces and the specimens were not sticking to the bottom and this was allowed to stand for 24 hours, to allow proper fixings. The fixed tissues were washed with running water for 24 hours to free them from excess fixatives.

(B.) Dehydration:

All water was removed from the tissue before embedding the tissue in paraffin. The dehydration was achieved by immersing the thin sections of the tissue in automatic tissue processor containing 12 jars.

The first three (3) jars contained 70, 90 and 95% alcohol respectively. This was done to remove the water content in the tissues. The absolute alcohol reduced the shrinking that occurred in the tissue. The time allowed for each step was 30 minutes. A second change of absolute alcohol was included to ensure complete removal of water. This was achieved in the second three (3) jars of the automatic tissue processor. This is called a well-refining step.

(C.) Clearing:

Solutions of xylene were used for clearing the tissue sections. This step was achieved in the third jar of the automatic tissue processor. This is because the alcohol (ethanol) used for dehydration would not dissolve or mix with molten paraffin, the tissue was immerse in xylene solution which was miscible with both alcohol and paraffin before infiltration could take place.

Clearing removes opacity from dehydrated tissue, making them transparent. A period of 15 minutes was allowed to elapse before the tissue was removed from the solution for infiltration with paraffin.

(D.) Infiltration with Paraffin:

Paraffin wax with a melting point of 50 – 52^{O}C (range) was used to infiltrate the tissue. The tissue was transferred directly from the clearer to a water bath containing melted paraffin. After 30 – 60 minutes incubation in the first bath, the tissue was then removed to a fresh dish of paraffin contained in the fourth three jars of the automatic tissue processor for a similar length of time.

(E.) Embedding (Blocks) with Paraffin:

As soon as the tissue was thoroughly infiltrated with paraffin, it (paraffin) was ready to be embedded. The paraffin was allowed to solidify around and within the tissue.

The tissue was then placed in a small container already filled with melted paraffin and was cooled rapidly with water to embed the tissue sections.

(F.) Paraffin Sectioning:

The embedded blocks were trimmed into squares and mixed in the microtome knives for sectioning after which the sections were floated on a water bath.

(G.) Mounting:

Glazing slides were thoroughly cleaned and a thin smear of albumen fixative was made on the slides. The albumenised slide was used to collect the required section from the rest of the ribbon in the water. The section on the glass slide was kept moist before staining.

(H.) Staining with Haematoxylin:

The slides were passed through a series of jars containing alcohols of decreasing strength and various staining solutions in the following order:

Table 2.9: Staining Order for Histopathological Examination

1	Xylene	3 minutes
2	Absolute	2 – 3 minutes
3	95% Alcohol	2 minutes
4	70% Alcohol	2 minutes
5	Lugol Solution	3 minutes
6	Running Water	3 minutes
7	5% Sodium thiosulphate	3 minutes
8	Running Water	3 minutes
9	Delafield haematoxylin	5 minutes
10	Running Water	3 minutes
11	Scott Solution	9 minutes
12	Running Water	3 minutes

The counterstaining of the tissue with eosin was followed in the order (1 – 6) as stated below (Table 2.10):

Table 2.10: Counterstaining Order with Eosin

1	70% Alcohol	1 dip
2	95% Alcohol	2 dips
3	Absolute Alcohol	3 minutes
4	Absolute Alcohol – Xylene (1:1)	3 minutes
5	Xylene	3 minutes
6	Mounting Medium: The section was kept with xylene while cover glass was added on the glass slide	

Microscopic Observation of Slide

The slides prepared were mounted on photomicroscope, one after the other and were then viewed at different magnification power of the microscope. Photograph of each of the slides was taken. The results obtained are as shown.

2.8 STATISTICAL ANALYSIS

The results were analysed for statistical significance by ANOVA, and was further subjected to Fischer LSD post hoc test using the SPSS Genstat Release (Windows 98) software package version 11. All data were expressed as mean ± SD, n=4. Differences between means were considered significant at $P<0.05$.

CHAPTER THREE

RESULTS

3.1 EXTRACT YIELD

When a 500 g quantity of the chipped dry roots of *Sarcocephalus latifolius* was subjected to hot aqueous extraction, the yield was 11.52% of the starting material. The hot aqueous extract of *Sarcocephalus latifolius* and *Daniella oliveri* dry roots in combination (SDA) gave a yield of 7.46%. The ethanol extract of *Sarcocephalus latifolius* dry root chips (SLE) weighed 59.0 g, giving a yield of 11.8%.

3.2 PHYTOCHEMICAL ANALYSIS OF THE EXTRACTS (SLA, SDA AND SLE)

The results of the qualitative phytochemical analysis of the three different extracts (SLA, SDA and SLE) is shown in Table 3.1. The table shows that the bioactive compounds that are found in all include flavonoids, alkaloids, resins, saponins, tannins, proteins, carbohydrates, reducing sugar, glycosides, steroids, terpenoids, fats and oils and acidic substances.

Table 3.1: Phytochemical analysis of SLA, SDA and SLE

Bioactive substances	**SLA**	**SDA**	**SLE**
Flavonoids	-	+++	+
Alkaloids	+++	+++	+++
Resins	-	-	+
Saponins	+++	+++	+
Tannins	+++	++	
Proteins	+++	+	++
CHO	+++	+++	+++
Reducing sugar	+++	+++	++
Glycosides	++++	++++	+++
Steroids	++	+++	++
Terpenoids	+++	+++	+++
Fats and oils	-	-	-
Acidic substances	-	-	+

\+ Present in small concentration
++ Present in moderately high concentration
+++ Present in very high concentration
– Absent
++++ Abundantly present

3.3 TOXICITY STUDIES OF THE EXTRACTS

3.3.1 ACUTE TOXICITY (LD_{50})

Twenty four (24) hour toxicity testing on mice showed that the three different extracts up to 5 g/kg elicited no significant modification in the general behaviour of the animals. This concentration is the maximum allowable dose by the Organisation for Economic Co-operation and Development (OECD) Guideline 423 for the testing of chemicals (OECD, 2000).

3.3.2 SUB ACUTE TOXICITY STUDIES OF SLA, SDA AND SLE

Results of sub-acute toxicity studies of the three extracts to establish their safety profiles are shown in Table 3.2. At the end of 30 days of the study period, no statistically significant differences were seen in the mean WBC and RBC counts, Hb, HCT, MCV, MCH, serum AST activity and ALT activity as compared to the normal control. It was observed that the mean WBC counts of the groups that received 250 mg/kg SLA and 500 mg/kg SDA were significantly lower than the normal control. The group that received 250 mg/kg of SDA showed a significantly lower ($P<0.05$) serum AST activity than normal control.

Table 3.2: Sub-acute toxicity studies (Feeding the extracts to normal rats for 30 days

Parameter	Control	SLA-250	SLE-250	SDA-250
WBC ($\times10^6$/µl)	6575±1438	3850±838	4925±736	6875±218
RBC ($\times10^3$/µl)	528±73.6	565.5±84.5	565.5±7.75	537.5±41.9
Hb (g/dl)	14.39±1.36	13.81±1.37	13.57±1.75	14.54±0.43
Hct (%)	47.5±4.51	51.0±6.22	48.0±5.09	48.0±1.41
MCV (fl)	0.91±0.08	0.91±0.03	0.85±0.03	0.90±0.09
MCH (pg)	0.27±0.02	0.25±0.04	0.24±0.03	0.27±0.26
AST (iu/l)	100.0±16.57	103.5±13.00	102.0±9.93	64.7±26.73
ALT (iu/l)	26.75±2.50	28.00±2.83	29.75±2.63	25.75±2.06
ALP (iu/l)	89.65±5.15	98.56±5.12	81.48±6.66	88.63±4.01

3.4 EFFECT OF THE EXTRACTS ON MEAN FASTING BLOOD GLUCOSE LEVELS OF NORMOGLYCAEMIC RATS

Table 3.3 shows the effects of the three different extracts, distilled water and glibenclamide on mean fasting blood glucose levels of non-diabetic rats monitored for 12 hours. The extracts invariably caused no significant change ($P>0.05$) in the blood glucose levels of the rats from the first to the twelfth hour of administration. Glibenclamide, on the other hand, significantly lowered the blood glucose levels ($P<0.05$) three hours post dosing.

Table 3.3: Effect of SLA on FBGL of normal rats

Treatment	Dose (mg/kg)	Initial FBG	Fasting Blood Sugar at Time (h) after Treatment								
			1	2	3	4	5	6	8	10	12
SLA	250	55.50 ± 7.76	61.50 ± 6.55	57.75 ± 2.50	57.70 ± 5.43	55.75 ± 1.70	58.00 ± 3.55	53.50 ± 3.20	54.25 ± 4.50	57.30 ± 7.76	55.00 ± 8.16
SDA	250	54.00 ± 2.82	59.73 ± 5.90	61.75 ± 4.57	63.50 ± 5.19	60.75 ± 3.68	58.80 ± 0.96	61.80 ± 4.34	56.75 ± 1.71	57.00 ± 6.87	54.25 ± 5.56
SLA	500	65.20 ± 2.21	67.11 ± 8.26	66.71 ± 3.27	66.34 ± 6.60	66.04 ± 4.20	65.10 ± 5.80	65.00 ± 1.25	66.00 ± 7.20	65.75 ± 3.10	65.35 ± 4.10
SDA	500	59 ± 3.70	59.90 ± 3.15	60.11 ± 4.12	60.00 ± 4.02	60.30 ± 8.15	60.80 ± 1.10	58.21 ± 8.26	59.00 ± 6.33	59.50 ± 4.28	60.20 ± 5.56
SLE	500	61.02 ± 2.10	61.10 ± 7.71	61.15 ± 6.23	59.30 ± 5.20	59.81 ± 4.33	60.01 ± 3.65	60.44 ± 2.19	60.60 ± 1.45	60.92 ± 9.10	62.01 ± 4.28
Glib.	2	68.50 ± 4.87	62.0 ± 5.02	62.00 ± 5.02	41.25 ± 1.75*	44.25 ± 1.49*	50.25 ± 1.75	53.50 ± 2.95	53.80 ± 3.10	56.20 ± 1.42	58.20 ± 2.21
Control (Distilled Water)	5(ml/kg)	62.20 ± 4.18	63.0 ± 3.50	63.00 ± 3.50	57.60 ± 3.58	65.00 ± 3.27	63.40 ± 3.65	61.20 ± 3.15	62.15 ± 2.17	60.50 ± 5.10	59.25 ± 1.65

* Significant change observed ($P>0.05$).

3.5 DOSE RESPONSE STUDY OF *Sarcocephalus latifolius* AQUEOUS EXTRACT (SLA) ON THE FBGL OF ALLOXAN-INDUCED DIABETIC RATS

Figure 3.1 illustrates the effect of different doses of the extract (SLA) on the fasting blood glucose levels of alloxan induced diabetic rats. The maximum reduction (80.0%) in the amount of blood glucose occurred at a dose of 250 mg/kg body weight. This dose of the extract significantly ($P<0.05$) lowered the blood glucose level in fasted diabetic rats from a mean value of 400 mg/dl at 0 hours to about 80 mg/dl in 6 hours.

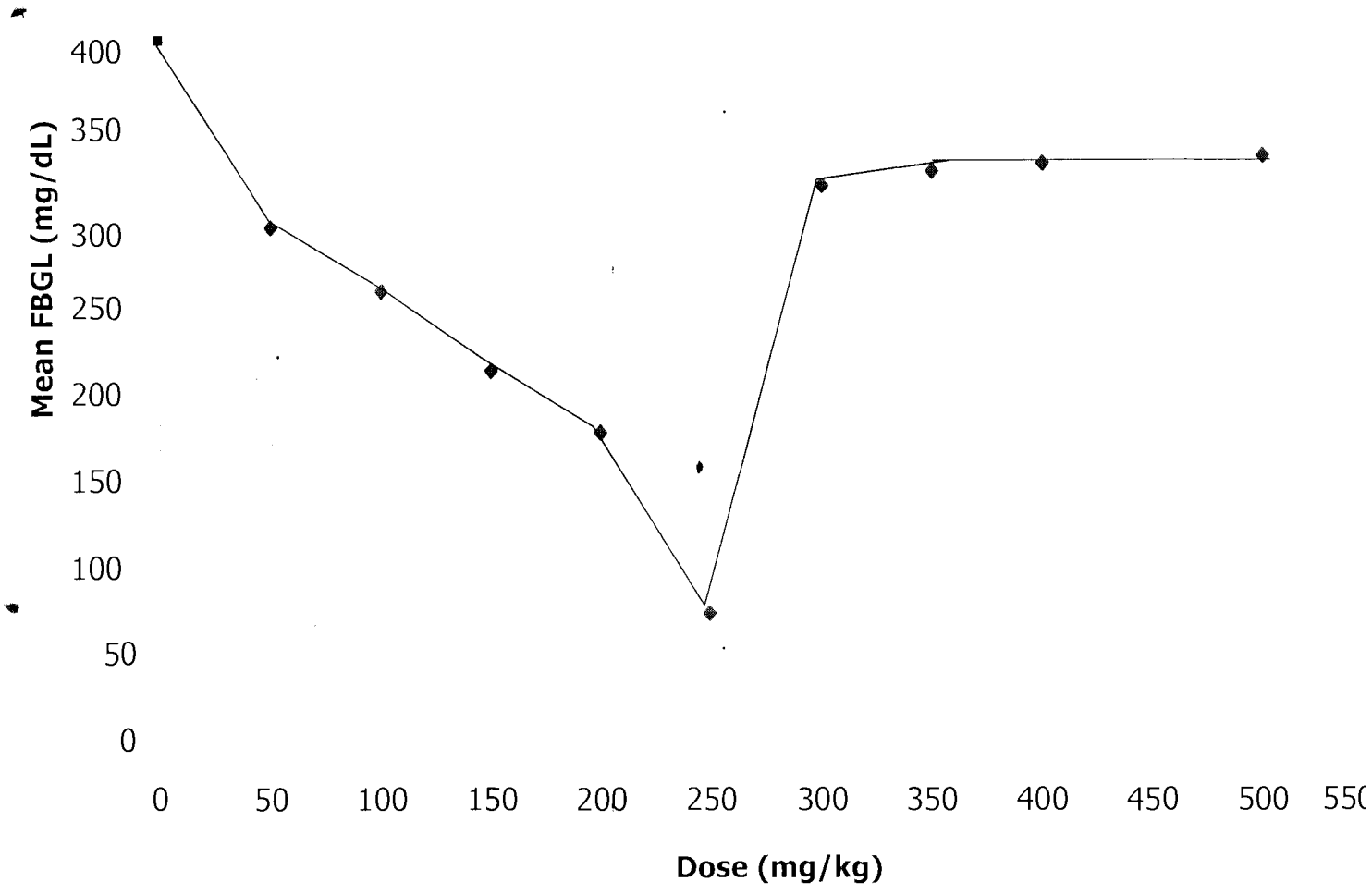

Fig. 3.1: Dose-response relationship of SLA on fasting blood glucose level in alloxan-induced diabetic rats

3.6 EFFECT OF THE EXTRACTS SLA, SDA AND SLE ON MEAN FASTING BLOOD GLUCOSE LEVELS OF ALLOXAN-INDUCED DIABETIC RATS

The data in Table 3.4 depict the respective effects of 250 mg/kg body weight of the three extracts on mean fasting blood glucose levels of diabetic Sprague Dawley (SD) rats. At this dose, SLA and SDA significantly ($P<0.05$) lowered blood glucose levels of diabetic rats relative to the control from means of 311±4.26 mg/dl and 261±3.02 mg/dl respectively to 73.60±2.23 mg/dl and 65±5.40 mg/dl within 6 hours. On the other hand, diabetic rats treated with SLE did not have any significant change in their fasting blood glucose level (FBGL). However, the reference drug glibenclamide significantly ($P<0.05$) reduced the FBGL of the rats to 85.5 ± 2.29.

Table 3.4: Effect of the extracts on the FBG levels of alloxan-induced diabetic rats

Treatment	Dose (mg/kg)	Initial FBGL	FBGL (mg/dl) at Time (h) after Treatment					
			1	2	3	4	5	6
SLA	250	311 (4.26)	220 (3.36)	175.00 (24.95)	120.8 (7.02)	98.60 (10.07)	92.20 (9.27)	73.60 (2.23)
SDA	250	261.0 (3.02)	268.00 (3.33)	259.00 (4.10)	172.00 (4.67)	101.00 (6.21)	77.00 (5.01)	65.00 (5.40)
SLE	250	346.0 (10.22)	369.0 (6.02)	350.2 (9.28)	392.3 (4.20)	358.25 (7.28)	327.5 (13.07)	271.25 (11.05)
Glibenclamide	5	247.25 (5.84)	161.75 (8.07)	123.60 (8.68)	84.75 (6.15)	80.50 (6.65)	66.00 (10.80)	85.50 (2.29)
Control	5ml/kg	293.6 (4.73)	300.20 (3.85)	289.60 (25.24)	285.6 (6.07)	295.20 (3.61)	280.40 (6.75)	276.60 (25.27)

Key:
Figures in bracket represent Std Deviation

3.7 EFFECT OF 21 DAYS OF REPEATED ORAL ADMINISTRATION OF THE EXTRACTS ON ALLOXAN-DIABETIC SD RATS

The overall changes in fasting blood glucose concentration from the first day (Day 1) to the last day (Day 21) in the normal and diabetic treated rats after repeated administration of the different extracts (twice daily) are shown in Appendix 1. There was a significant ($P<0.05$) increase in FBGL of each of the groups treated with the extracts within the first one week of treatment. Continued daily administration of the extracts SLA and SDA for 21 days caused a significant reduction ($P<0.05$) in the blood glucose

levels when compared with the diabetic control group of rats and day 1 values. Similarly, repeated administration of glibenclamide (5 mg/kg twice daily) for 21 days produced a significant reduction ($P<0.05$) in the fasting blood glucose level in alloxan-diabetic SD rats when compared with the diabetic control and day 1 values. SLE did not show a remarkable reduction in the FBGL of diabetic rats even after the 21 days of treatment.

Table 3.5: Effect of 21-day treatment with the extracts on fasting blood glucose (FBG) levels of diabetic rats

Group	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Normal control	75.25±13.88	85.75±8.18	79.25±12.20	72.25.5±8.62
Diabetic control	228.5±32.89	256.25±15.50	258.50±16.05	285.50±13.03
SLA-250	263.0+99.68	296.75±8.90	236.00±10.50	120.75±19.05
SLE-250	307.25±48.19	424.00±58.70	326.00±13.10	249.75±8.72
SDA-250	302.75±79.62	341.50±12.82	206.00±5.90	119.00±20.34
Glibenclamide	256.25±65.79	167.25±9.90	129.00±5.50	120.00±5.16

levels when compared with the diabetic control group of rats and day 1 values. Similarly, repeated administration of glibenclamide (5 mg/kg twice daily) for 21 days produced a significant reduction ($P<0.05$) in the fasting blood glucose level in alloxan-diabetic SD rats when compared with the diabetic control and day 1 values. SLE did not show a remarkable reduction in the FBGL of diabetic rats even after the 21 days of treatment.

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Diabetic control	228.5±32.89	256.25±15.50	258.50±16.05	285.50±13.03
SLA-250	263.0±99.68	296.75±8.90	236.00±10.50	120.75±19.05
SLE-250	307.25±48.19	424.00±58.70	326.00±13.10	249.75±8.72
SDA-250	302.75±79.62	341.50±12.82	206.00±5.90	119.00±20.34
Glibenclamide	256.25±65.79	167.25±9.90	129.00±5.50	120.00±5.16

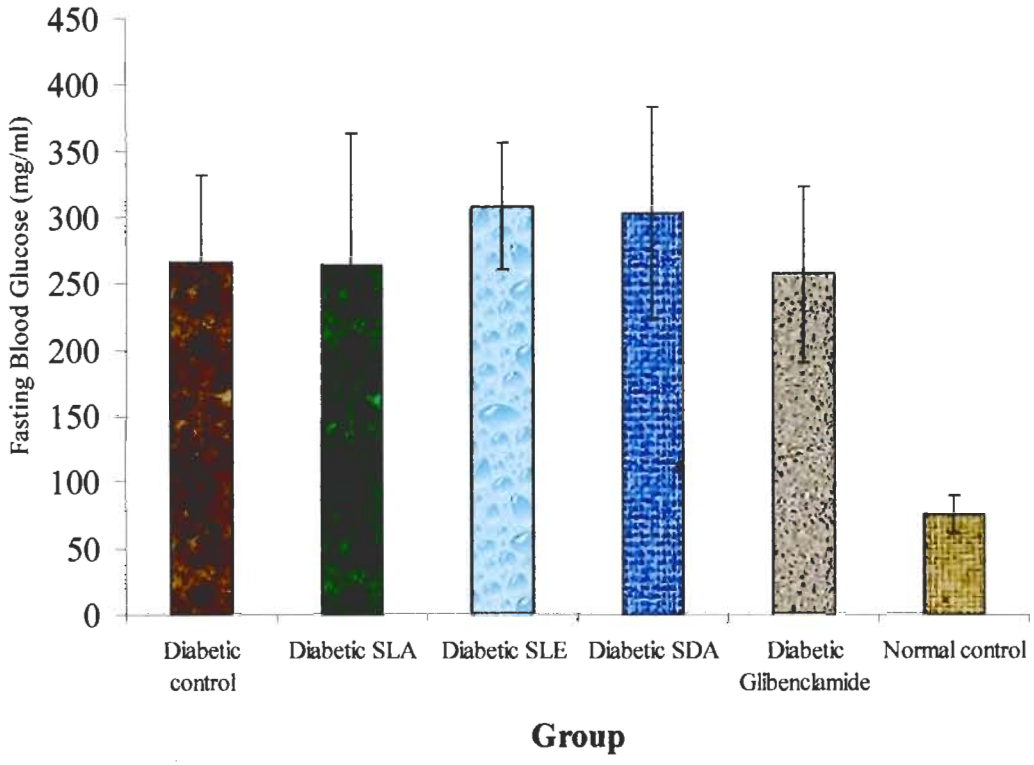

Fig. 3.2: Fasting blood glucose concentration on day 0 in normal control and diabetic rats.

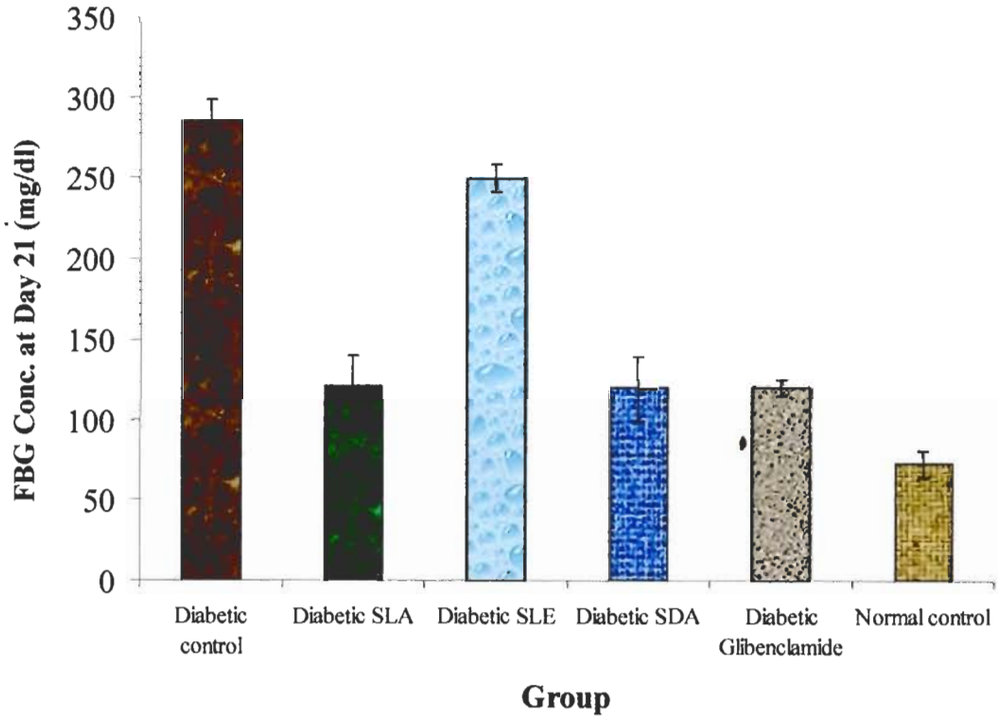

Fig. 3.3: Fasting blood glucose concentration at day 21 in normal control and diabetic rats.

3.8 PROTEIN CONCENTRATION OF THE LIVER AND KIDNEY FRACTIONS

The results represented in Figures 3.4 and 3.5 and in Table 17 (Appendix 2) shows the protein concentrations of the post mitochondrial fractions of the organs (liver and kidney).

Table 3.6: Absorbance of Standard Bovine Serum Albumin (BSA)

Stock BSA (2 mg/ml)	Normal Saline (ml)	Mean Absorbance	Protein Conc (mg/ml)
0.1	0.9	0.060	1.0
0.2	0.8	0.141	2.0
0.3	0.7	0.220	3.0
0.4	0.6	0.300	4.0
0.5	0.5	0.371	5.0
0.6	0.4	0.451	6.0
0.7	0.3	0.530	7.0
0.8	0.2	0.601	8.0
0.9	0.1	0.692	9.0
1.0	0.0	0.780	10.0

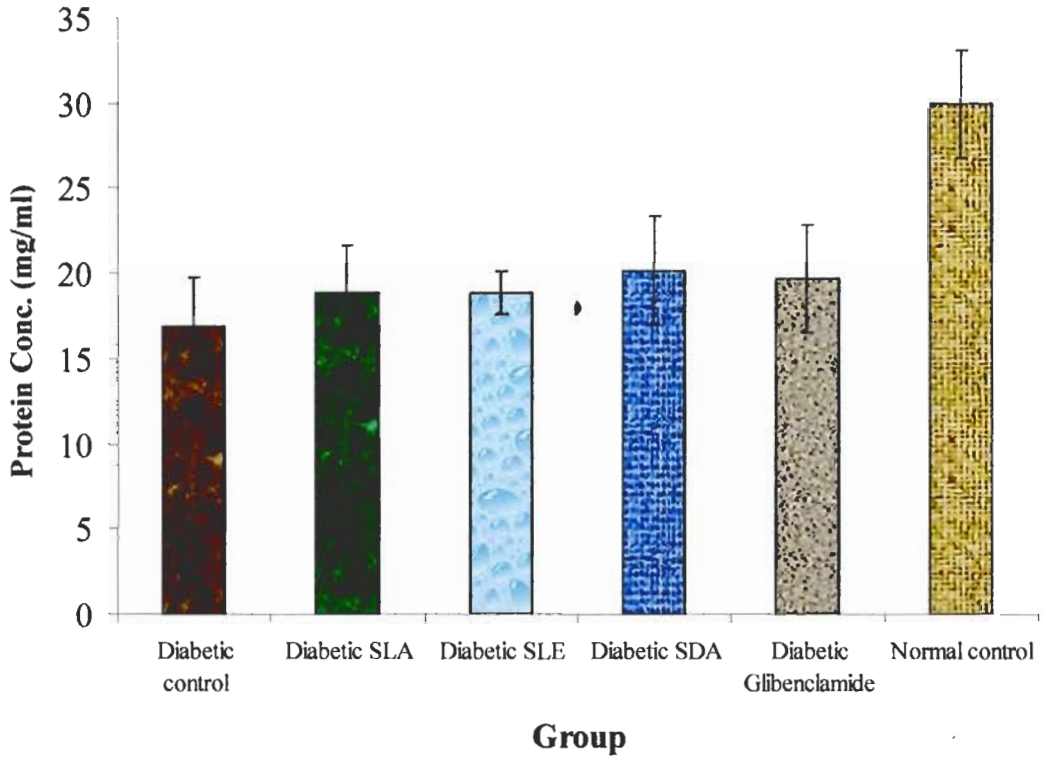

Fig. 3.4: Protein concentration in liver homogenate of normal control and diabetic rats.

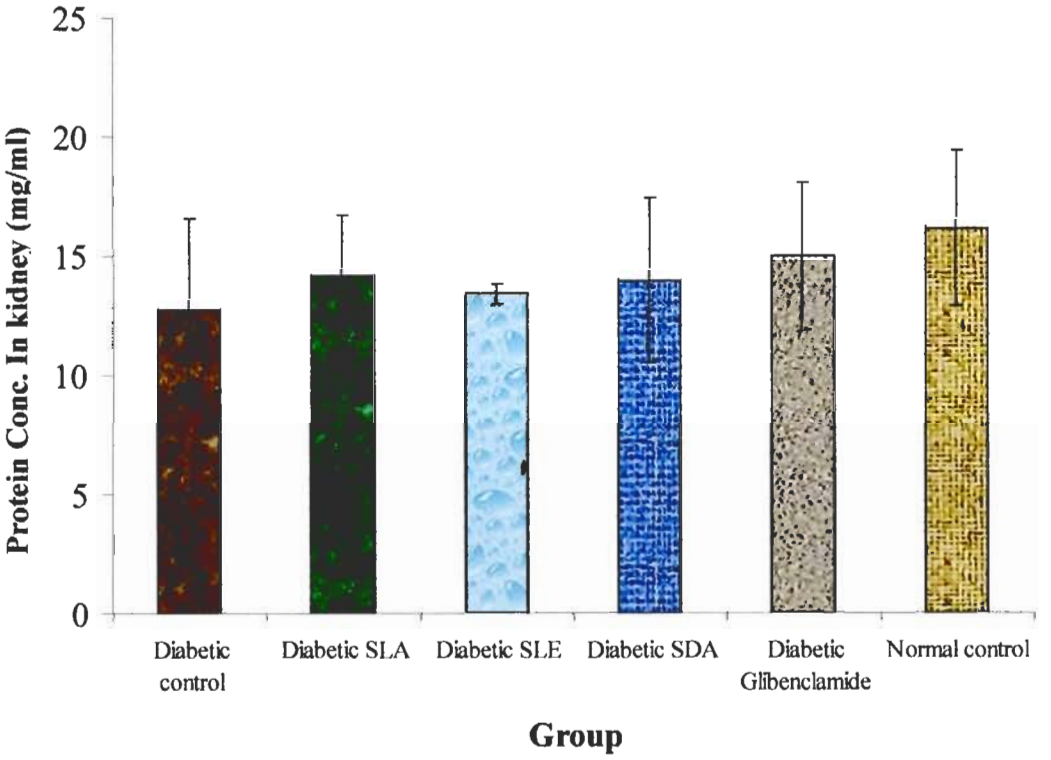

Fig. 3.5: Protein concentration in the kidney of normal control and diabetic rats.

3.9 EFFECT OF THE EXTRACTS ON THE ACTIVITIES OF HEPATIC ENZYMES OF GLUCOSE METABOLISM

The activities of hepatic hexokinase (HK), glucokinase (GK) and phosphofructokinase (PFK) determined in non-diabetic and diabetic treated rats are shown in Figures 3.26, 3.27 and 3.28 and summarised in Appendix 10.

As compared to the normal control values, the mean levels of the enzymes (hexokinase, glucokinase and Phosphofructokinase) decreased in the diabetic control animals. Glucokinase was significant at $P<0.05$. Treatment with SDA, SLA and glibenclamide led to a rise in the activities of these enzymes when compared with the normal control. On the other hand, SLE did not elicit an increase in the activities of GK and PFK.

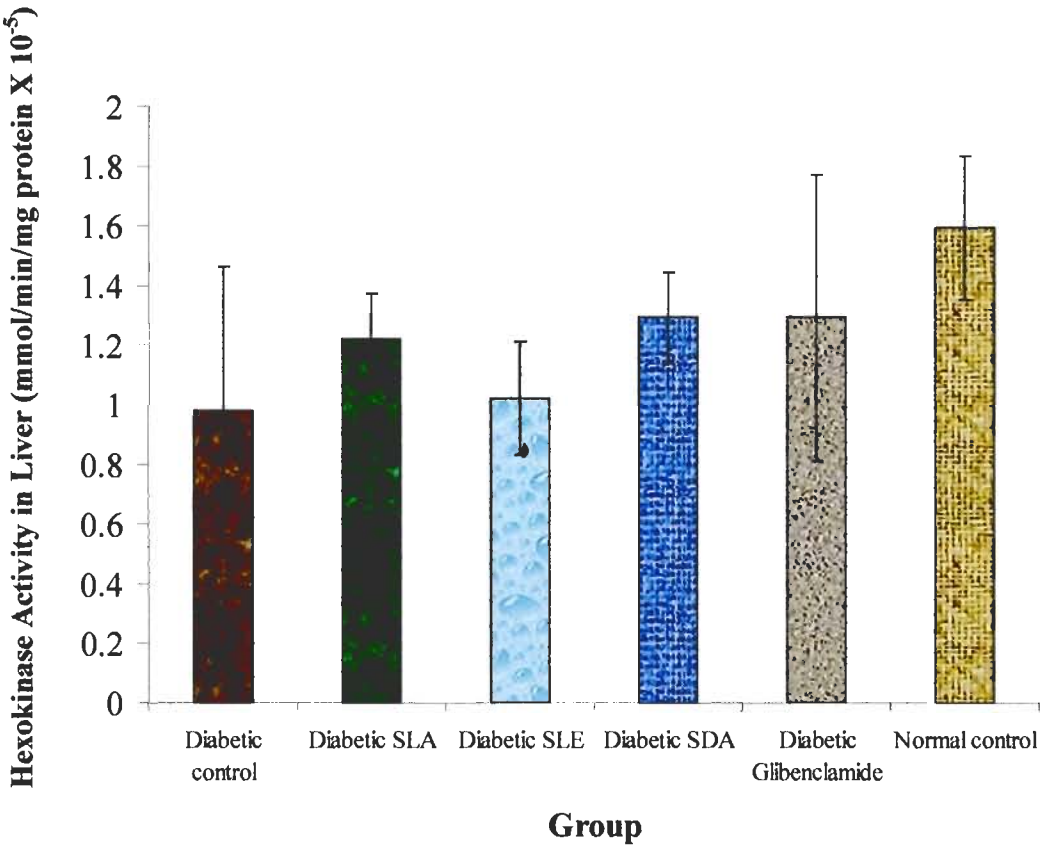

Fig. 3.6: Effect of extracts on hexokinase activity in the liver of normal control and diabetic rats.

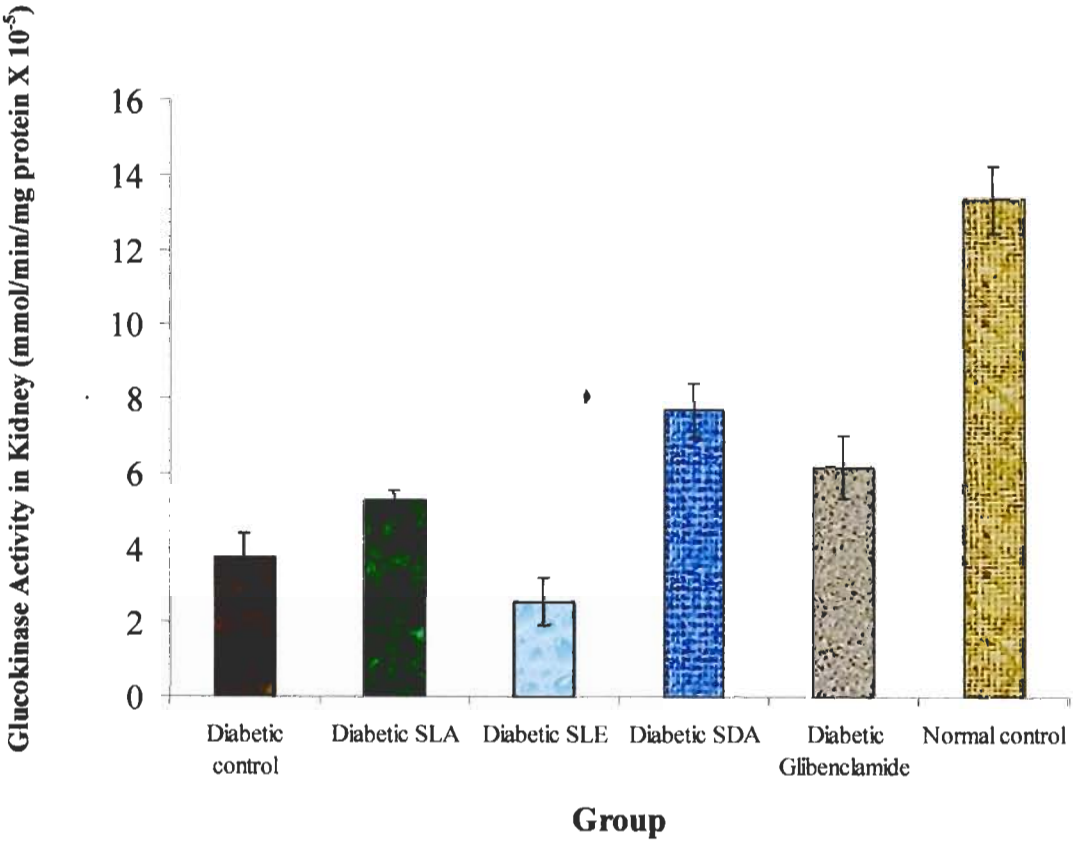

Fig. 3.7: Effect of extracts on glucokinase activity in the liver of normal control and diabetic rats.

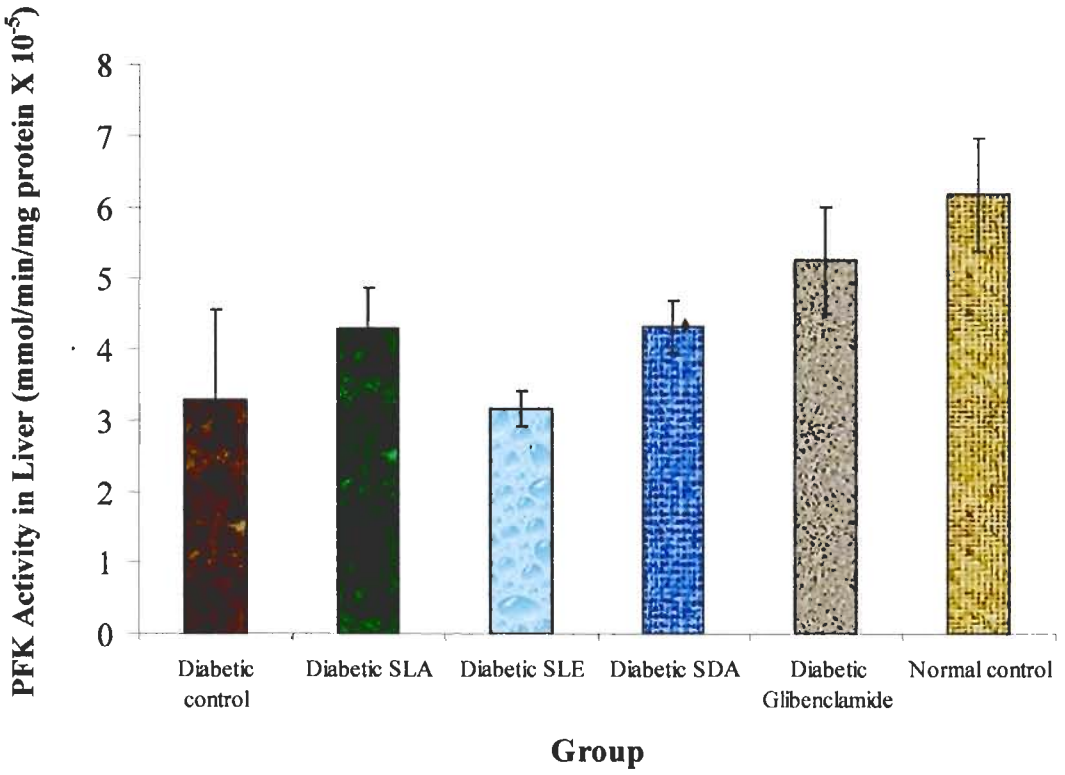

Fig. 3.8: Effect of extracts on phosphofructokinase activity in the liver of normal control and diabetic rats.

3.10 EFFECT OF THE EXTRACTS ON HEPATIC GLYCOGEN CONTENT

Figure 3.29 and Appendix 11 show the hepatic glycogen content in the non-diabetic and alloxan-induced diabetic rats after 21 days of repeated oral administration of distilled water, SLA, SLE, SDA and glibenclamide. In diabetic control animals, hepatic glycogen content decreased significantly when compared with the normal controls. Treatment with the extracts and glibenclamide led to increase in the glycogen content with SDA showing the highest increase.

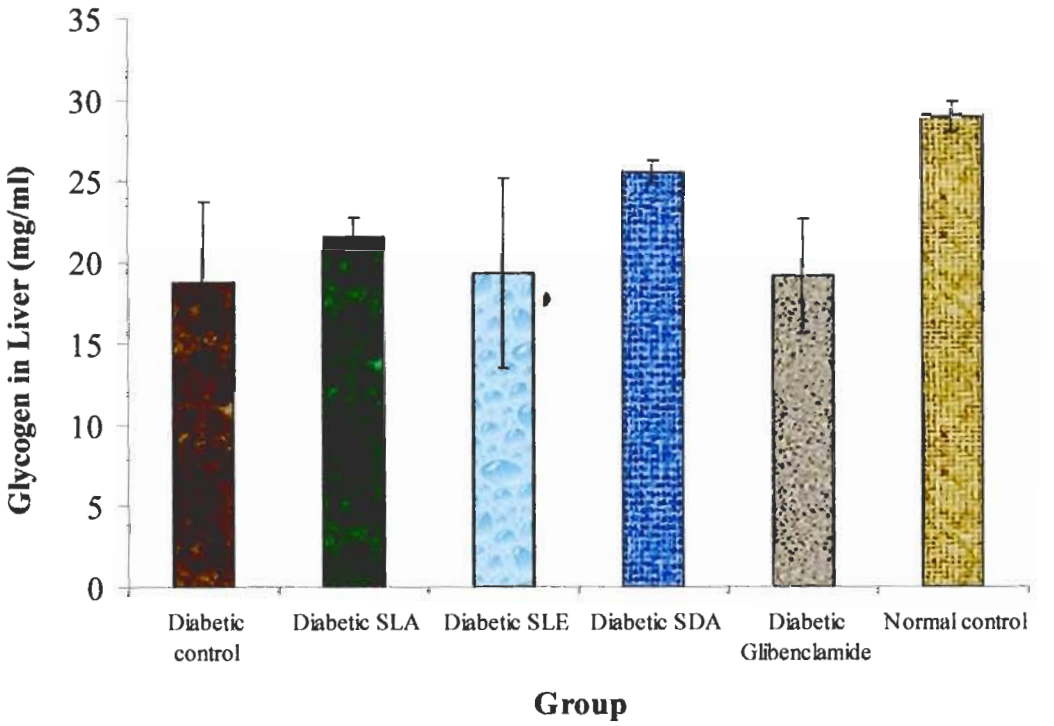

Fig. 3.9: Effect of extracts on glycogen content in the liver of normal control and diabetic rats.

3.11 EFFECT OF THE ROOT EXTRACTS ON MDA LEVELS (LIPID PEROXIDATION LEVEL)

Figures 3.16 and 3.17 show the extent of lipid peroxidation in the liver and kidney of rats treated with the extracts and glibenclamide (Table 21 in Appendix 6). For the liver fractions, a significantly elevated ($P<0.05$) level of MDA was observed in diabetic control rats. Also the groups treated with SLA and SLE showed elevated MDA level when compared with the diabetic control rats. In the group administered SDA and glibenclamide, significant reduction ($P<0.05$) of the MDA level relative to the diabetic control groups was observed. The SDA returned to normal.

For the kidney samples, the diabetic control group showed a significant ($P<0.05$) increase in the MDA level when compared with the normal control group. Treatment with SDA or glibenclamide elicited remarkable decrease in the MDA levels while the groups treated with SLA and SLE showed no significant difference between the normal and the diabetic control group.

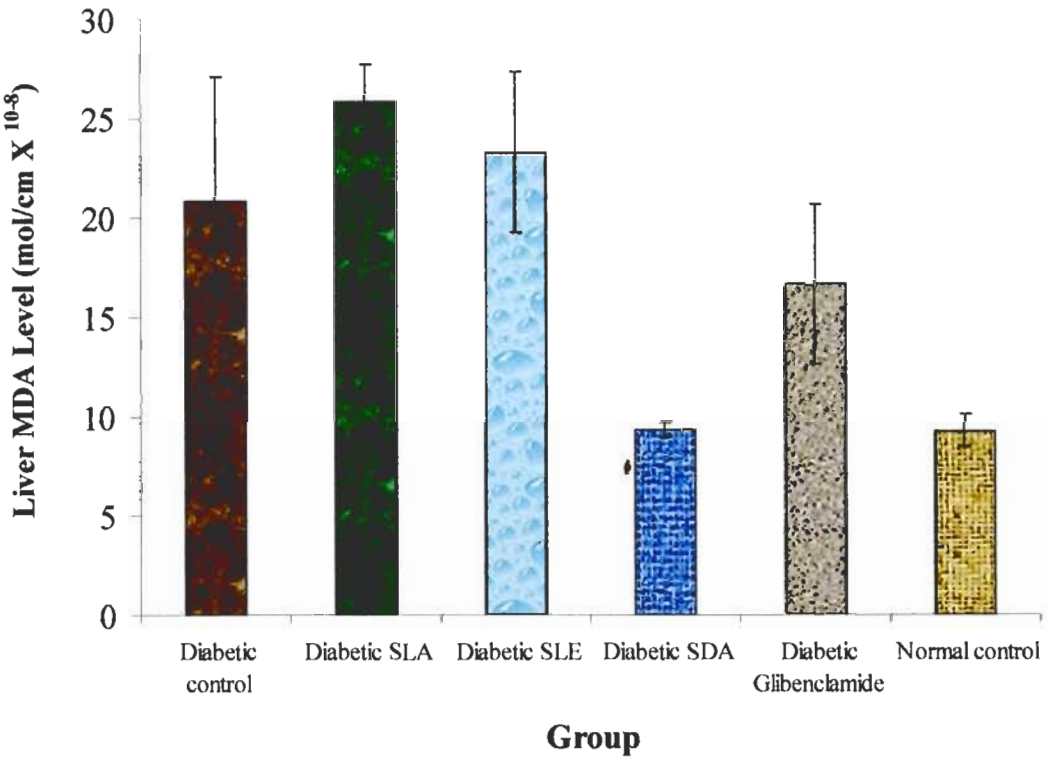

Fig. 3.10: Effect of extracts on MDA levels in the liver of normal control and diabetic rats

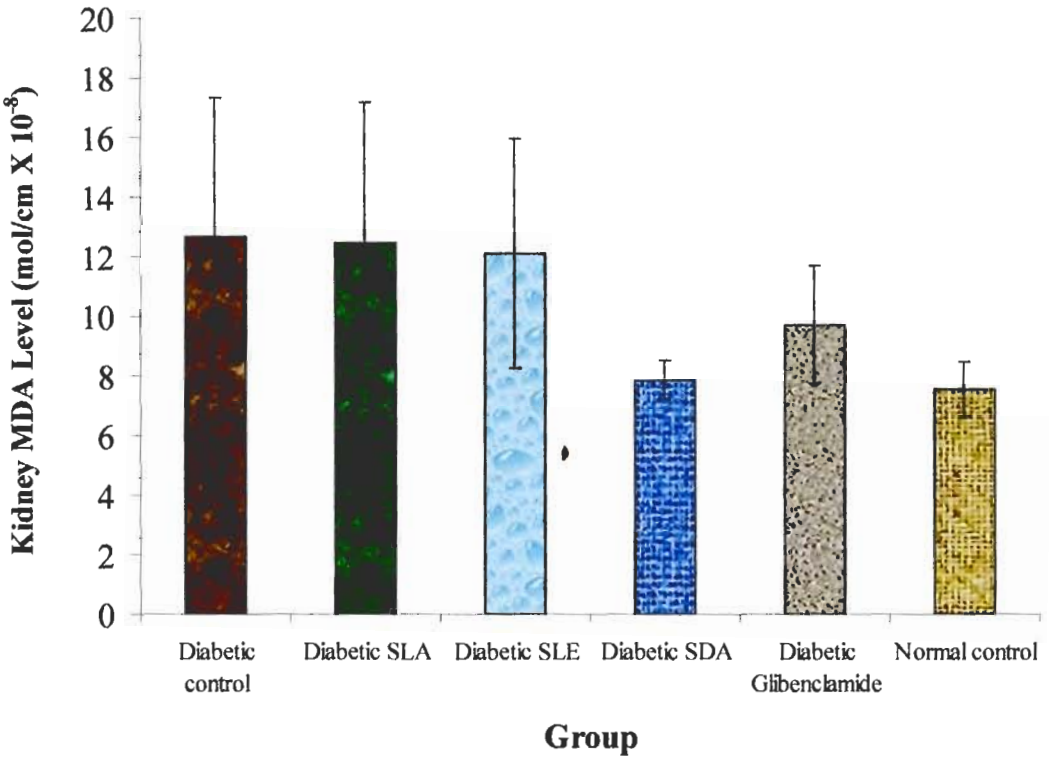

Fig. 3.11: Effect of extracts on MDA levels in the kidney of normal control and diabetic rats.

3.12 EFFECT OF THE EXTRACTS ON REDUCED GLUTATHIONE CONCENTRATION

The levels of reduced glutathione (GSH) in the liver and kidney of treated diabetic rats are shown in Figures 3.18 and 3.19 and also represented in Appendix 22. In both organs, there was a significant decrease ($P<0.05$) in the GSH concentration of diabetic control groups when compared with the normal control group. Treatment with the extracts raised the concentration of GSH. Glibenclamide treatment also caused a significant increase in the GSH level when compared with the diabetic control. While glibenclamide caused significant ($P<0.05$) elevation in GSH concentration, treatment with the respective extracts produced effects not significantly different from the normal controls.

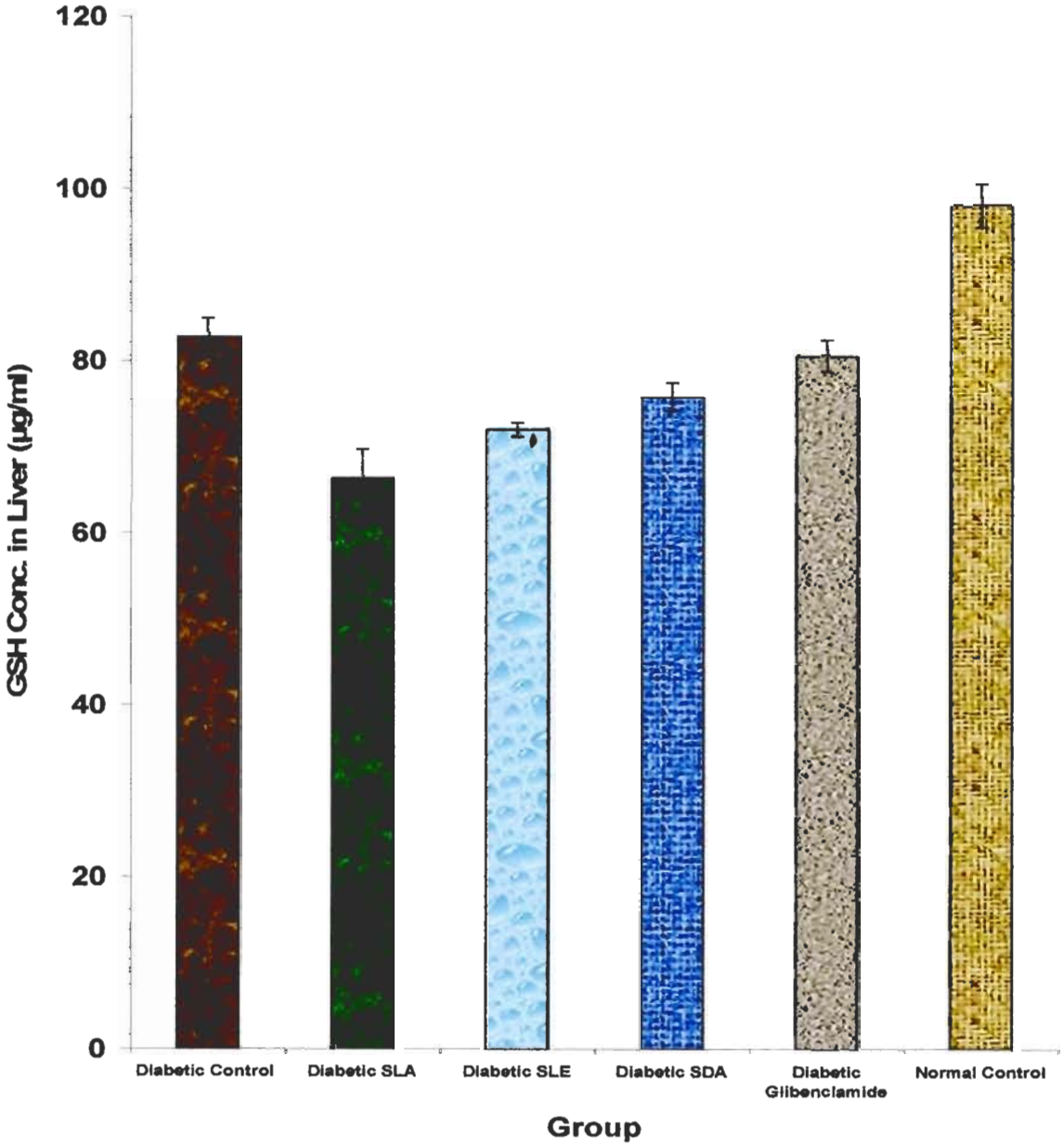

Fig. 3.12: Effect of extracts on GSH concentration in the liver of normal control and diabetic rats.

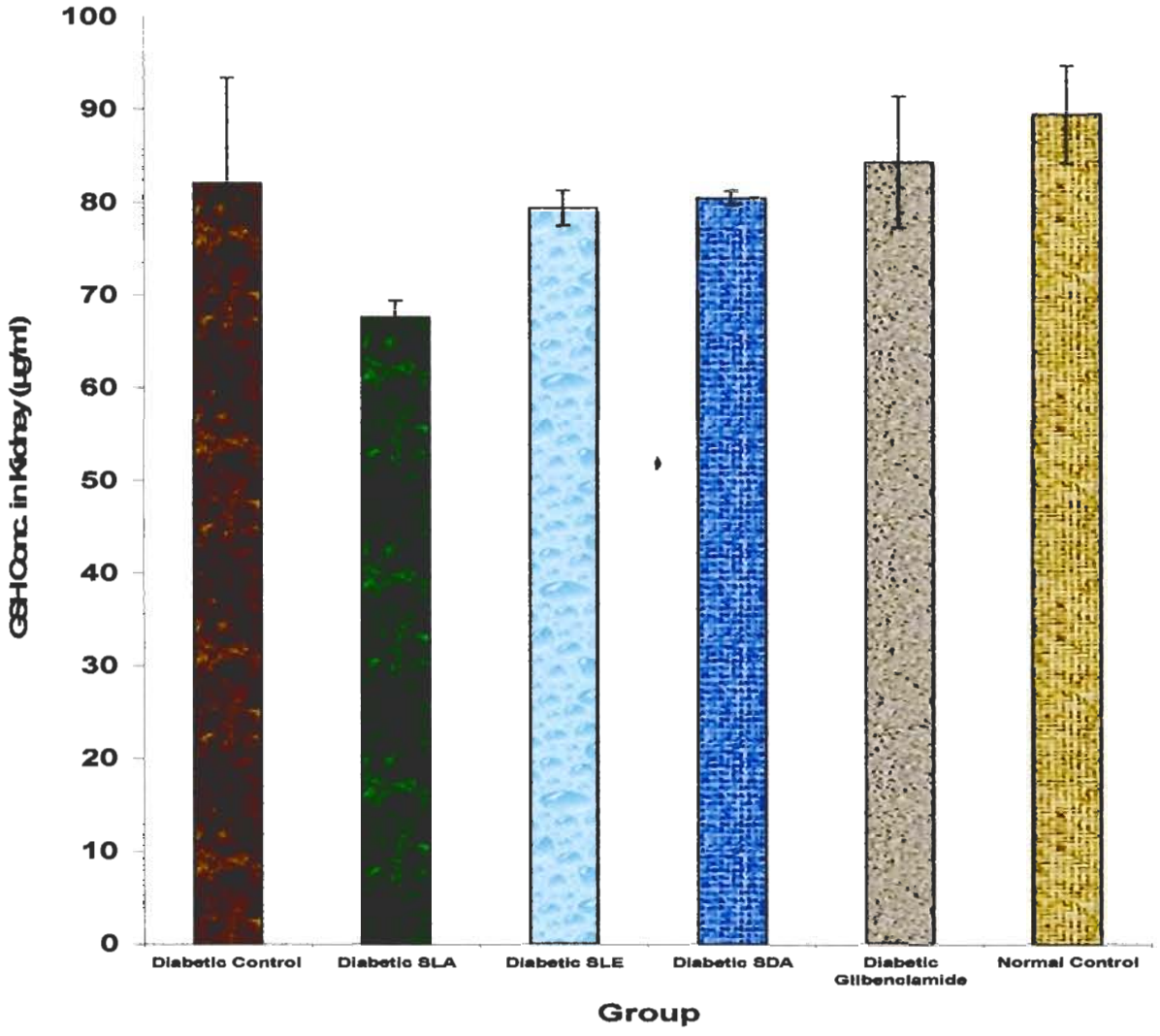

Fig. 3.13: Effect of extracts on GSH levels in the kidney of normal control and diabetic rats.

3.13 EFFECT OF THE EXTRACTS ON CATALASE ACTIVITY

The activities of catalase in the liver and kidney of diabetic treated animals are shown in Figures 3.20 and 3.21. In both organs, it was observed that catalase activity in the diabetic control animals was significantly higher ($P<0.05$) than those of the normal control groups. The activity of catalase in the diabetic treated animals were significantly lower ($P<0.05$) than that of the diabetic control animals but compared well with that of the normal control. These results are also represented in Appendix 23.

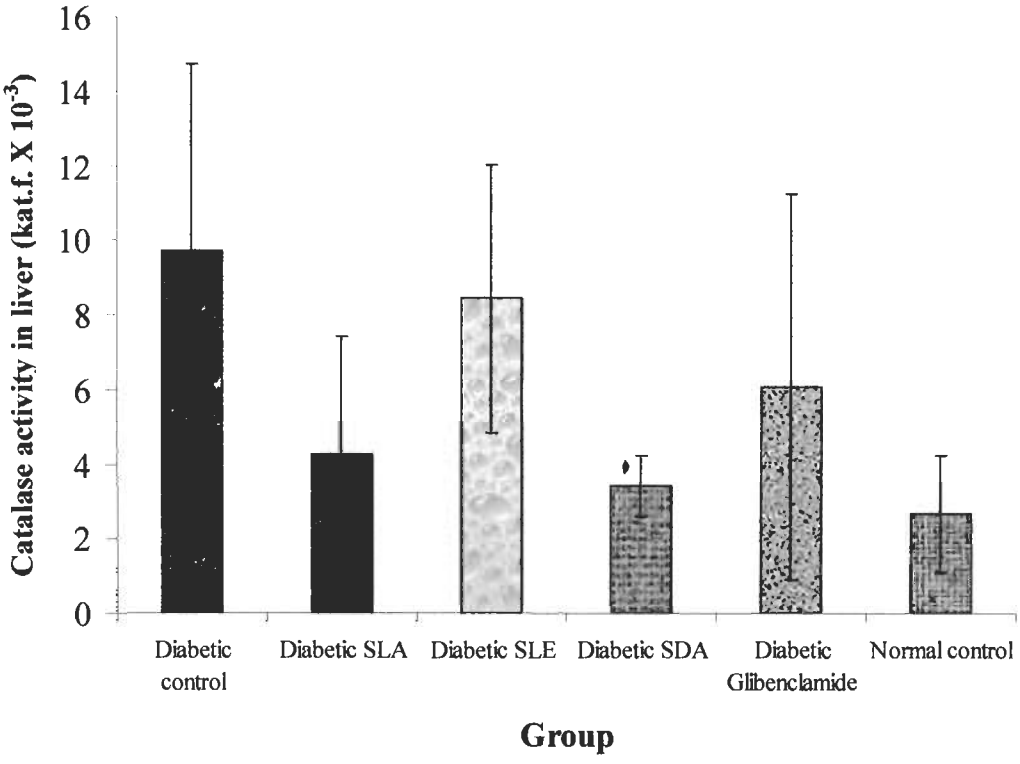

Fig. 3.14: Effect of extracts on catalase activity in the liver of normal control and diabetic rats.

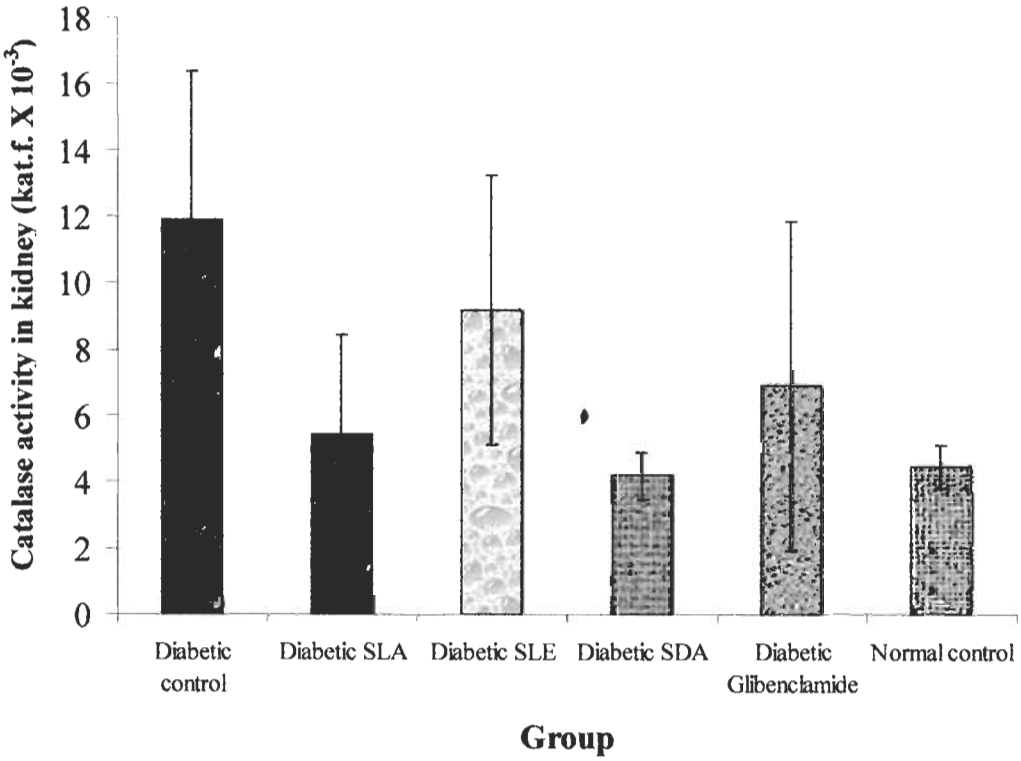

Fig. 3.15: Effect of extracts on catalase activity in the kidney of normal control and diabetic rats.

3.14 EFFECT OF THE EXTRACTS ON SUPEROXIDE DISMUTASE (SOD) ACTIVITY

Figures 3.22 and 3.23 (Appendix 24) show the activities of SOD in the liver and kidney of various treated diabetic groups of rats. The SOD activity in the liver of the diabetic control animals increased significantly ($P<0.05$) compared with the normal control group. Treatment with the extracts and glibenclamide lowered the increase in SOD activity with the least activity shown in the group that was treated with SDA.

In the kidneys, the SOD activity also increased significantly ($P<0.05$) in the diabetic control group relative to the normal control animals. SDA and SLA showed non-significant decreases ($P>0.05$) in the activity of SOD compared with the diabetic control animals. SLE increased the activity of SOD while there was no difference between the glibenclamide treated group and the diabetic control group.

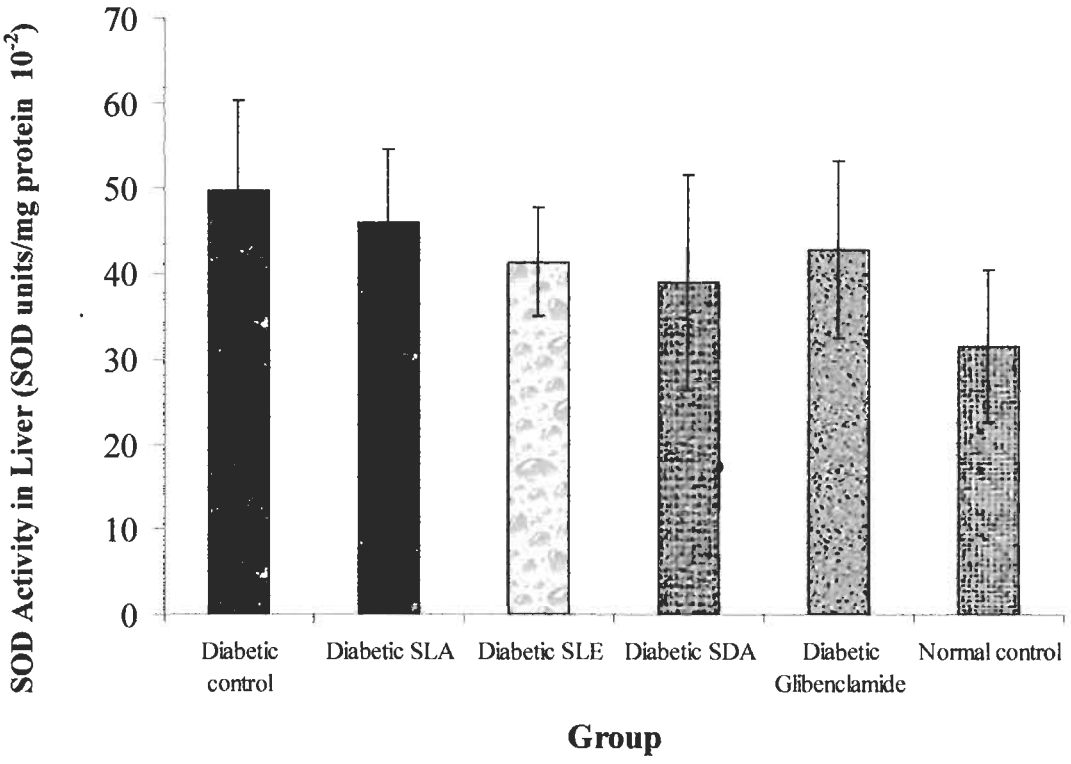

Fig. 3.16: Effect of extracts on SOD activity in the liver of normal control and diabetic rats.

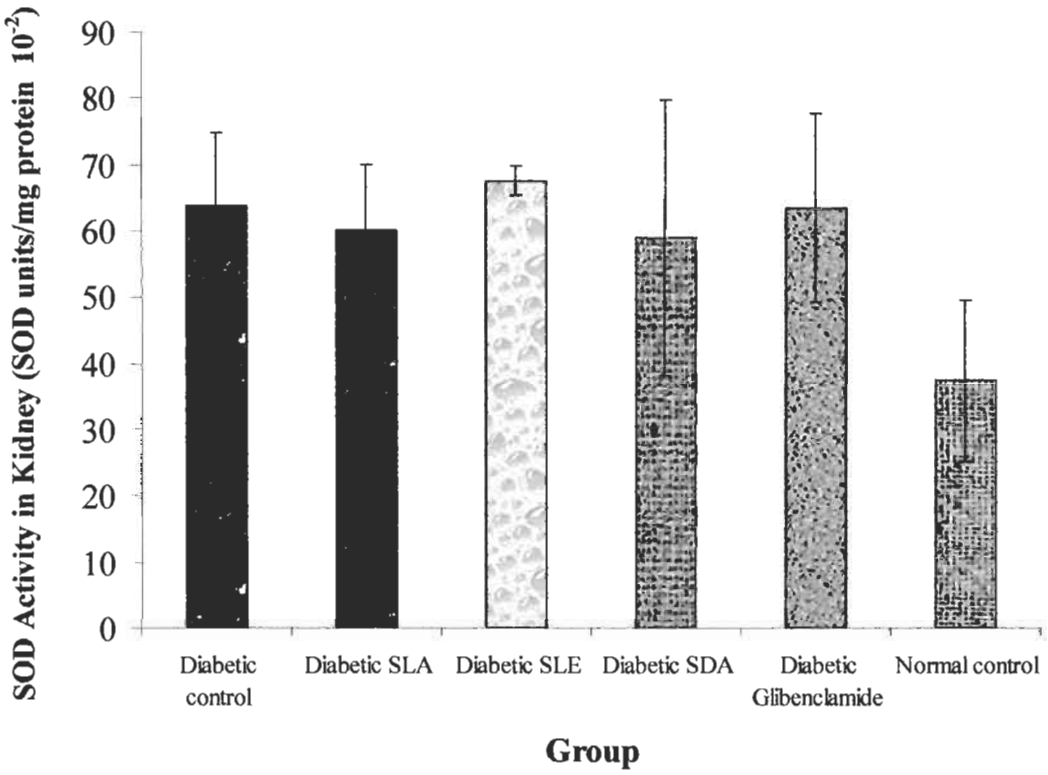

Fig. 3.17: Effect of extracts on SOD activity in the kidney of normal control and diabetic rats.

3.15 EFFECT OF THE EXTRACTS ON GLUTATHIONE S-TRANSFERASE ACTIVITY

Figures 3.24 and 3.25 show that in both liver and the kidney, the different extracts significantly reduced ($P<0.05$) diabetic induced increase in GST activity. For the liver and kidney, there was also a significant difference ($P<0.05$) between the GST activities of the diabetic control animals and the normal control animals. The raw values are represented in Appendix 25.

3.15 EFFECT OF THE EXTRACTS ON GLUTATHIONE S-TRANSFERASE ACTIVITY

Figures 3.24 and 3.25 show that in both liver and the kidney, the different extracts significantly reduced ($P<0.05$) diabetic induced increase in GST activity. For the liver and kidney, there was also a significant difference ($P<0.05$) between the GST activities of the diabetic control animals and the normal control animals. The raw values are represented in Appendix 25.

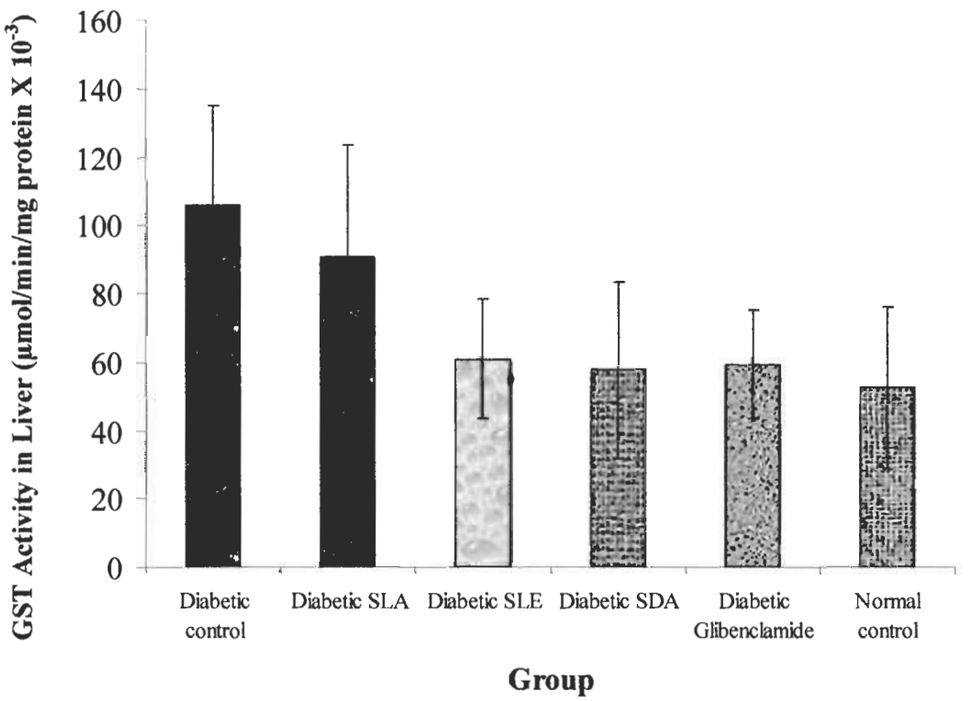

Fig. 3.18: Effect of extracts on GST activity in the liver of normal control and diabetic rats

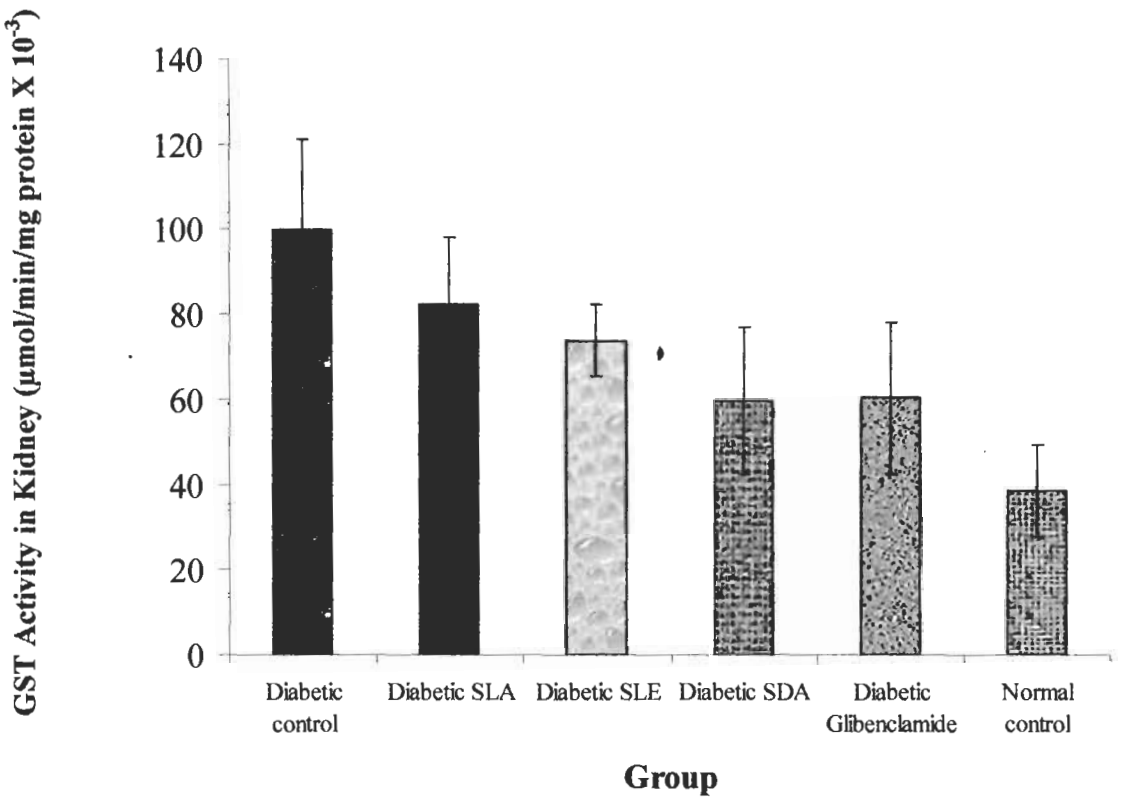

Fig. 3.19: Effect of extracts on GST activity in the kidney of normal control and diabetic rats.

3.16 EFFECTS OF THE EXTRACTS ON SERUM LIPID PROFILE OF TREATED DIABETIC AND NON-DIABETIC RATS

Serum triglyceride concentrations (Fig. 3.6) and total cholesterol levels (Fig. 3.7) were significantly reduced ($P<0.05$) while a significant increase in HDL-cholesterol ($P<0.05$) (Fig. 3.9) was recorded in all the extract treated rats when compared with the diabetic control rats. However, serum LDL (Fig. 3.8) concentration was significantly lower ($P<0.05$) in diabetic control rats compared with the treated diabetic and normal control rats. The daily administration of glibenclamide (5mg/kg twice a day) for 21 days to alloxan-diabetic rats caused a significant decrease in the serum triglyceride and total cholesterol levels ($P<0.05$) when compared with the diabetic control group rats, however. This treatment did not increase the HDL-cholesterol.

3.16 EFFECTS OF THE EXTRACTS ON SERUM LIPID PROFILE OF TREATED DIABETIC AND NON-DIABETIC RATS

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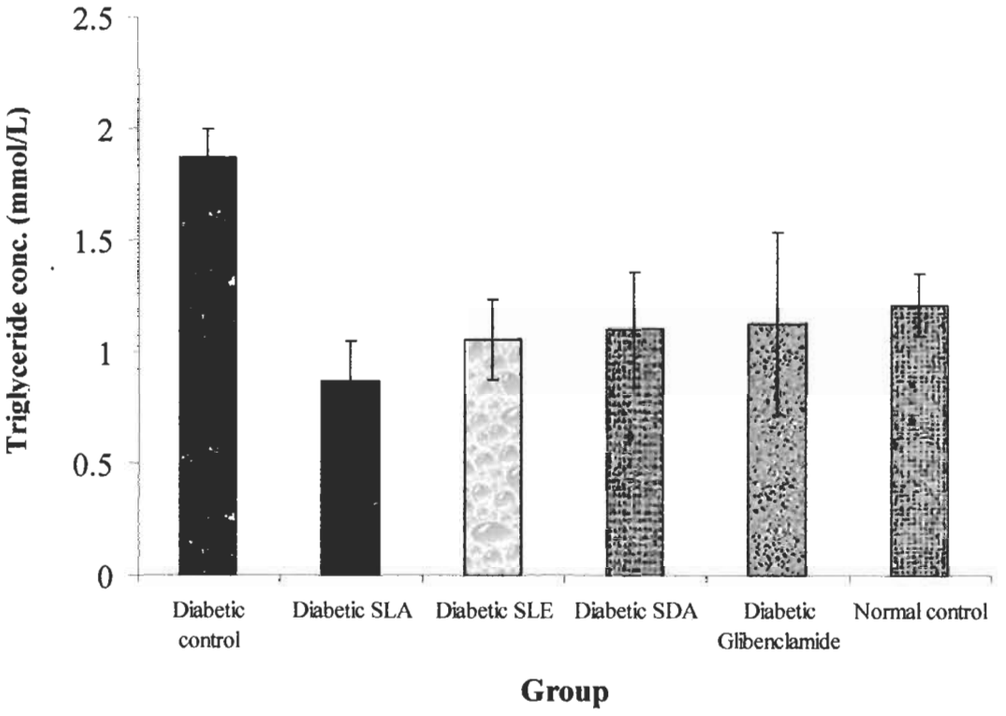

Fig. 3.20: Triglyceride concentrations of diabetic rats and normal control rats after 21 day treatment with the extracts.

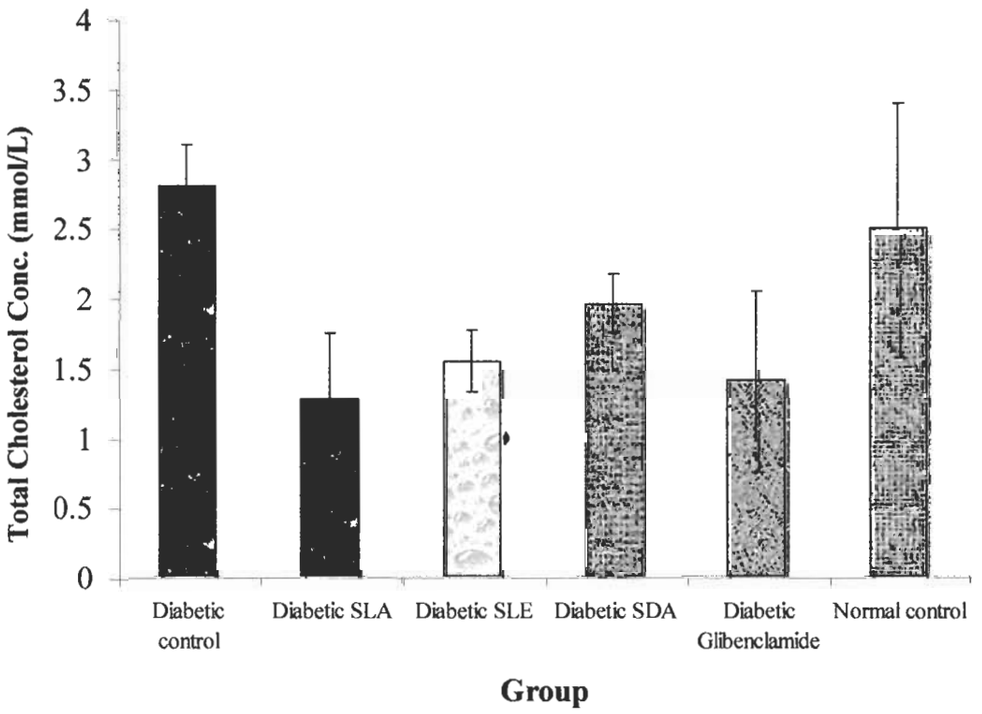

Fig. 3.21: Effect of the extracts on total cholesterol concentrations of normal control and diabetic rats.

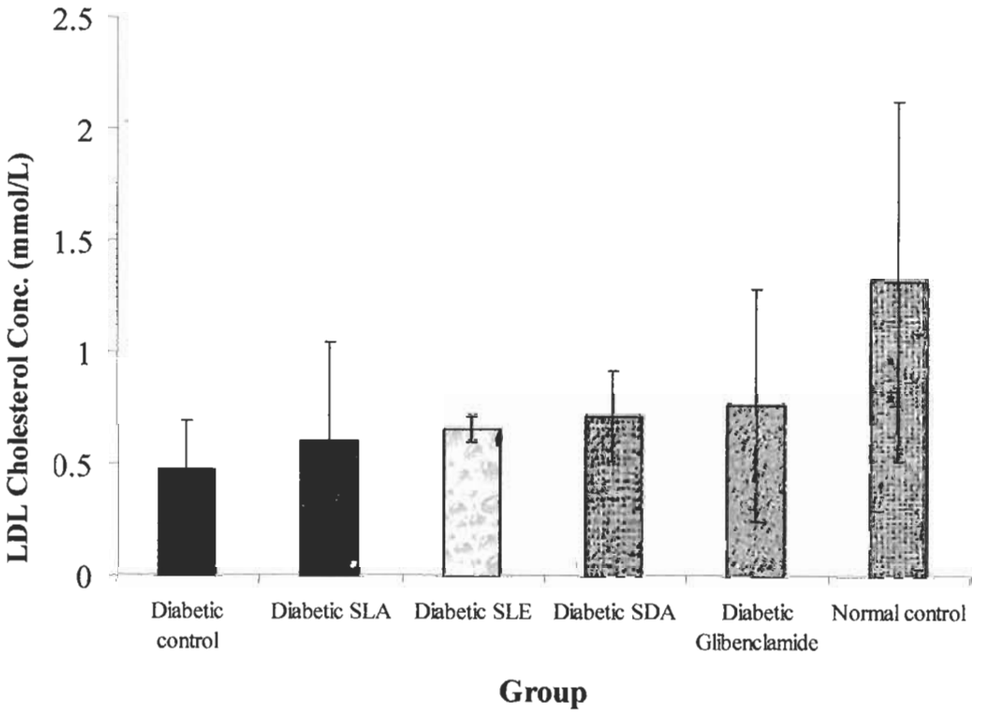

Fig. 3.22: Effect of extracts on low density lipoprotein (LDL) concentration of normal control and diabetic rats.

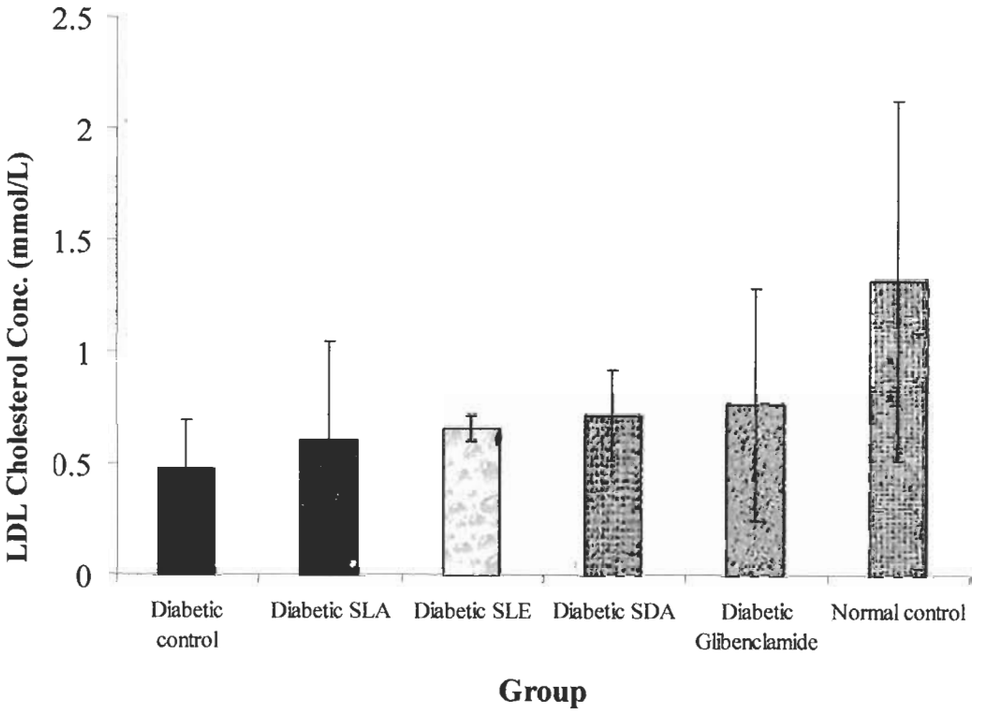

Fig. 3.22: Effect of extracts on low density lipoprotein (LDL) concentration of normal control and diabetic rats.

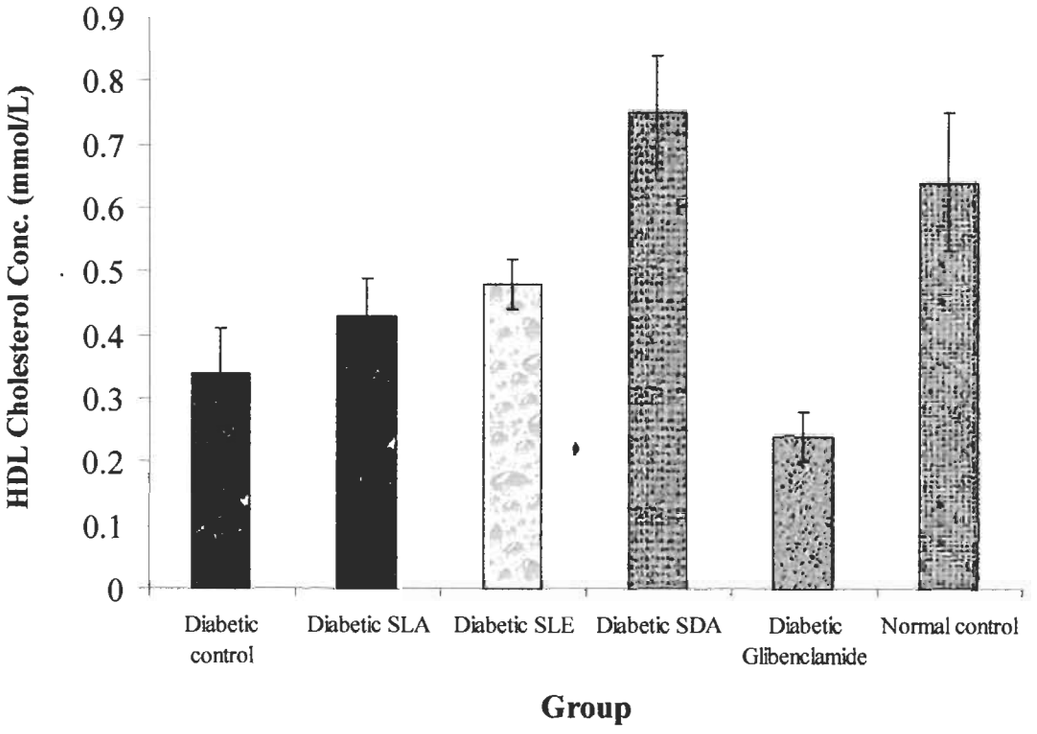

Fig. 3.23: Effect of extracts on high density lipoprotein (HDL) concentrations of normal control and diabetic rats.

3.17 EFFECT OF THE EXTRACTS (SLA, SLE AND SDA) ON THE HAEMATOLOGICAL PARAMETERS OF TREATED DIABETIC RATS

The haematological parameters of the different diabetic animal groups treated with the extracts and glibenclamide are shown in Figures 3.10, 3.11, 3.12 and 3.13 (or Table 19) and Appendix 4. There were insignificant ($P<0.05$) decreases in the haemoglobin and haematocrit contents of the diabetic control group of animals when compared with those in the normal control group. Treatment with the extracts as well as glibenclamide increased these parameters. No significant difference ($P>0.05$) was recorded in the red blood cell (RBC) count of all the groups. However, the white blood cell (WBC) counts of the groups that received SLA, SLE and glibenclamide showed significant increases ($P<0.05$) when compared with the diabetic control group, while the group that received SDA showed no difference from the normal control.

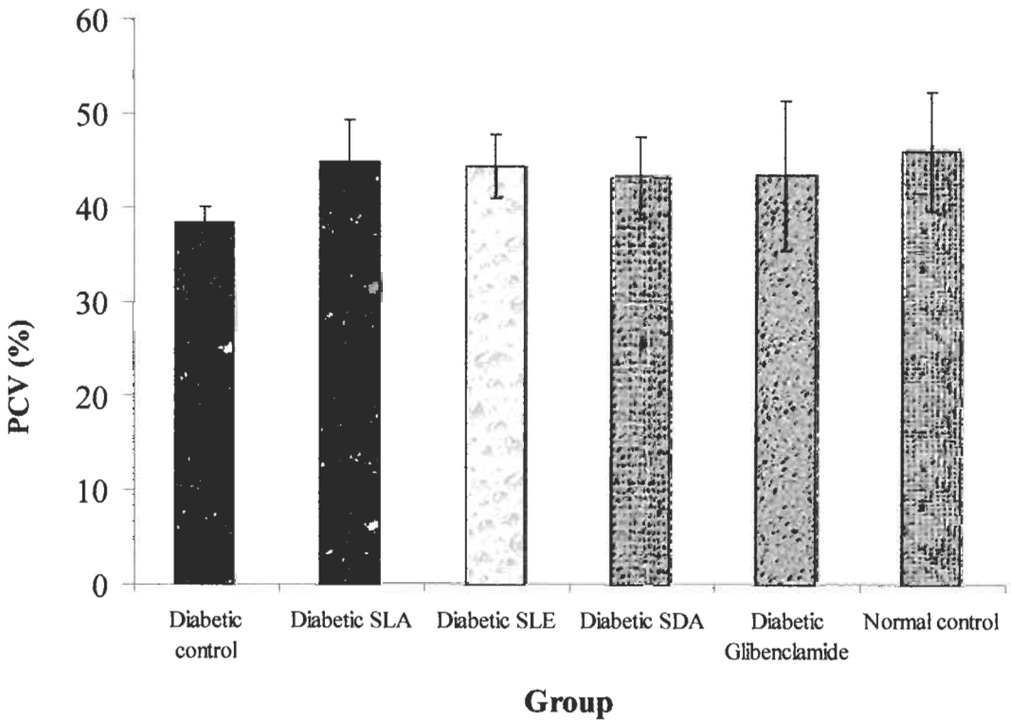

Fig. 3.24: Effect of extracts on packed cell volume (PCV) of normal control and diabetic rats.

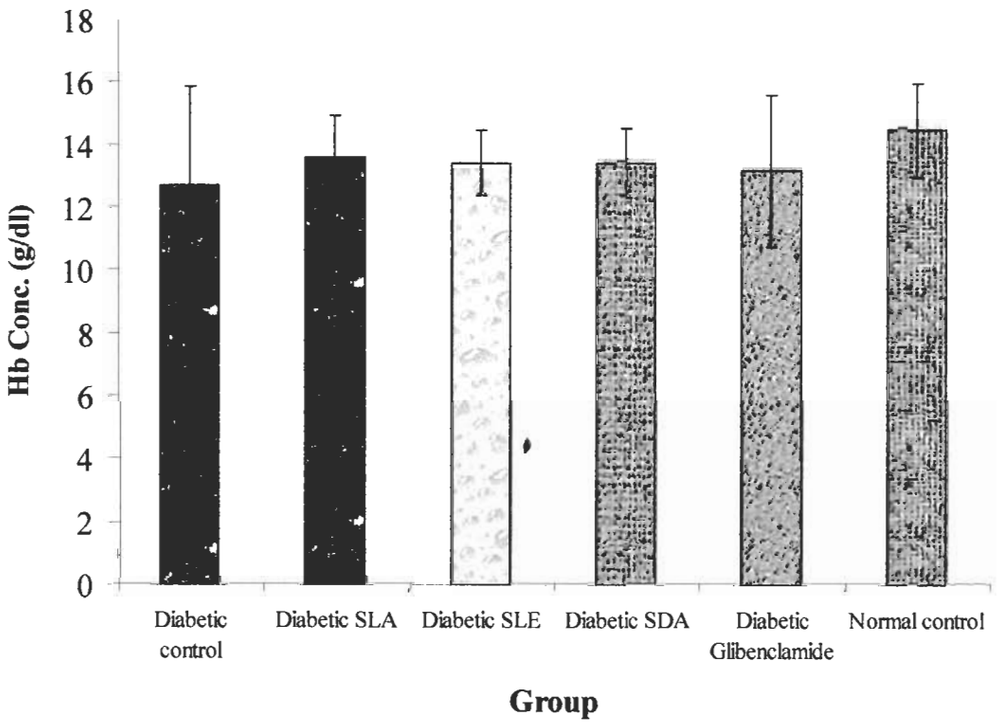

Fig. 3.25: Effect of extracts on haemoglobin (Hb) concentration in normal control and diabetic rats

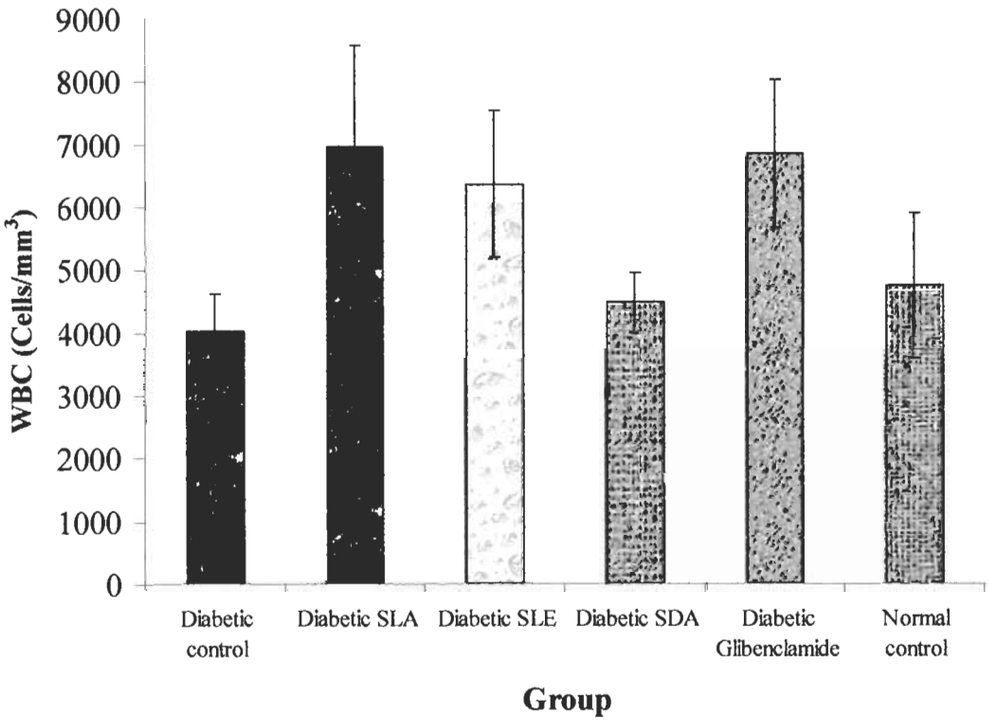

Fig. 3.26: Effect of extracts on white blood cell (WBC) count in normal control and diabetic rats.

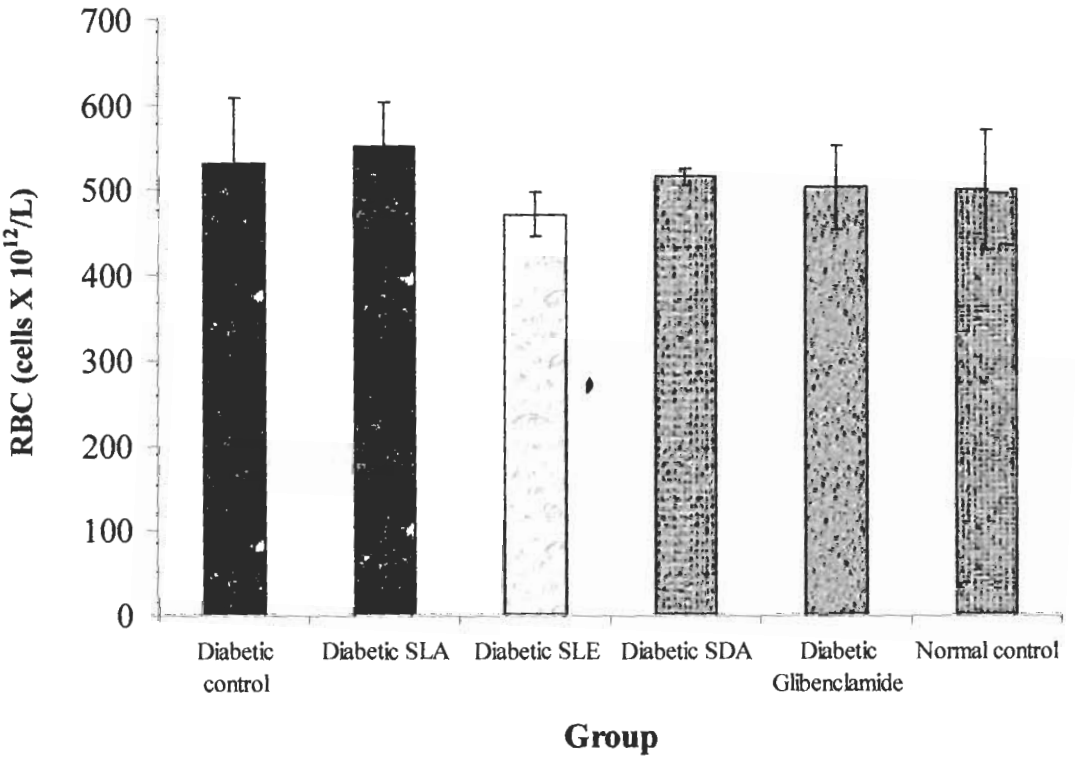

Fig. 3.27: Effect of extracts on red blood cell (RBC) count in normal control and diabetic rats.

3.18 EFFECT OF THE EXTRACTS AND GLIBENCLAMIDE ON THE ACTIVITIES OF SERUM ALANINE AMINOTRANSFERASE (ALT) AND ASPARTATE AMINOTRANSFERASE (AST)

The activities of ALT and AST in the sera of the animals are shown in Figures 3.14 and 3.15 (Appendix 5). There was no significant difference ($P>0.05$) in the activity of AST in the sera of the animals treated with the extracts when compared to that of the diabetic control animals and the normal control animals. However, the animals that received SLE and glibenclamide showed slight increases in AST activity. The activity of ALT in the sera of the diabetic control animals was significantly increased ($P<0.05$) when compared with the normal control animals. Treatment with SDA significantly ($P<0.05$) reduced ALT activity while the other extracts showed very slight increases in ALT activity when compared with the diabetic control animals.

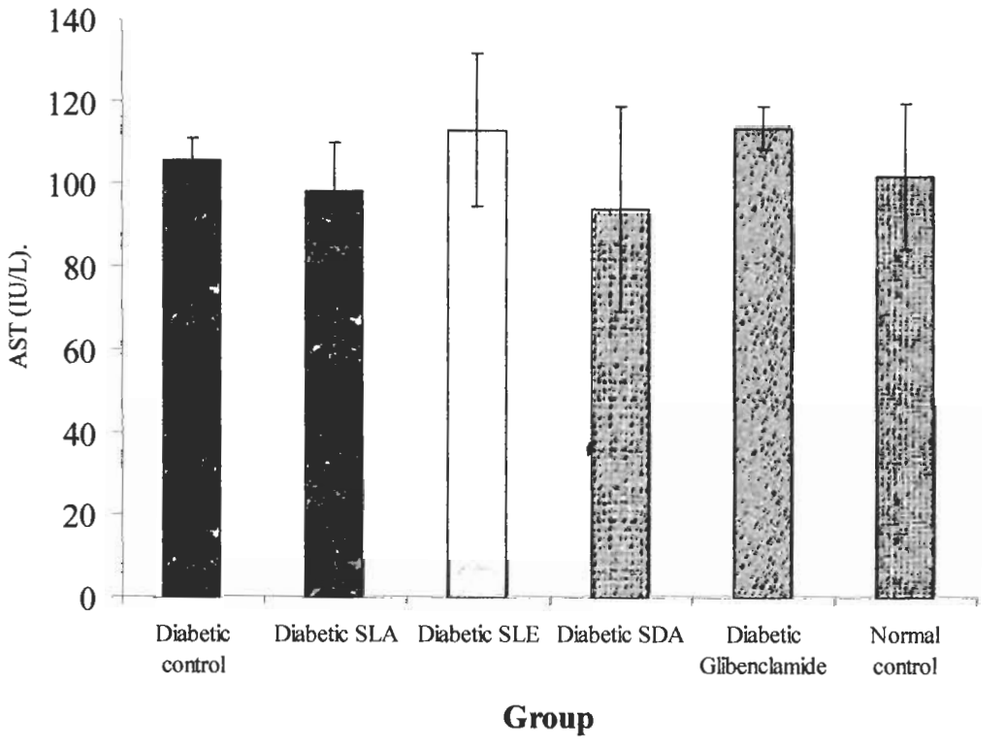

Fig. 3.28: Effect of extracts on aspartate aminotransferase (AST) in normal control and diabetic rats.

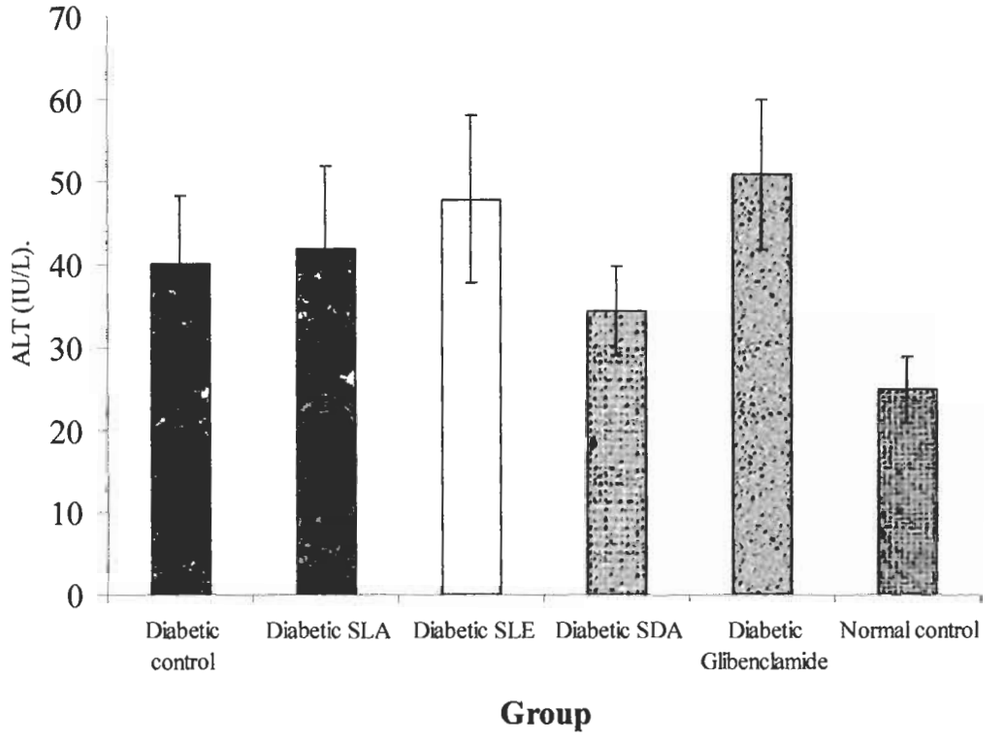

Fig. 3.29: Effect of extracts on alanine aminotransferase (ALT) in normal control and diabetic rats.

3.19 HISTOPATHOLOGY CHANGES

Characterised predominantly circulatory disturbances; degenerative/necrotic and inflammatory changes. Liver sections of non-diabetic, untreated rats were devoid of lesions and showed the normal histologic features with the hepatocytes, radiating from the central vein of a lobule to the periphery (portal area) containing the bile ducts, lymphatics and blood vessels (Plate 3.1).

Liver sections of diabetic, untreated rats were mildly to moderately hyperurimic/haemorrhagic with mild haemosidemin deposits in the centrilobular areas and the periportal areas. There were moderate areas of hepatocytes degeneration and necrosis in the centrilobular and periportal areas, with massive inflammatory exudates consisting of mononuclear leucocytes and plasma cell in the areas. There was also kupffer cells hypertrotry and proliferation; and biliary.

Liver sections of diabetic animals treated with glibenclamide showed variable degrees of hyperaemia/haemorrhage; but the hypatocytes in both centrilobular and portal areas only showed very mild degeneration/necrosis; and without infiltration of inflammatory cells (Plate 3.3).

Of the other groups of diabetic rats treated with the different extracts, only liver sections of rats with SDA demonstrated a level of hepatoprotection compared with what was observed for glibenclamide. There were only mild lobular foci of degeneration and necrosis, and with mild infiltration of inflammatory cells into the portal area (Plate 3.4).

In the subacute toxicity studies of the different types/dosages of the extracts; non-diabetic rats treated with the different extracts showed focally diffuse area of hepatocyte degeneration and necrosis with associated mononuclear leucocyte infiltration. The histopathologic lesion was more severe in the periportal areas. The SLA treated rats showed minute non-specific hepatocellular degeneration (Plate 3.5). SLE treated rats showed mild to moderate hepatocellular changes; while the hepatotoxicity of non-diabetic SDA treated rats showed a dose-dependent portion of lesions severity with the 500 mg/kg treated rats showing widespread areas of hepatocyte necrosis and severe mononuclear leucocytes infiltration. Kupffer cells proliferation was also severe just as was biliary epithelial cell proliferation (Plate 3.6). Rats treated with 125mg/kg of SDA

showed very mild hepatic degenerative and necrotive changes; and mild MNL infiltration of the portal areas (Plate 3.7).

Kidney sections of untreated, non-diabetic rats showed the normal histologic features for the mammalian kidney with clearly discernible renal corpuscles (glomeruli) located in the cortex: radially arranged tubules of different characters running from cortex to medulla (Plate 3.8). Kidney sections of diabetic, untreated rats showed severe degeneration and necrosis of tubular epithelial cells and inflammatory cell (mononuclear leucocytes and plasma cells) infiltration of the interstitial spaces and peri-glomerular spaces (Plate 3.9). Kidney of diabetic rats treated with glibenclamide showed mild to moderate hyperaemia but with little or no degeneration and necrosis of tubular epithelial cells: and without mononuclear leucocyte infiltration.

Kidney sections of rats treated with the different extracts in the subacute toxicity study did not show the typical histological features (tubular degeneration and necrosis) associated with diabetes. The different extracts (SLA, SLE and SDA) appeared to have produced variable degrees of nephrotoxicity in the treated rats. The nephrotoxicity appeared to have been dose-dependent in the case of SDA; in which tubular degeneration was associated with focally diffuse, but mild mononuclear cells infiltration of the interstices and the peri-glomerular spaces (Plate 3.10)

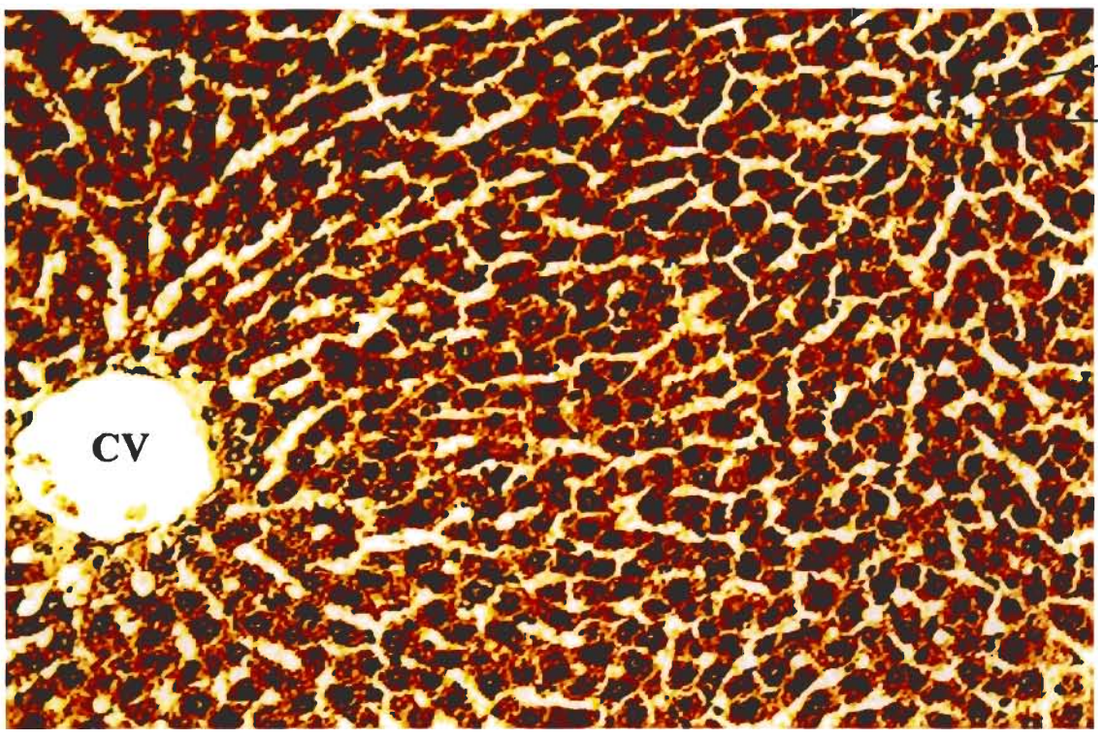

Plate 3.1: Liver section of non-diabetic, untreated rat showing normal histologic features of the liver. Note central vein (CV) and hepatocytes radiating to the portal area containing bile duct (Bd) and hepatic portal vein (HPV).
H&E Stain: × 200

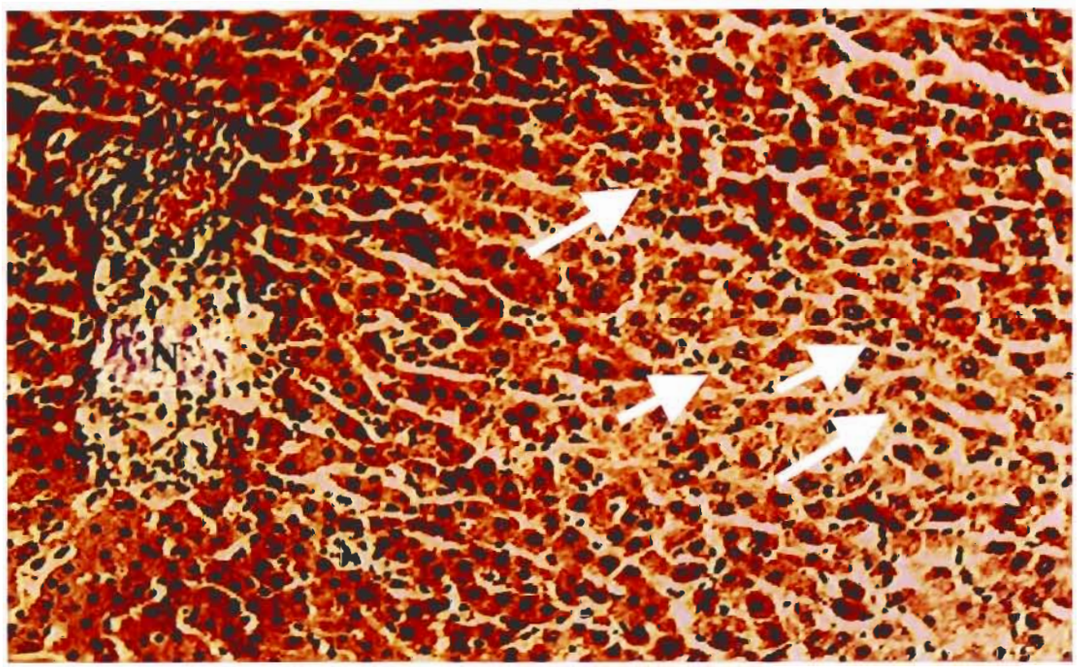

Plate 3.2: Liver section of diabetic, untreated rat showing mononuclear leucocytes around the central vein (CV) in the portal area; focal area of periportal hepatocytes necrosis (N) and widespread lobular hepatocyte degeneration and necrosis (arrows).
H&E Stain: × 200

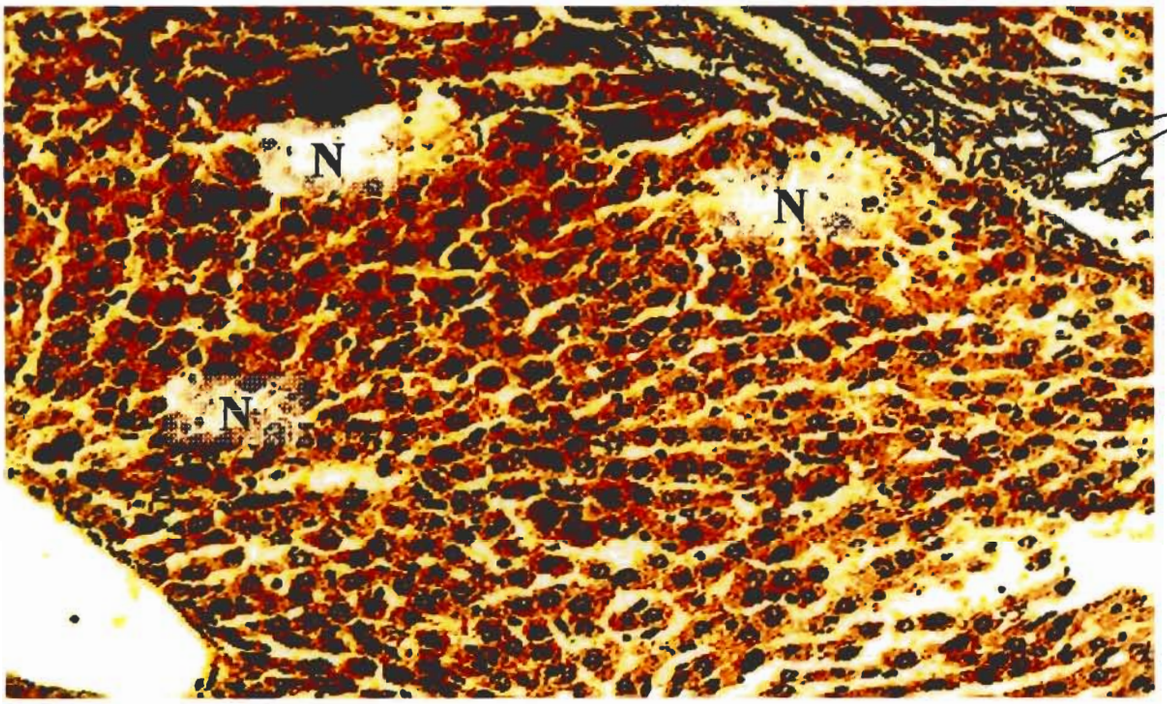

Plate 3.3: Liver section of diabetic rat treated with Glibenclamide (5mg/kg b.w.) showing focally diffuse areas of lobula hepatocytes degeneration and necrosis (N) but without mononuclear cells unfiltration of the portal areas (P).

H&E Stain: × 200

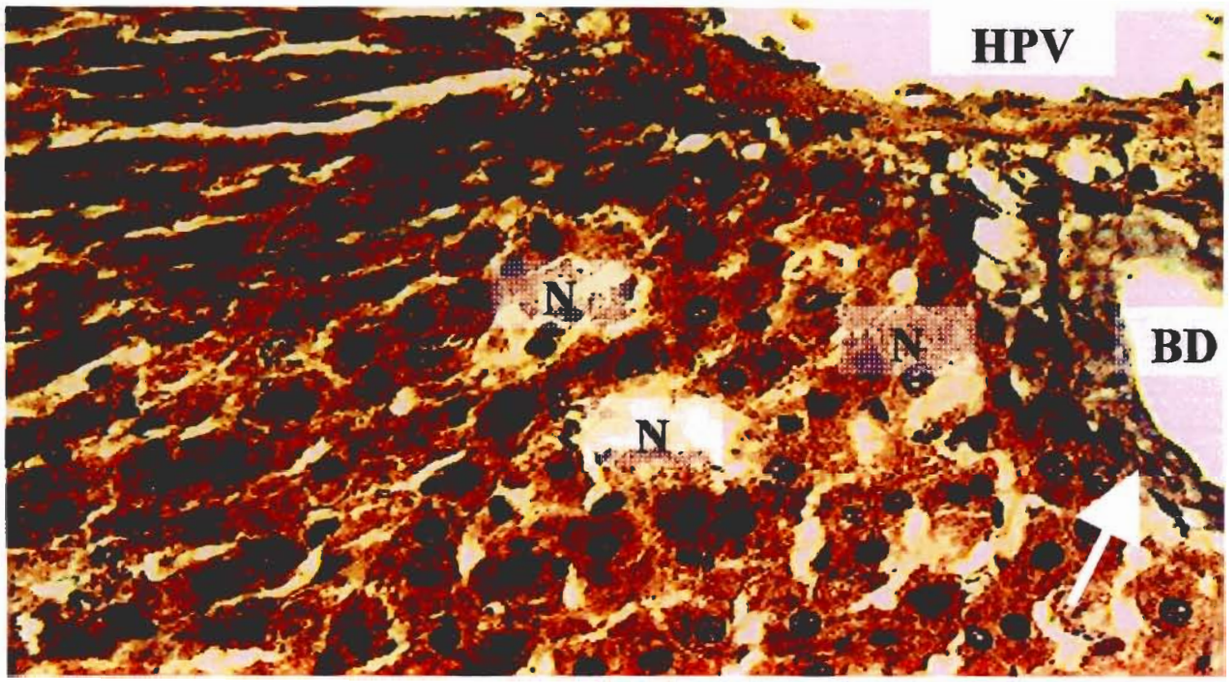

Plate 3.4: Liver section of diabetic rats treated with SDA (150mg/kg) showing lobular foci of hepatocytes degeneration/necrosis (N), and mild mononuclear leukocytes infiltration of the portal area (arrow). Note Hepatic Portal Vein (HPV) and Bile Duct (BD).

H&E Stain: × 400

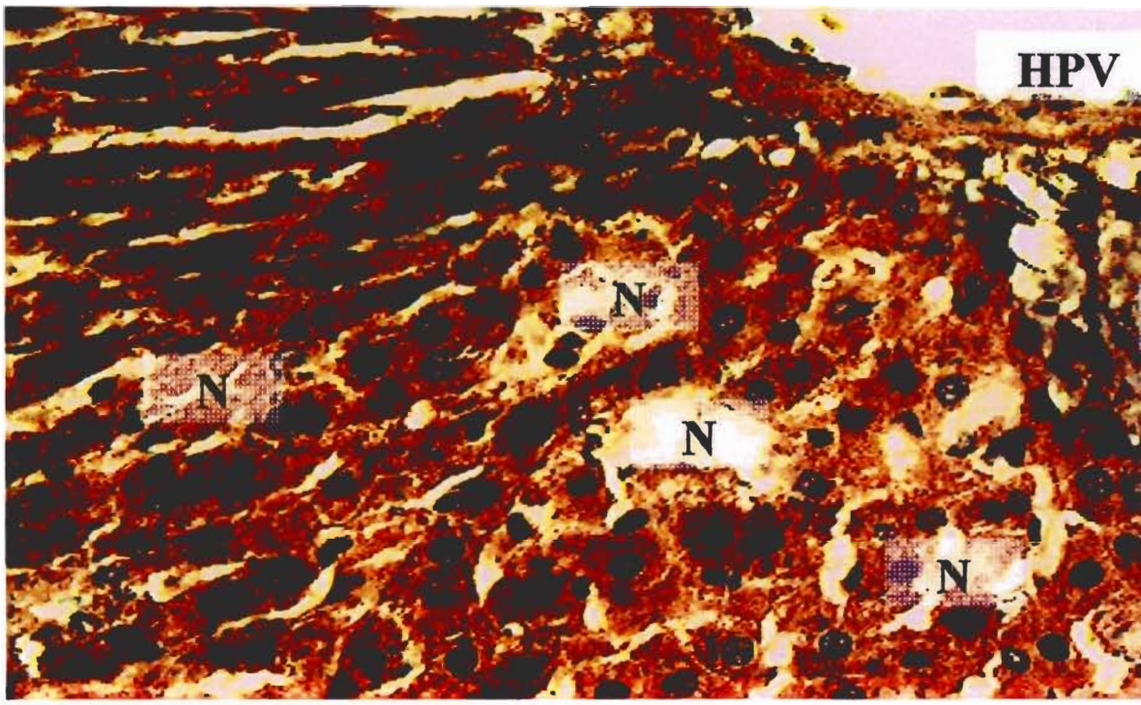

Plate 3.5: Liver section of non-diabetic rat treated with SLA (250mg/kg) in subacute toxicity study showing tiny foci of hepatocytes degeneration and necrosis (N). Note absence of mononuclear leukocytes and Hepatic Portal Vein (HPV) and Bile Duct (BD). H&E Stain × 400

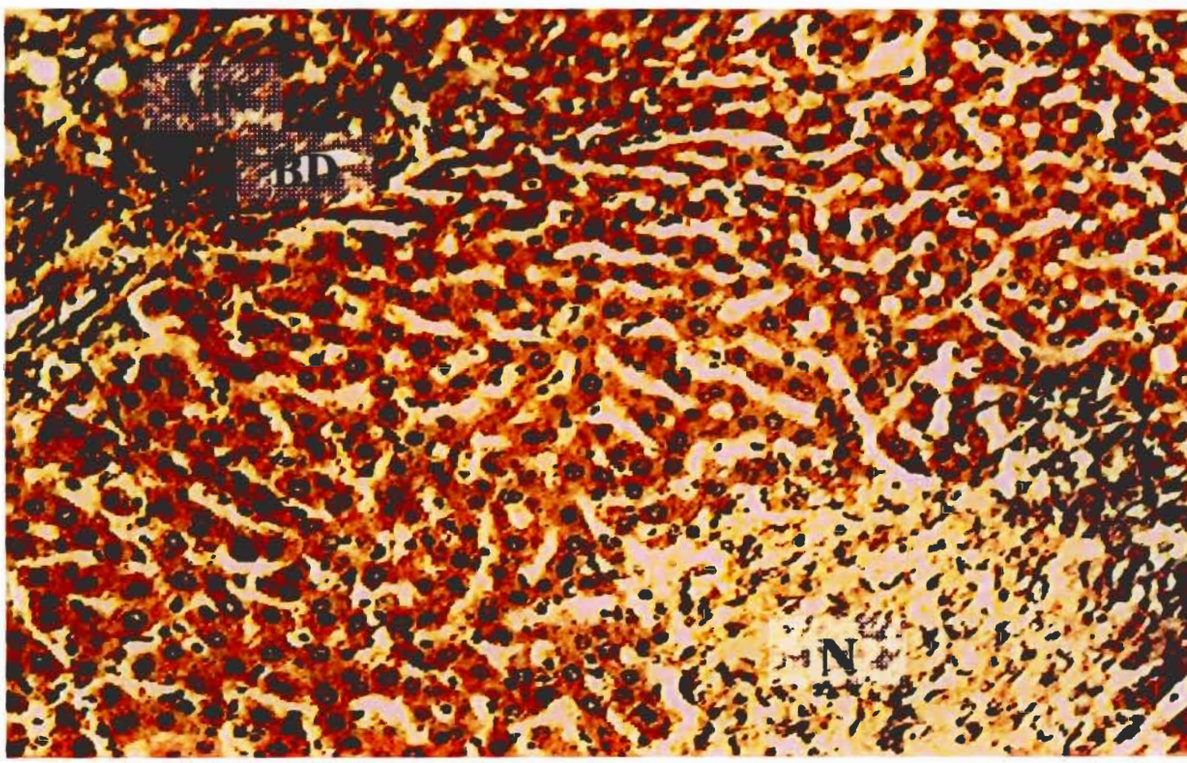

Plate 3.6: Liver section of non-diabetic rats treated with SDA (500mg/kg) in subacute toxicity study showing wide area of hepatocytes necrosis (N) and severe mononuclear leukocytes (MNL) infiltration of the portal area. Note: Bile Duct (B.D).
H&E Stain: × 200

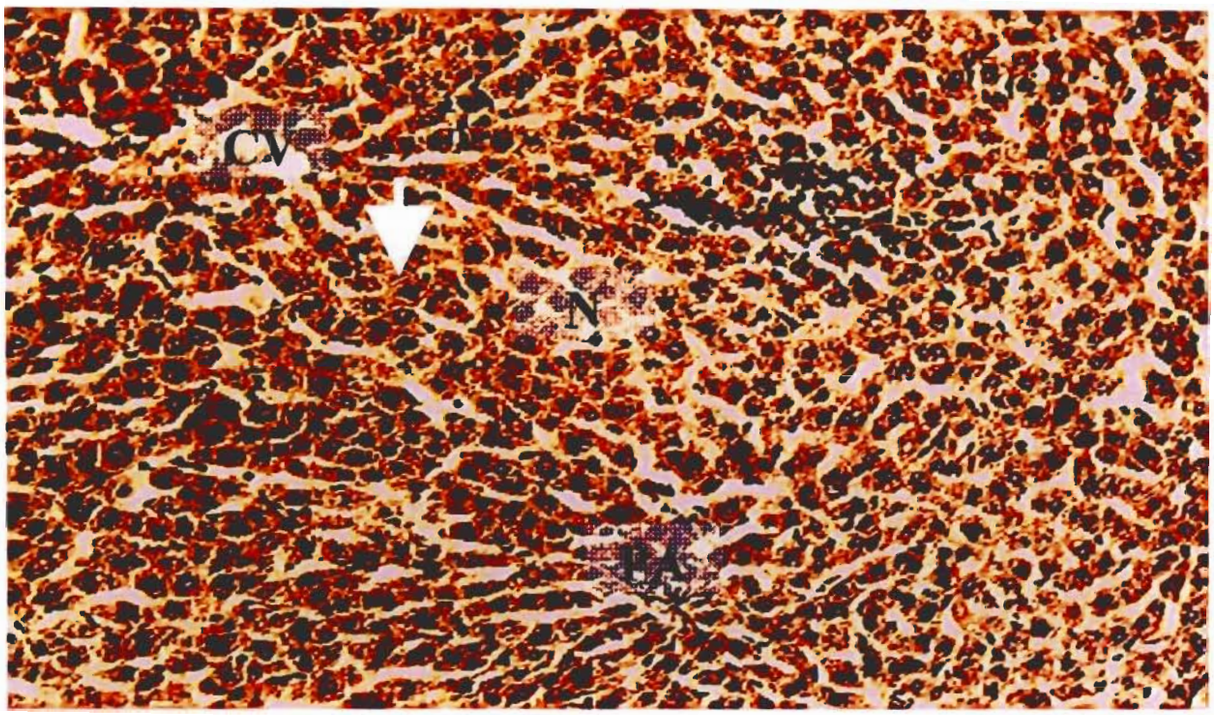

Plate 3.7: Liver section of nondiabetic rat treated with SDA (125mg/kg) in subacute toxicity study showing mild paulobular hepatocytes degeneration (arrows). Note Central Vein (CV) and Portal Area (PA) of a lobule.

H&E Stain: × 200

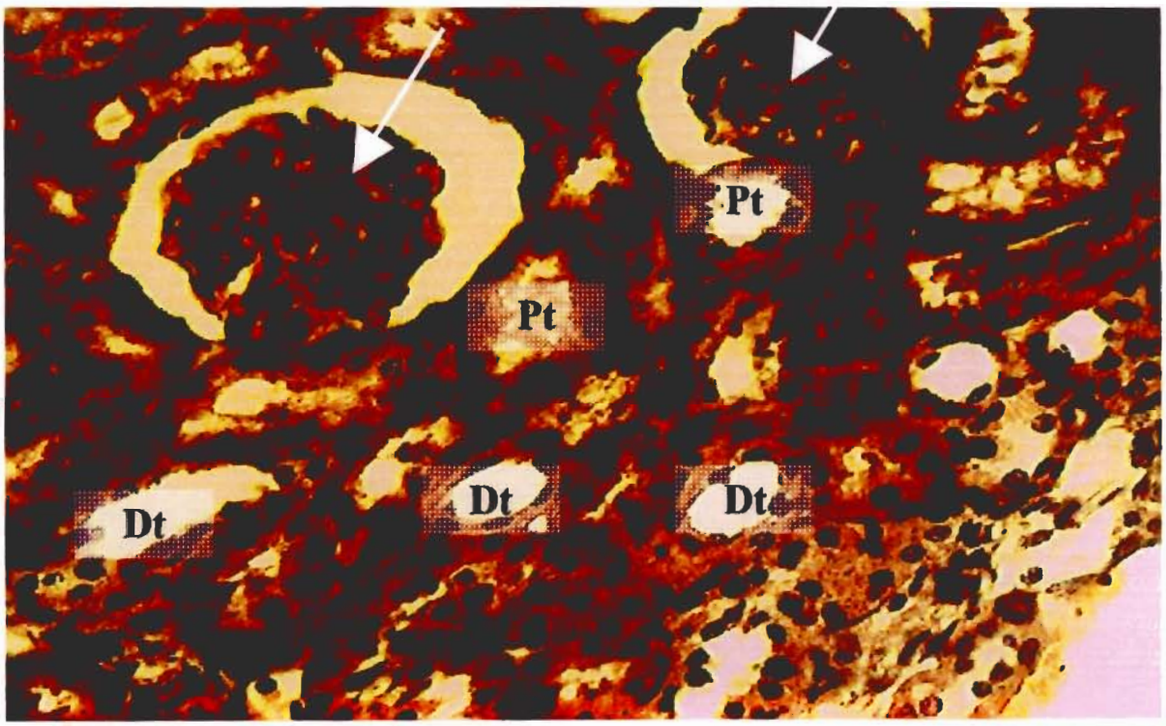

Plate 3.8: Kidney section of non-diabetic, untreated rat showing Renal Corpuscles (arrow), Proximal tubules (Pt) and Distal tubules (Dt) with normal histologic features. H&E Stain: × 200

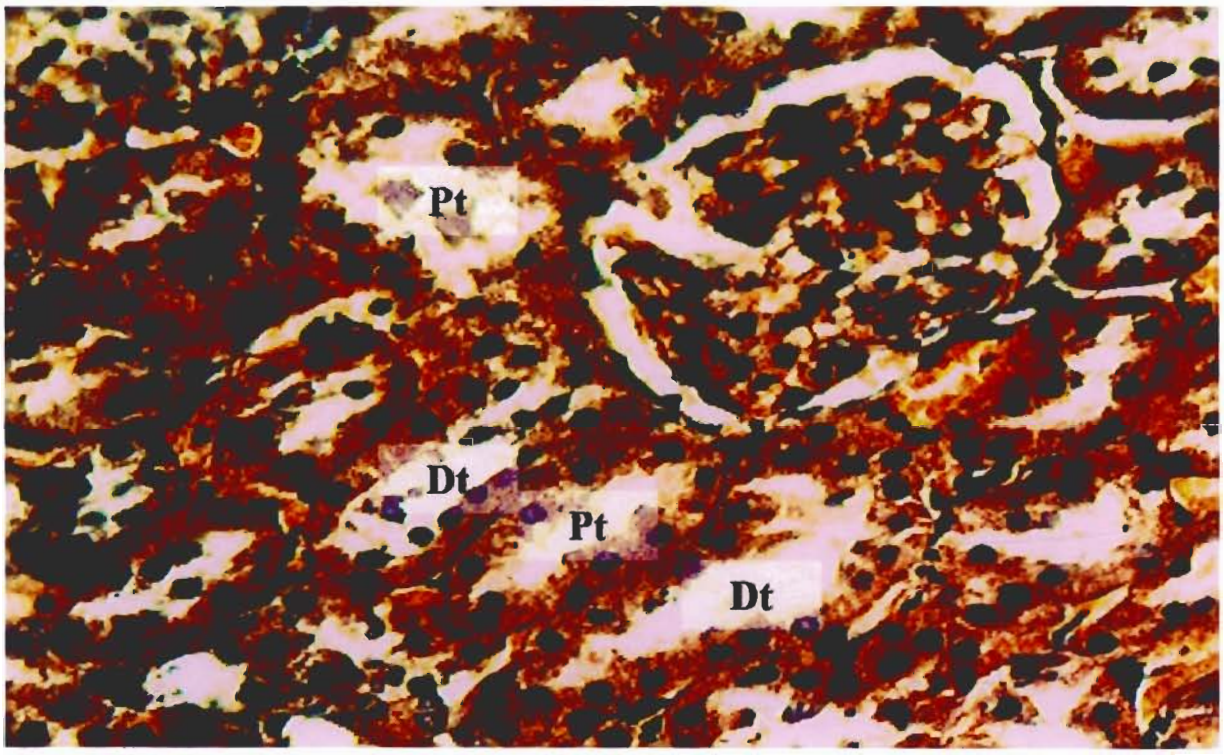

Plate 3.9: Kidney section of diabetic, untreated rat showing widespread degeneration of endothelial cells of the renal corpusules (arrow), Proximal tubule epithelia (Pt) cells and Distal tubule epithelial (Dt) cells.

H&E Stain: × 200

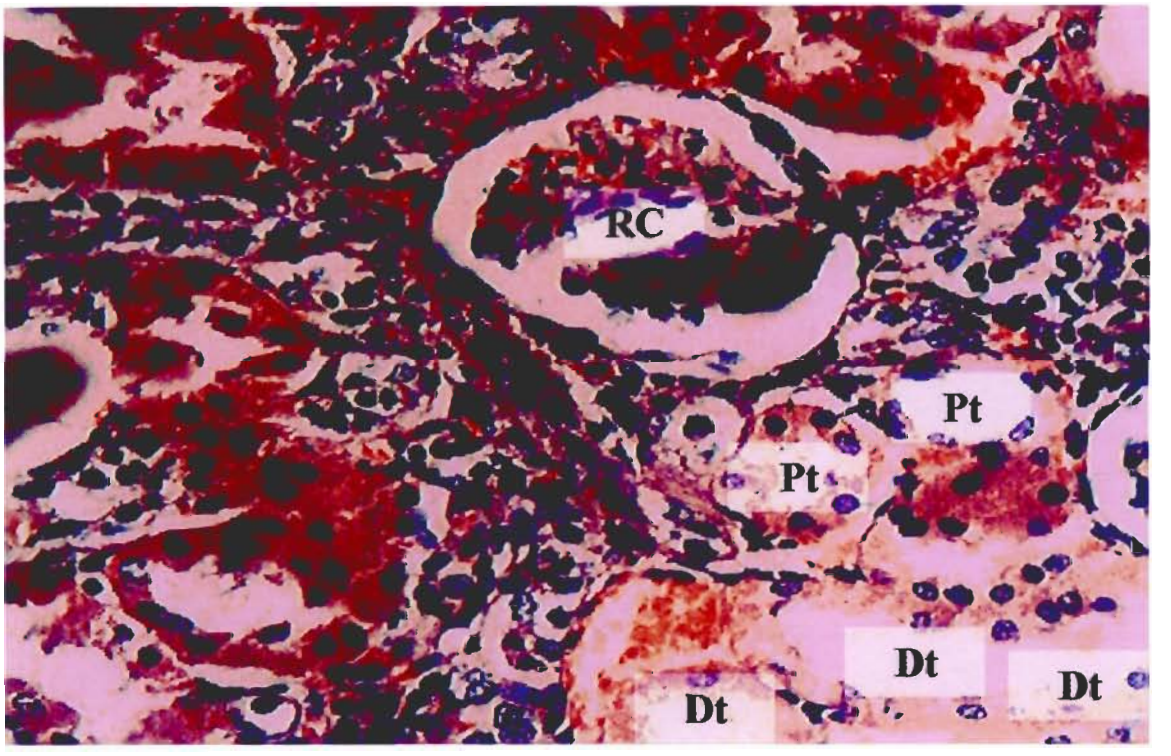

Plate 3.10: Kidney section non-diabetic rat treated with 500mg/kg of SDA showing focal area of Proximal tubules (Pt) and Distal tubules (Dt) degeneration and necrosis. Note Renal Corpuscle (RC) with endothelial cells degeneration and mild mononuclear leukocytes aggregation around the Corpuscle (arrow).
H&E Stain: × 200

CHAPTER FOUR
DISCUSSION

Diabetes mellitus which is about the fastest growing metabolic disease worldwide is characterised by a reduced capacity of the ß-cells of the pancreas to release sufficient insulin to induce the activity of glucose metabolising enzymes. The ß-cells may be destroyed as in the type 1 diabetes (IDDM) or intact as in type 2 diabetes (NIDDM) (Panneerselvam and Govindaswamy, 2002). With an increasing knowledge of its multifactorial nature, there is a need for safer and more effective therapies.

In this study, the three different extracts (SLA, SDA and SLE) possess pharmacologic action and show varied potential as antihyperglycaemic agents. The results also provide a pharmacological basis for the use of the plants in traditional medicine for the management of diabetes mellitus. Even though the ethanol extract showed the highest yield percentage, at the dose tested, it exhibited the least anti-hyperglycaemic activity. This may be as a result of the fact that ethanol may have reacted with one or more of the active constituents to reduce the activity or due to ethanol toxicity.

At the dose tested, the combined extract (SDA) exhibited better anti-hyperglycaemic activity than glibenclamide (5 mg/kg) in both the acute and chronic situations. Studies carried out to access the safety of these plant extracts using mice revealed an oral LD_{50} greater than 5 g/kg. Since 5 g/kg is the maximum allowable dose by the Organisation for Economic Co-operation and Development (OECD) Guideline 423 for the testing of chemicals (OEDC, 2000), it was not quite necessary administering higher doses. The result of a preliminary dose response study using SLA indicated that 250 mg/kg is the most effective dose showing a biphasic effect ranging between 50 mg/kg and 250 mg/kg. This suggests that beyond the peak dose of 250 mg/kg, the extract may no longer be safe.

Pharmacological investigation of these extracts, indicated that SLA and SDA caused

at a dose of 250 mg/kg, while increase in the dose to 500 mg/kg did not show a significant change in the fasting blood glucose levels (FBGL).

The effect of standard oral hypoglycaemic agent (glibenclamide) was compared with the extracts: in the normoglycaemic rats glibenclamide produced a noticeable hypoglycaemic activity, significant at both the 3rd and 4th hours of administration. In the diabetic rats, the effect of glibenclamide on FBGL compared well with that of the extracts (SLA and SDA), but the extracts showed a slight initial increase in FBGL before the progressive and marked reduction.

Alloxan produces hyperglycaemia by selective cytotoxic effect on pancreatic β-cells (Szkudelski, 2001), causing permanent destruction of β-cells. The dose 65 mg/kg of alloxan used in this study, caused moderate diabetes (Grover *et al.*, 2000). It has been reported that glibenclamide was not very effective when complete destruction of β-cells has occurred and hence more effective in moderately diabetic rats than in severe diabetes (Sharma *et al.*, 2000). The acute hypoglycaemic effect of glibenclamide (sulphonylureas) has been shown to be by the stimulation of insulin production by the residual β-cells of the pancreas in addition to enhancement of glucose utilisation (Moller, 2001).

This suggests that the extracts may have a similar mechanism of action with glibenclamide and may in addition possess an insulinomimetic effect on peripheral tissues (i.e. extrapancreatic mechanism) either by promoting glucose uptake and metabolism or inhibiting hepatic gluconeogenesis (Djomeni *et al.*, 2006). This postulation correlates with that of Farjou *et al.* (1987) on the work with *Artemisia*

The antihyperglycaemic activities of the extracts SLA and SDA were quite considerable and in fact the activity of SDA exceeded that of glibenclamide and hence the inference that this extract may be more efficacious as more than one component may be responsible for activity.

Daily administration of SLA and SDA (250 mg/kg) and glibenclamide (5 mg/kg) to the diabetic rats twice daily for three (3) weeks resulted in a significant reduction

(54%, 61% and 53% respectively) in fasting blood glucose levels. The reduction was considerably less (19%) with SLE. These observations suggest that the active principle(s) may be more hydrosoluble than solvensoluble. Repeated administration of these extracts twice daily elicited an increase in the fasting blood glucose levels of the diabetic rats within the first week which subsequently (week 2) decreased up to the third week. This finding may explain the assertions of tradomedical practitioners: that the combination therapy demonstrates an initial rise in FBGL which goes down after sometime and then stabilises. In view of this, unlike the sulphonylureas, the onset of action of these extracts is slow and so the extracts cannot be used for the management of emergency situations.

The protein concentrations of the organs were not significantly affected in the liver and kidney fractions based on the total protein assay. This does not rule out the possible presence of modification and damages to the proteins as a result of oxidative processes. Protein modification is usually more evident from about the 12^{th} week of hyperglycaemia (Wolff *et al.*, 1991).

Diabetes mellitus is known to be associated with a reduced capacity of the β-cells of the pancreas to release sufficient insulin to induce the activity of glucose metabolising enzymes in both type 1 and type 2 diabetes mellitus (Panneerselvan and Govindaswamy, 2002). The results of this investigation indicate that the activities of hexokinase (EC 2.7.1.2), the first enzyme of glycolysis, glucokinase (EC 2.7.1.3) and the rate-limiting enzyme of glycolysis (EC 2.7.1.11) were significantly depressed in untreated diabetic rats. These results are consistent with the reports of other researchers for the activities of Hexokinase and glucokinase (Hikino *et al.*, 1989; Grover *et al.*, 2000; Panneerselvan and Govindaswamy, 2002; Mahmood *et al.*, 2003; Gupta *et al.*, 1999; Stanley *et al.*, 2000) and phosphofructokinase (Sochor *et al.*, 1991; Grover *et al.*, 2000).

Treatment of the diabetic rats with the extracts SLA and SDA showed an increased hexokinase, glucokinase and phosphofructokinase activities; while SLE depressed the activities of glucokinase and phosphofructokinase but increased hexokinase activity. Furthermore, reduced glucokinase activity favours the release into the circulation of

glucose synthesised via gluconeogenesis. This suggests why SLE exhibited a very poor antihyperglycaemic effect. The mechanism(s) of action of SLA and SDA are not yet known exactly, but from their effect on these glycolytic enzymes, they seem to increase flux of glucose into the glycolytic pathway in an attempt to reduce high blood-glucose concentration. Reduction in these enzyme activities in diabetic animals has been reported to give rise to a depletion of liver (Iynedjian *et al.*, 1988; Seoane *et al.*, 1996) and muscle glycogen (Hikino *et al.*, 1989).

It has been previously demonstrated that the hepatic glycogen content in untreated diabetic rats was higher than in treated and untreated non-diabetic rats (Anderson and Stowring, 1973; Chattopadhyay, 1998 and Ugochukwu and Babaday, 2003). In this study, the hepatic glycogen content was reduced significantly in diabetic controls as compared to the normal control animals. This result correlates these earlier findings and in addition is in agreement with the findings of Welihinda and Karunanayake, 1986; Grover *et al.*, 2000; and Bollen *et al.*, 1998 who demonstrated that glycogen deposition from glucose is impaired in diabetic animals and in proportion to the severity of insulin deficiency (Gannon and Nuttal, 1997; Stalmans *et al.*, 1997). Some workers (Pushparaj *et al.*, 2001) have found no differences, though.

Treatment of the diabetic rats with the extracts (SDA and SLA) inhibited this depletion in glycogen content with SDA almost returning it to normal while the effect of SLE was quite negligible. This is possibly due to either the stimulation of insulin release from β-cells (Lolitkar and Rao, 1996) or insulinomimetic activity of the extracts giving rise to direct peripheral glucose uptake or combination of the two.

In this study, we observed a significant increase in malondialdehyde (MDA) levels in both the liver and the kidney of diabetic rats when compared to normal control rats; hence reaffirming the claim that lipid peroxidation is induced by diabetic condition (Cho *et al.*, 2002; Ayidin *et al.*, 2001). Repeated treatment of the diabetic rats significantly reduced the MDA concentrations while surprisingly SLA and SLE slightly increased MDA concentration in both organs.

Several studies with human and animal models using TBARS (MDA) assay have shown increased lipid peroxidation in liver and kidney membranes (Feillet-Coudray *et al.*, 1999; Kakkar *et al.*, 1997 & 1998; Obrosova, 2003) of diabetic rats. Free radicals are formed disproportionately in diabetes by glucose oxidation, glycation of proteins and the subsequent oxidative degradation of glycated proteins (Martini *et al.*, 2003). These reactive compounds in turn cause lipid peroxidation, resulting in the formation of hydroperoxy fatty acids and endoperoxides which increase the formation of malondialdehyde and thromboxaneB_2 (TxB_2). The accumulation of TxB_2 and TxA_2 can cause platelet aggregation and promote thrombosis (Sushil *et al.*, 1998). The significant reduction in MDA by SDA could lead to a decrease in oxidative stress in both the liver and the kidney, especially in the latter and hence a possible deceleration in the progression of diabetic complications in the kidney.

Numerous studies have demonstrated that the reduced glutathione (GSH) concentration in both the diabetic kidney (Lee *et al.*, 2000; Obrosova *et al.*, 2003) and liver (Ugochukwu and Babady, 2002) are found to be significantly reduced. This suggests that the decreased GSH concentration may play a role in the development of diabetic complications. In this study, there was a significant increase in GSH concentration in the diabetic rats treated with the extracts compared to the diabetic control rats. These increases could be one of the factors responsible for the reduced oxidative stress in the diabetic treated rats.

The activity of glutathione S-transferase (GST) was highest in the organs from the diabetic animals. This could be as a result of the induction of enzyme synthesis in diabetics. The high level of glutathione S-transferase activity in the diabetic control animals correlates with the decreased level of glutathione in the group. The different extracts tested have different levels of modulatory activity on the glutathione content and glutathione S-transferase activity of the different organs.

Oxidative stress has been known to induce the production of Reactive Oxygen Species (ROS) scavenging enzymes (Niskanem *et al.*, 1995, Noumoz-Zadeh *et al.*, 1997). From these results, the enzymes superoxide dismutase (SOD) and catalase activities were observed to increase in the diabetic control rats compared to the

normal rats in both organs. However, treatment with SDA and SLA significantly reduced the catalase activity in the hepatocytes and in the kidney with SDA almost normalizing the activity. SLE showed a very insignificant effect on the enzyme activities. These results were consistent with other studies for liver SOD and kidney SOD (Yadav *et al.*, 1997;) as well as liver catalase (Ugochukwu *et al.*, 2003) and kidney catalase (Ugochukwu and Coboume, 2003).

In contrast, some other studies have reported a decrease in the activity of SOD in diabetic control animals after four (4) weeks (Gumieniczek, 2003). These enzymes were probably responsible for the compensatory mechanism in an attempt to maintain homeostasis. The increased SOD activity may result from increased dismutation of superoxide anions due to their rapid production (Cho *et al.*, 2002). The increased catalase activity in the diabetic control rats could be as a result of high hydrogen peroxide (H_2O_2) concentrations, since catalase scavenges H_2O_2 (Ugochukwu *et al.*, 2003).

The decreased level of glutathione that was recorded in the diabetic control animals could equally be a compensatory mechanism by the animals to overcome the effects of oxidative stress that might have resulted from decreased activity of catalase and SOD. Glutathione participates in widely differing metabolic pathways and functions as an antioxidant through two distinct mechanisms (Fernandez-Checa *et al.*, 1997). First, glutathione can transfer hydrogen ions directly to oxidants without the intervention of an enzyme thereby quenching the activity of the oxidant. Secondly, glutathione can donate hydrogen atoms to assist an enzyme that converts hydroperoxide to water; the glutathione peroxidase, (Fernandez-Checa *et al.*, 1997). In this way, glutathione is involved in the quenching of different reactive oxygen species; and therefore acts as a compensatory antioxidant to both SOD and catalase. Glutathione depletion is a positive indicator of tissue degeneration and the magnitude of depletion parallels the severity of the damage (Anderson, 2004).

Lipids play a very vital role in the pathogenesis of diabetes mellitus. Lipid profile which is altered in the serum of diabetic patients (Orchard, 1990 and Betteridege, 1994), appears to be a significant risk factor in the development of premature

atherosclerosis and coronary heart disease (Minorova *et al.*, 2000). This entails an increased triglyceride and total cholesterol concentrations. Hyperlipidaemia is a recognised consequence of diabetes mellitus (Pushparaj *et al.*, 2000; Pepato *et al.*, 2003; Sharma *et al.*, 2000).

In this study, the extracts significantly reduced raised triglyceride and total cholesterol concentrations in treated diabetic rats when compared with the untreated diabetic group of rats. Similar observations were reported by Pushparaj *et al.* (2000); Ugochukwu *et al.* (2003) and Momo *et al.* (2006) on the respective effects of *Averrhoea bilimbi*, *Gongronema latifolium* and *Laportea ovatifolia* on diabetes and dyslipidaemia. The abnormal high concentration of serum lipids in diabetes is mainly due to the increase in the mobilisation of fatty acids from the peripheral depots, since insulin inhibits the hormone sensitive lipase. The extracts might have reduced the triglycerides in the diabetic animals by decreasing the serum non-esterified fatty acids (NEFA) similar to masoprocol (Reed *et al.*, 1999).

Results showing the effect of the extracts on LDL–cholesterol level revealed a significant suppression of LDL–cholesterol by alloxan treatment. Much as the different extracts elevated it, the elevation was slight and non-significant. The reason for this observation was not known or investigated.

Furthermore, it was also observed that single extract treatments (SLA and SLE) increased HDL–cholesterol levels relative to the diabetic untreated group of rats, but the combination therapy, SDA significantly elevated the HDL–cholesterol level even above the normal control level. This is of interest, since SDA remarkably elevated HDL–cholesterol, it significantly decreased atherosgenicity (increased anti-atherogenic index). SDA therefore has the potential to prevent the formation of atherosclerosis and coronary heart disease which are secondary diabetic complication (Fontbonne *et al.*, 1989; Cho *et al.*, 2002).

The effect of the extracts on the haematological parameters showed insignificant decreases in the haemoglobin (Hb) and haematocrit contents of the diabetic control group of rats when compared with the normal control group. Treatment with the

extracts increased these parameters. Haematocrit or packed cell volume (PCV) can be used as a screening tool for anaemia. No significant difference was recorded in the red blood cell (RBC) count of all the groups.

Since Hb, PCV and RBC reflect the total population of red blood cells in the blood, it implies that the extracts may affect incorporation of haemoglobin into red blood cells and the morphology/osmotic fragility of red blood cells produced.

However, the increase in these parameters suggests that the extracts may improve the population of red blood cells produced from the bone marrow (Cheeke, 1998; Seeley *et al.*, 1998).

Moreso, the white blood cell (WBC) counts of the groups that received SLA, SLE and glibenclamide showed significant increases when compared with the diabetic control group. The group that received the combination therapy (SDA) compared well with the normal control group. These findings suggest that alloxan may have caused destruction or impaired production of white blood cells. It has been reported that granulocyte – macrophage colony stimulating factor and interleukins IL-2, IL-4 and IL-5 regulate the proliferation, differentiation and maturation of committed stem cells responsible for the production of white blood cells (Ganong, 2001; Guyton and Hall, 2000). Alloxan may have interfered with either of these steps in the production of WBC; thereby predisposing to infection. Since the extracts improved the status of the white blood cell counts, they may possess the potential of causing progressive but selective bone marrow activation.

The sub-acute toxicity studies using 250 mg/kg of the extract were carried out for 30 days to further assess the safety profile of these extracts. The results revealed no adverse effect on the haematological parameters which includeWBC and RBC counts, Hb, Hct, MCV and MCH. The extracts also showed no deleterious effect on the serum liver enzymes, AST and ALT. Thus, these plant extracts can be presumed to be free from toxicological effect on these parameters at the dose 250 mg/kg used in the study.

The activities of the serum enzymes: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were elevated in the diabetic animal groups when compared with the normal control animals. The extract SDA reduced the levels of AST and ALT while SLA reduced only AST but not ALT in the serum. Although ALT and AST are markers of hepatocellular injury, their serum levels also are also elevated in situations of injury to other organs like kidney, heart and muscle (Bain, 2003; Valentine *et al.*, 1990). Therefore, the increased levels of ALT and AST in the serum of the diabetic untreated animals could be a result of liver or kidney damage; since the animals were under oxidative stress that also affected the kidneys.

Phytochemical analysis of the extract also revealed the presence of constituents known to posses a single or a diverse range of biological activities.

4.1 CONCLUSION

In summary, the results of this study have shown that roots of *Sarcocephalus latifolius* alone and in combination with *Daniella oliveri* possess antihyperglycaemic, showed capacity to reduce both lipid levels and oxidative stress. The onset of hypoglycaemic action is late as compared to that seen with insulin or the sulfonylureas and hence the extracts will be preferable in long-term treatment or as a diabetic maintenance therapy. Although both extracts showed protective effects against oxidative stress induced by hyperglycaemia, the degrees vary. Actually, SDA i.e the combination extract, is a better option than SLA since it not only showed better glucose lowering activity, but also elicited a more potent antioxidative and anti-lipidemic activities.

4.2 SUGGESTIONS FOR FURTHER STUDIES

Further biochemical and pharmaceutical investigations are required to elucidate the nature of the active compound in both roots in isolation and in combination and use them to carry out bioassay-directed experiments to determine their mechanism(s) of action.

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APPENDICES

Appendix 1: Protein Concentration (mg/ml) in the Organs

GROUP	LIVER MEAN±S.D	KIDNEY MEAN±S.D
Normal Control	29.90±3.17	16.15±3.18
Diabetic Control	16.90±2.83	12.77±3.76
SLA-250	18.85±2.78	14.17±2.51
SLE-250	18.87±1.25	13.37±0.42
SDA-250	20.12±3.17	13.97±3.41
Glibenclamide	19.65+3.14	14.95±3.08

Appendix 2: Effect of 3-week repeated administration of the extracts on the serum lipid profile of diabetic rats[a]

Group	**Total Cholesterol (mMol/L)**	**Triglycerides (mMol/L)**	**HDL-Cholesterol (mMol/L)**	**LDL-Cholesterol (mMol/L)**
Normal Control	2.49±0.89	1.21±0.14	0.64±0.11*	1.32±0.81
Diabetic Control	2.80±0.30	1.87±0.13	0.34±0.07	0.48±0.22
SLA-250	1.27±0.47*	0.87±0.18*	0.43±0.06	0.61±0.44
SLE-250	1.54±0.22*	1.06±0.18*	0.48±0.04*	0.66±0.06
SDA-250	1.95±0.21*	1.10±0.26*	0.75±0.09*	0.72±0.20
Diabetic Glibenclamide	1.41±0.63*	1.13±0.41*	0.24±0.04	0.77±0.52

a values are given as mean ± S.D. for n=4

Appendix 3: Effect of the extracts on haematological parameters

Treatment Group	HCT (%)	Hb (g/dl)	WBC (cells/mm3)	RBC (×1012/L)
Normal Control	46.00±6.32	14.47±1.48	4725±1152.89	497.50±69.46
Diabetic Control	38.50±1.73	12.76±3.11	4025±590.90	528.75±77.07
SLA-250	45.00±4.32	13.63±1.31	6950±1611.42	548.75±51.70
SLE-250	44.50±3.42	13.44±1.03	6350±1167.62	568.75±25.94
SDA-250	43.25±4.27	13.42±1.12	4475±457.35	512.50±9.57
Diabetic glibenclamide	43.50±7.94	13.18±2.41	6825±1178.62	500.00±48.98

Appendix 4: Effect of the extracts on the activities of the liver enzymes:- AST and ALT

Treatment Group	AST (IU/L)	ALT (IU/L)
Normal Control	102.00±17.66	24.75±3.95
Diabetic Control	106.00±5.42	40.00±8.28
SLA-250	98.50±11.84	41.75±10.21
SLE-250	113.25±18.46	47.75±10.14
SDA-250	94.00±24.73	34.25±5.44
Diabetic Glibenclamide	113.50±5.19	50.75±9.07

Appendix 5: Liver peroxidation level (MDA) (mol $cm^{-1} \times 10^{-8}$)

Group	Liver mean ± SD	Kidney mean ± SD
Normal Control	9.25±0.81	7.54±0.93
Diabetic Control	20.86±6.23	12.66±4.69
SLA-250	25.83±1.84	12.45±4.71
SLE-250	23.25±4.03	12.11±3.84
SDA-250	9.29±0.37	7.87±0.63
Gilbendamide	16.65±3.94	9.71±1.99

Appendix 6: Effect of repeated administration of the extracts on GSH concentration (µg/ml)

Group	Liver mean ± SD	Kidney mean ± SD
Normal Control	9.25±0.81	7.54±0.93
Diabetic Control	20.86±6.23	12.66±4.69
SLA-250	25.83±1.84	12.45±4.71
SLE-250	23.25±4.03	12.11±3.84
SDA-250	9.29±0.37	7.87±0.63
Gilbendamide	16.65±3.94	9.71±1.99

Appendix 7: Effect of catalase activity (Kat.f. $\times 10^{-3}$)

Group	Liver mean ± SD	Kidney mean ± SD
Normal Control	2.67±1.56	4.48±0.66
Diabetic Control	9.72±5.02	11.93±4.46
SLA-250	4.27±3.17	5.43±3.01
SLE-250	8.44±3.60	9.20±4.06
SDA-250	3.42±0.82	4.18±0.71
Gilbendamide	6.07±5.18	6.90±4.94

Appendix 8: Effect of catalase activity (SOD units/mg protein $\times 10^{-2}$)

Group	Liver mean ± SD	Kidney mean ± SD
Normal Control	31.50±8.90	37.28±12.13
Diabetic Control	49.74±10.52	63.71±11.14
SLA-250	45.95±8.66	59.94±10.06
SLE-250	41.32±6.40	67.49±2.09
SDA-250	38.96±12.57	58.96±20.56
Gilbendamide	42.75±10.33	63.34±14.29

Appendix 9: Effect of repeated administration (21 days) of the extracts on the activities of the hepatic enzymes of carbohydrate metabolism in diabetic rats[a]

Group	**mMol/min/mg protein) Hexokinase × 10^{-5}**	**mMol/min/mg protein) Glucokinase × 10-5**	**mMol/min/mg protein) Phosphofractokinase × 10**
Normal Control	1.59 ± 0.24	13.32 ± 0.91	6.17 ± 0.79
Diabetic Control	0.98 ± 0.48*	3.75 ± 0.66*	3.29 ± 1.26*
SLA-250	1.22 ± 0.15	5.25 ± 0.28*	4.29 ± 0.58
SLE-250	1.02 ± 0.19	2.56 ± 0.65*	3.16 ± 0.25
SDA-250	1.29 ± 0.15	7.67 ± 0.72*	4.31 ± 0.37
Gilbenclamide	1.29 ± 0.48	6.15 ± 0.86*	5.25 ± 0.75*

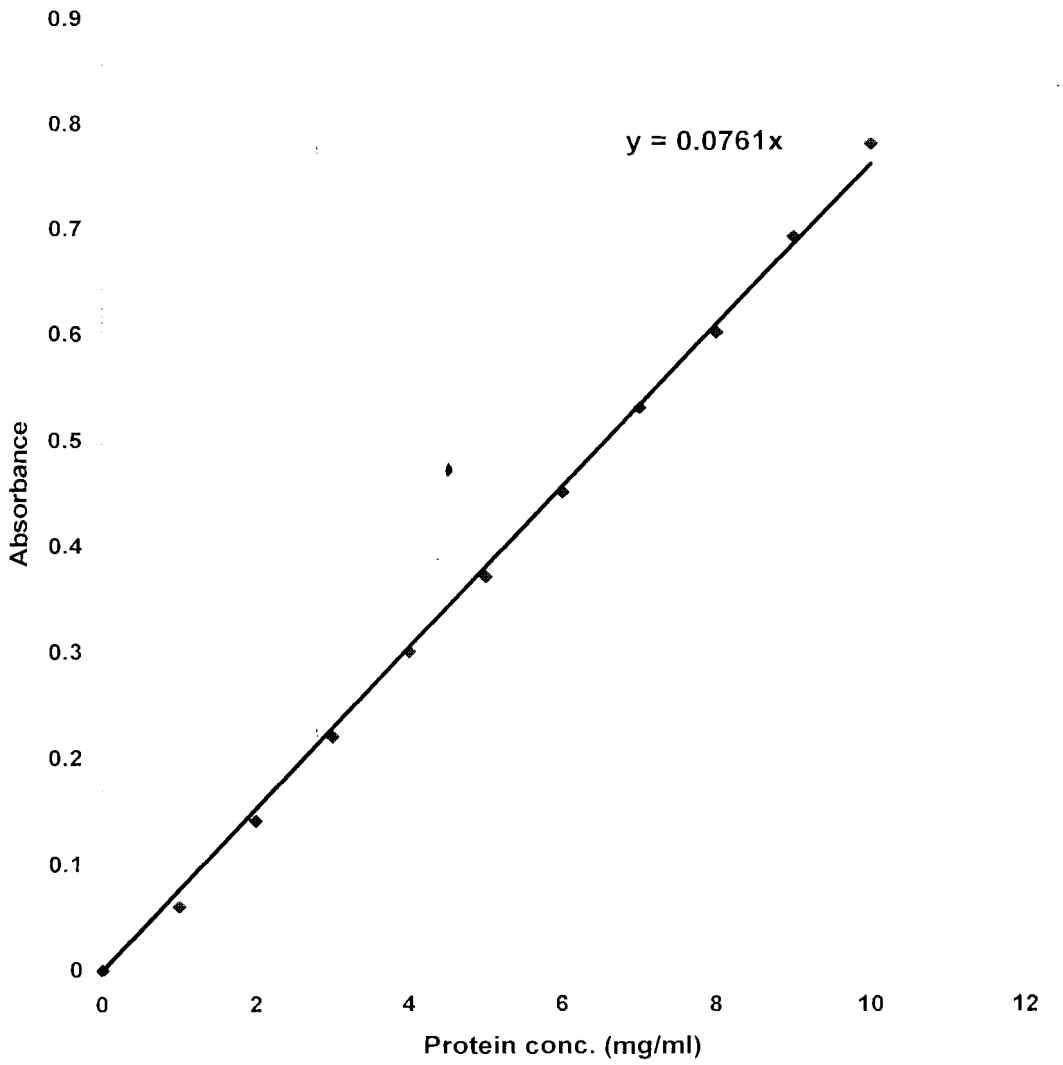
a values are given as mean ± S.D. for groups of 4 animals each. Diabetic control was compared with the normal and the diabetic treated groups

$P<0.05$

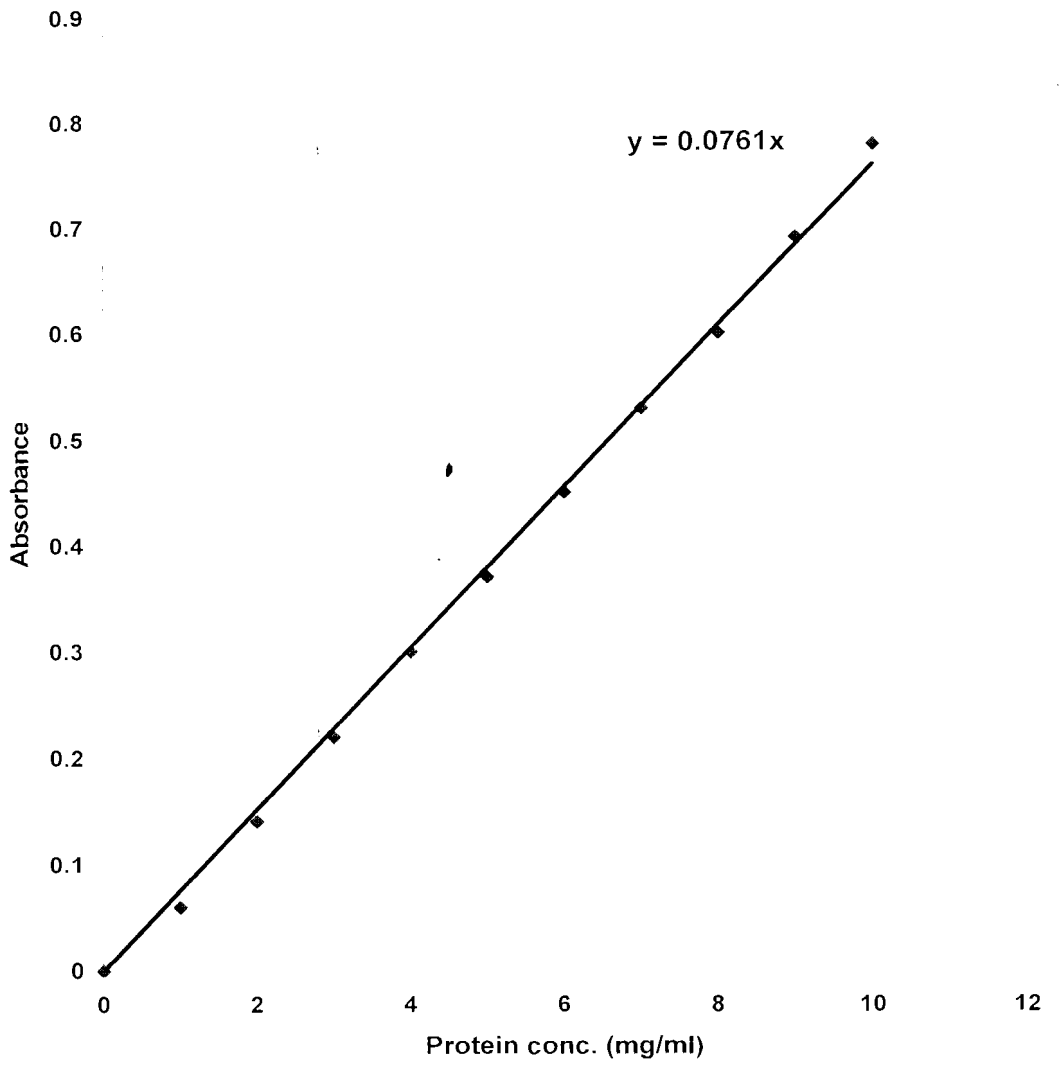
Appendix 10: Effect of administration of 21 days of the extracts on liver glycogen content of diabetic rats[a]

Group	**Liver glycogen (mg/g tissue)**
Normal control	28.95 ± 0.85
Diabetic control	18.80 ± 4.92
SLA-250	21.55 ± 1.16
SLE-250	19.33 ± 5.83
SDA-250	25.55 ± 0.62
Glibenclamide	19.15 ± 3.51

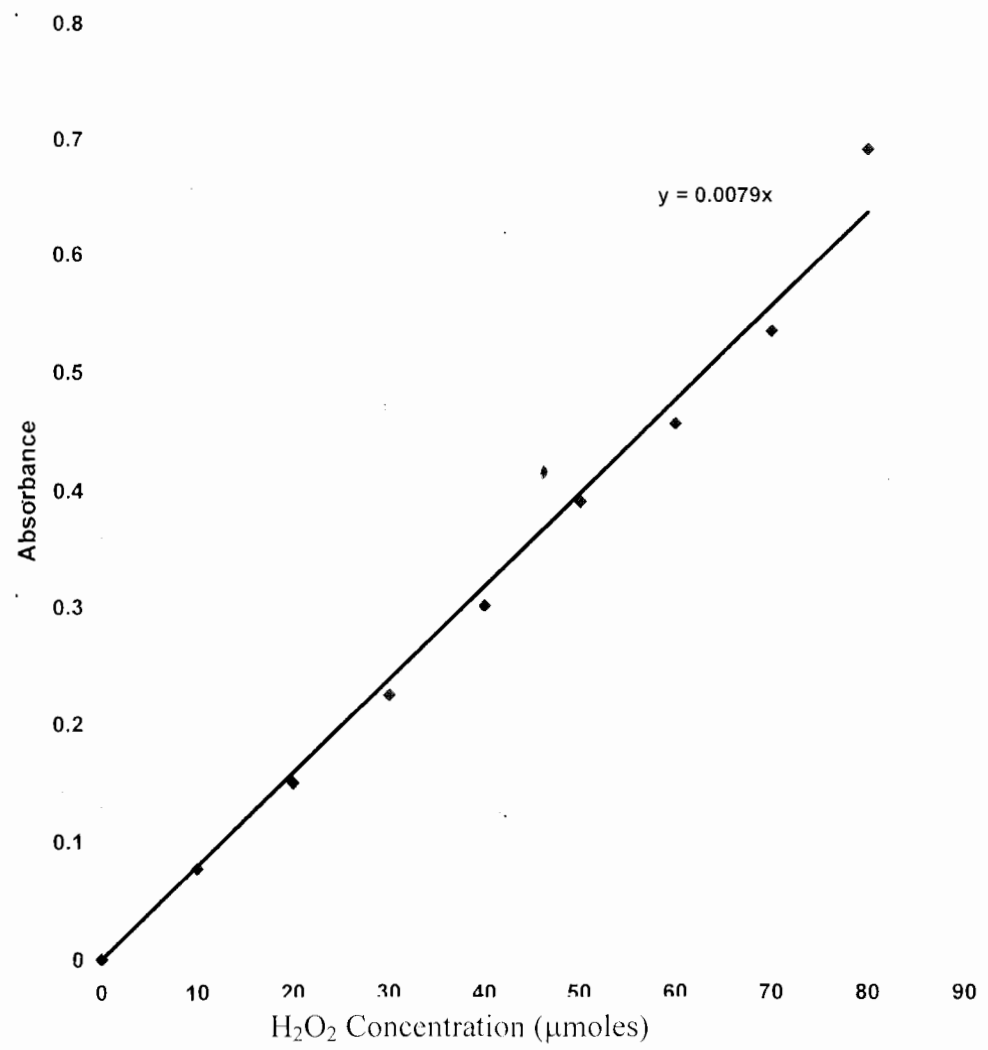
a values are given as mean ± S.D. for groups of 4 animals each



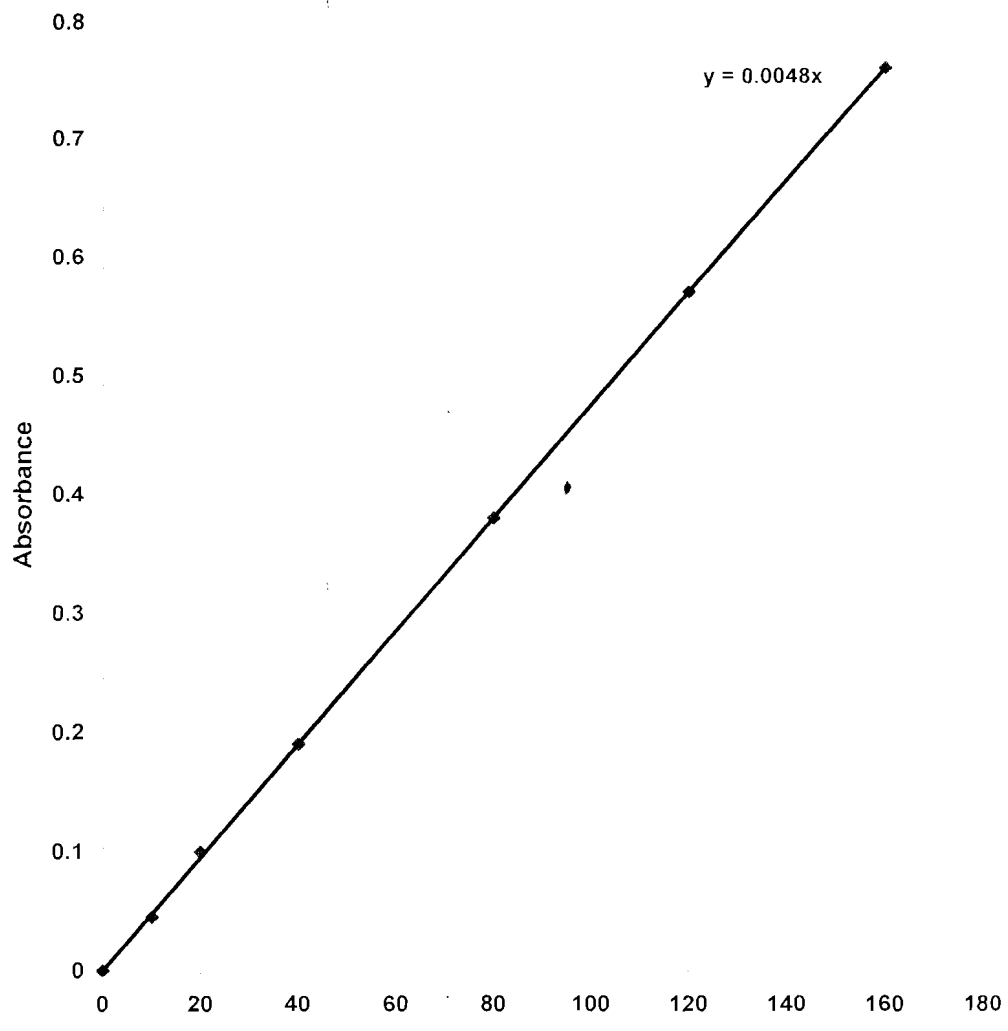
Appendix 11: Protein (BSA) Standard Curve



Appendix 11: Protein (BSA) Standard Curve

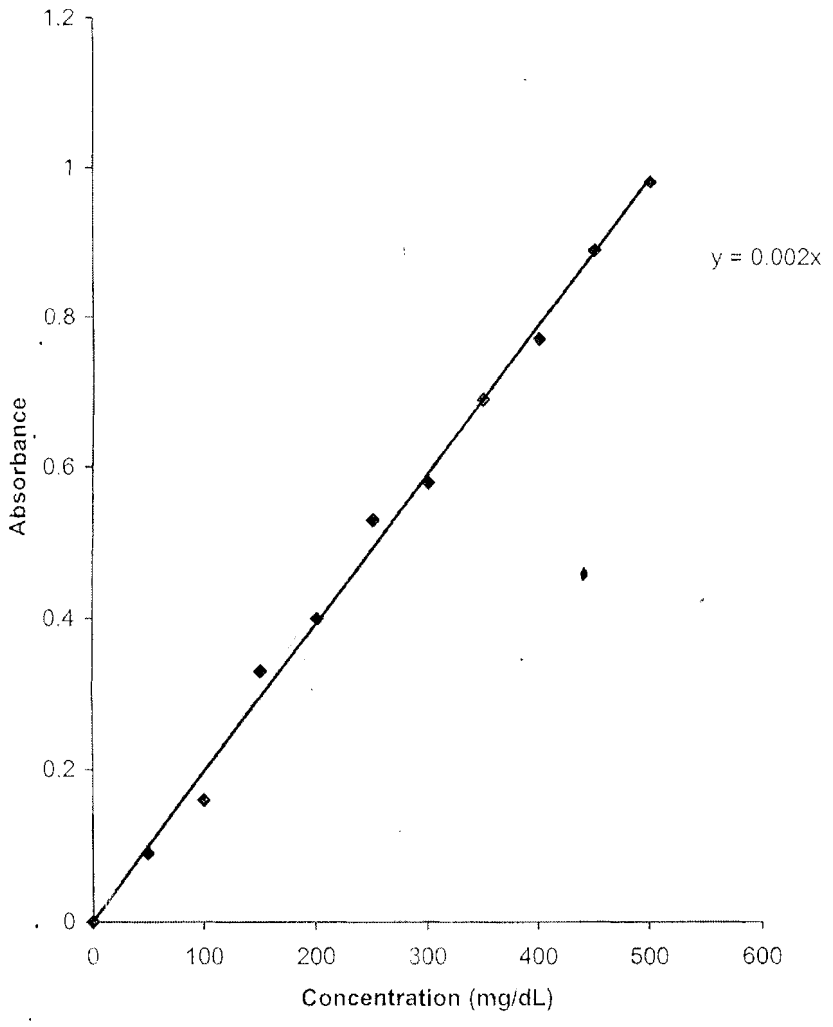


Appendix 12: Standard Hydrogen Peroxide (H_2O_2) Curve



GSH Concentration (µg/ml)

Appendix 13: Standard Glutathione (GSH) Curve



Appendix 14: Standard Glucose Curve